The role of decreased protein synthesis in delaying ageing – emphasis on translation accuracy

Victoria Eugenia Martínez Miguel
2018

Cancer Institute, University College London,
72 Huntley St, WC1E 6DD, London
UK
Declaration

I, Victoria Eugenia Martínez Miguel, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed

Dated
Las moscas

Vosotras, las familiares,
inevitables golosas,
vosotras, moscas vulgares,
me evocáis todas las cosas.
¡Oh, viejas moscas voraces
como abejas en abril,
viejas moscas pertinaces
sobre mi calva infantil!
Moscas de todas las horas,
de infancia y adolescencia,
de mi juventud dorada;
de esta segunda inocencia,
que da en no creer en nada,
de siempre.
Inevitables golosas,
que ni labráis como abejas,
ni brilláis cual mariposas;
pequeñitas, revoltosas,
vosotras, amigas viejas,
me evocáis todas las cosas.

The flies

You, the familiar,
unavoidably insatiable,
you, common flies,
you bring all back to me.
Oh, old voracious flies,
like bees in April,
old pertinacious flies,
so intent over my infant scalp!
Flies from all eras,
from Infancy and adolescence,
from my Golden youth;
and now from this second innocence,
that offers nothing to believe in,
always flies.
Unavoidably insatiable,
you don’t work as bees do,
you don’t glitter the way butterflies glitter,
tiny, unruly beings,
you old friends,
bring it all back to me.

Antonio Machado, (26th July 1875 – 22nd February 1939)
Abstract

Ageing is a malleable process and outstanding discoveries demonstrate that: 1) a single point mutation can double the lifespan of model organisms, 2) these long-lived animals are healthier and 3) the effect of these interventions is evolutionary conserved from yeast to mammals. This offers the possibility that the insights gained from research in ageing could be translated to improve health in the elderly and to prevent age-related diseases. Major interventions leading to longevity and health improvements include dietary restriction, mild down-regulation of insulin and target-of-rapamycin (TOR) signalling. Interestingly, a common underlying characteristic of all these lifespan-extending treatments is that they all lead to a reduction of protein synthesis and, in addition, directly lowering translation can also improve longevity. However, the underlying mechanism of this effect on longevity is largely elusive. The aim of this project was to test a largely neglected hypothesis that lowered translation improves fidelity of the proteins synthetized, thereby leading to improved health and longevity. To explore this, *Drosophila melanogaster* was used as a model organism in which a focused longevity RNAi longevity screen was performed, and we found that downregulating translation-associated factors can extend lifespan in flies. Furthermore, a dual-reporter luciferase assay was developed in *Drosophila*-derived S2R+ cells to study translation fidelity and we have found that several drugs known to extend lifespan decrease the level of translation errors. In addition, a mutant with a single residue substitution in a ribosomal protein was generated by CRISPR/Cas9 and we found that directly improving translation fidelity can extend lifespan and improve healthspan in flies. Thus, *in vitro* and *in vivo* techniques have been used to understand the molecular and cellular mechanisms behind the effect of translation, especially its fidelity, in ageing.
Impact statement

The field of biogerontology had a major breakthrough when it was found that a single-gene mutation could double the lifespan of *C. elegans* (Kenyon et al., 1993). This led to discovering many more mutations that could not only extend lifespan, but also improve healthspan and prevent age-related diseases. Importantly, using model organisms, it was found that these anti-ageing interventions are evolutionarily conserved from yeast to mammals. After decades of studying, we already know many of the molecular mechanisms behind ageing, and how they can be pharmacologically targeted to improve longevity. This is not only important for the sake of science, but it also opens the possibility of creating drugs for humans that can ameliorate the health decline that inherently comes with ageing. This is especially important in the 21st century, as there is a great socioeconomic impact rising from the fact that the proportion of elderly has been rising, and is predicted to keep increasing, in both less and more developed countries (United Nations, 2017). Therefore, it is crucial to find ways of tackling age-related diseases. There are already drugs being investigated for their use in humans, but we are still in need of more and better ones (Barzilai et al., 2016, Longo et al., 2015). Thus, in my research I have studied how these drugs affect the process of translation in *Drosophila*-derived cells and I have found a simple correlation between drugs that can extend lifespan in flies and decrease mRNA translation errors, which has the potential to become a way of screening for anti-ageing drugs.

In addition, I have studied a long-standing question in biology, does translation fidelity affect ageing? In 1963, L. Orgel theorized that ageing was caused by the translation apparatus, which had the potential of causing an ‘error catastrophe’ if it was erroneous (Orgel, 1963). There were many studies at the time that showed that errors in translation did not increase with age or that directly inducing translation inaccuracy could not lead to ageing. Nevertheless, the theory was not totally refuted, as the techniques at the time were limited, and it was still argued that maybe the errors had not been properly detected. This has meant that more recently, with the advancement in the techniques now available to measure transition errors, more laboratories are trying to understand the real relationship between translation fidelity and longevity (Shum et al., 2018, Ke et al., 2018). This has led us to adapt for *Drosophila* the gold-standard technique at the moment to measure translation errors, a dual-luciferase assay, and study the progress of accuracy with age flies. We are the first group to report the level of translation errors in an aged metazoan organism, as we have found that it dramatically increases with age in flies. For this reason, we generated using CRISPR/Cas9 the first metazoan organism that has a mutation in a ribosomal protein that leads to hyperaccuracy and we have found that these mutant flies live longer than the controls thanks to this mutation.
Acknowledgements

First, I want to show my gratitude to the Bill Lyon’s charitable trust, which generously sponsored my PhD through the Impact Studentship because without it I could not have done it. I also want to thank the Cancer Institute for having let me use their fantastic facilities and, having made the time of my PhD extra especial. I also want to acknowledge how UCL has not just been a facility provider but also its courses and teaching opportunities have formed me academically and personally.

At the Cancer Institute, I also want to say thank you to all my friends, as they supported me, both morally and intellectually, throughout these four years, in especial, Dimitra Georgopulu, Dubravka Pezic, José Ariza, Katerina Lambada, Ketty Kessler, Lucia Cottone, Manuel Beltrán, Ramazan Guendogdu, Teresa Sposito, Valeria Amodeo and Valentí Gómez. Also, I need to thank Duncan Kennedy because he has been a great help fixing Varioskan when I was in panic.

Obviously, I need to mention all my fantastic students in the lab, because their help was invaluable and because I have learnt a lot through teaching them: Laila Khazendar, Katie Wall, Sharmin Saleque and Justyna Ekert. Here, I need to add here Ellen McMullen and Noora Alomaira because they weren’t my students, but they were great to have around.

In my laboratory, huge thanks go to my best lab mates ever, Ellie Stead and Celia Lujan. Ellie has been a great partner in the lab, always willing to help with a smile and an incredibly brilliant scientist, what else could I ask for? And well, Celia Lujan, she knows she is the best technician of the year (holding the title for the last 5 years), and she has been such a crucial part of this project, that without her it wouldn’t have been possible. In addition, she has become one of my best friends and because she has helped me in all the ways possible, inside and outside the lab, there are not enough words in the world to thank her.

Outside the institute I want to thank my family. My grandparents, Etelvina and Mauricio, have been incredibly supportive, always asking if the anti-ageing remedy was already discovered for them (I wish it was and I could give them liters and liters). My sister, Minerva, who has been the best flat mate I ever had as she has helped me a lot (after a distressing day of work, everything becomes better if you have someone to talk to and laugh with). Luke, my partner, has always been great and loving, even though we have been seeing each other every few weekends, if I had to go to the lab, he has never hesitated to come with me, and even better, he has never complaint about it. And of course, I need to praise my parents, Fernando
and Maria Victoria, who have been incredible during this time, they have encouraged me a lot and have always been very caring and thoughtful. And obviously, I couldn’t have done so many lifespans without Drosoflipper, which wouldn’t exist without the great mind of my dad.

Finally, I want to thank my supervisor, Ivana Bjedov. She has been the most incredible supervisor and I am so happy I have gone through this journey with her. She has always been extremely considerate, taking care of me in the nicest way. It has been a wonderful experience having her as a teacher because she is a wonderful scientist and incredibly passionate about it. She has been so inspiring and a true role model, as she is one of the hardest-working people I know. I can only hope she has the most successful career, because she truly deserves it.

_Dedicated to my grandmother_

*Victoria Anaya Fernández (1931-2002),*  
Because I got her name and mitochondria,  
And I hope I get any other of her traits, as she was wonderful.
Table of Contents

Declaration .................................................................................................................. 2
Abstract ...................................................................................................................... 4
Impact statement ....................................................................................................... 5
Acknowledgements .................................................................................................... 6
List of figures .............................................................................................................. 13
List of tables .............................................................................................................. 16
List of symbols and abbreviations ........................................................................... 17
Chapter 1. Introduction ............................................................................................. 23
  1.1. Introduction to the biology of ageing ............................................................... 24
    1.1.1. Introduction to ageing ............................................................................... 24
    1.1.1.1. Relevance of anti-ageing interventions to societies ......................... 25
    1.1.2. Biology of ageing .................................................................................... 25
      1.1.2.1. Evolutionary theories ........................................................................ 26
      1.1.2.2. Mechanistic theories ......................................................................... 33
      1.2.3. Model organisms used in ageing research ........................................... 45
      1.1.3. Molecular mechanisms behind ageing .................................................. 49
        1.1.3.1. Insulin/IGF (insulin-like growth factor)-like signalling (IIS) ............ 49
        1.1.3.2. Dietary restriction ............................................................................. 52
        1.1.3.3. AMP kinase .................................................................................... 54
        1.1.3.4. Sirtuins .......................................................................................... 54
        1.1.3.6. Signals from the reproductive system ............................................... 55
        1.1.3.7. Epigenetics ...................................................................................... 55
        1.1.3.9. Involvement of translation ............................................................... 57
    1.1.4. Pharmacological treatments to ameliorate ageing ..................................... 57
  1.2. Introduction: Translation and translational fidelity ......................................... 59
    1.2.1. Translation ............................................................................................... 59
      1.2.1.1. tRNA charging ................................................................................... 59
      1.2.1.2. Initiation ............................................................................................ 60
      1.2.1.3. Elongation .......................................................................................... 65
      1.2.1.4. Termination and ribosome recycling ............................................... 66
      1.2.1.5. Ribosomal subunits .......................................................................... 68
      1.2.1.6. Mitochondrial translation .................................................................. 70
      1.2.1.7. Signalling pathways involved in translation ...................................... 70
    1.2.2. Translational fidelity .................................................................................. 72
1.2.2.1. Mutations and antibiotics affecting fidelity ........................................ 74
1.2.2.2. Ribosomal structures affecting decoding and fidelity ................................ 76
1.2.2.3. Translation fidelity studies in eukaryotes ........................................... 78
1.2.2.4. Stress and translation fidelity ......................................................... 80
1.2.2.5. Harmful outcomes of mistranslation ............................................... 81
1.2.2.6. Beneficial effects of translation inaccuracy ....................................... 82
1.2.3. Translation and ageing ................................................................. 83
1.2.3.1. Translation fidelity and ageing ....................................................... 86
1.3. Thesis Outline ....................................................................................... 88
1.4. Thesis aims and objectives ............................................................... 89
Chapter 2: Materials and Methods ............................................................. 90
2.1. *Drosophila* stocks and maintenance .................................................. 91
2.1.1. Fly maintenance ............................................................................... 91
2.1.2. *Drosophila* medium preparation ...................................................... 91
2.1.3. Stock list ............................................................................................ 94
2.1.4. Lifespan analysis .............................................................................. 96
2.1.5. Drugs used in lifespans ..................................................................... 97
2.2. Healthspan and behavioural analysis in *Drosophila* ................................ 98
2.2.1. Negative geotaxis or climbing assay .................................................. 98
2.2.2. Stress resistance assays ..................................................................... 99
2.2.3. Fertility assay ................................................................................... 100
2.2.4. Developmental assay ....................................................................... 100
2.3. Gene expression analysis ..................................................................... 100
2.3.1. Tissue preparation .......................................................................... 100
2.3.2. RNA extraction ............................................................................. 100
2.3.3. cDNA conversion .......................................................................... 101
2.3.4. RT-qPCR......................................................................................... 102
2.4. Protein purification and analysis ......................................................... 102
2.4.1. Protein extraction .......................................................................... 102
2.4.2. SDS-PAGE electrophoresis .............................................................. 103
2.4.3. Immunoblotting ............................................................................ 103
2.5 Overall translation measurements ......................................................... 103
2.5.1. Sample preparation ......................................................................... 104
2.6. Translation fidelity assay *in vivo* ........................................................ 104
2.6.1. Cloning of the translation fidelity reporters for *Drosophila* .................. 105
2.6.2. Fly generation and backcrossing of the translation fidelity reporters .... 106

Martínez Miguel, V.E.
Chapter 3: Genetic screen for translation-associated factors that can extend lifespan in Drosophila

3.1. Introduction .................................................................................................................. 130
3.2. Results .......................................................................................................................... 131

3.2.1. The effect of downregulation of translation-associated proteins by RNAi on lifespan and healthspan in Drosophila .................................................................................. 131

3.2.1.1. Downregulation of translation-associated proteins by RNAi in metabolic tissues can extend lifespan ............................................................................................................. 131

3.2.1.2. Downregulation of translation-associated proteins by RNAi in metabolic tissues can improve health in Drosophila but does not confer resistance to heat shock and tunicamycin stresses .......................................................................................................................... 140

3.2.1.3. Downregulation of translation-associated proteins by RNAi in metabolic tissues does not alter signalling pathways related to protein synthesis .......................................................................................................................... 146

3.2.1.4. Measurements of de novo protein synthesis in flies with downregulated translation-associated proteins by RNAi in metabolic tissues .......................................................................................................................... 148

3.2.1.5. Translation fidelity examination in flies with downregulated translation factors by RNAi in metabolic tissues .......................................................................................................................... 154

3.2.1.6. Epistasis measurements using paromomycin .......................................................................................................................... 154
5.2. Results

5.1 Introduction

3.3. Discussion

3.3.1. Ubiquitously downregulating certain translation-associated factors in adult flies can be detrimental, but their downregulation in the fat body and intestine can extend lifespan

3.3.2. Downregulation in the fat body and intestines of eIF4E extends lifespan but downregulation of eIF4G shortens it

3.3.3 Downregulation of eEF2 in the fat body and intestines extends lifespan and healthspan

3.3.4 Downregulation in the fat body and intestines of RPS23 extends lifespan

3.3.5 Translation fidelity in flies with downregulated eIF4E, eIF4G, eEF2 and RPS23 in the metabolic tissues

Chapter 4. Pharmacological screen for drugs that improve translation fidelity and their potential to be anti-ageing treatments in *Drosophila*

4.1. Introduction

4.2. Results

4.2.1. Drugs that extend lifespan improve translation fidelity *in vitro*

4.2.1.1. Validation of translation fidelity reporters for *Drosophila* derived S2R+ cells

4.2.1.2. Several drugs that extend lifespan decrease the level of translation errors

4.2.1.3. Effect on lifespan of the drugs tested on the translation fidelity assay

4.2.1.4. Conclusions about the effect of longevity-related drugs on translation fidelity

4.3. Discussion

4.3.1. The lifespan extending effect of the drugs tested correlates with increased translation fidelity

Chapter 5. Impact on development and longevity of a substitution point mutation that alters translation fidelity

5.1 Introduction

5.2. Results

5.2.1. Generation and characterisation of the first metazoan translation fidelity mutant

5.2.1.1. Design of the translation fidelity mutations by CRISPR Cas-9

5.3.1.2. Translation fidelity is improved in RPS23 K60R flies

5.3.1.3. Directly altering translation fidelity by a mutation in the ribosomal decoding centre delays development independently of the environment

5.3.1.4. Directly altering translation fidelity in *Drosophila* influences longevity in a condition-dependent manner

5.3.1.5. RPS23 K60R female flies have improved healthspan when reared under standard conditions

5.3.1.6. Homozygous RPS23 K60R female flies are sensitive to paromomycin but heterozygous RPS23 K60R flies were not
5.3.1.7. Conclusions about the characterisation of the translation fidelity mutant RPS23 K60R ............................................................................................................................................... 216

5.4. Discussion .................................................................................................................................................. 217

5.4.1. K60R reduces stop codon readthrough in *Drosophila* ........................................................................ 217

5.4.2. The level of stop codon readthrough increases with age in *Drosophila* ........................................ 220

5.4.3. K60R delayed development ................................................................................................................... 222

5.4.4. K60R can extend lifespan and improve healthspan in a condition-dependent manner ..................... 225

Chapter 6: General discussion .......................................................................................................................... 231

6.2 Future work .................................................................................................................................................. 239

References ......................................................................................................................................................... 242
List of figures

Chapter 1.

Figure 1.1. Mortality, fertility and survivorship in several organisms ranging from vertebrate to invertebrate, plants and algae.

Figure 1.2. Diagram of the reasoning behind the theories supporting that ageing evolved as a consequence of the force of natural selection declining and mutations accumulating with age.

Figure 1.3. Model of the modified mutation accumulation theory.

Figure 1.4. Orgel’s error catastrophe theory.

Figure 1.5. Ageing as a quasi-program of development.

Figure 1.6. Illustration of the different eye colour phenotypes associated to mutations in the third chromosome.

Figure 1.7. Interactions between the mTOR and IIS pathways and stress-sensing mechanisms.

Figure 1.8. Mechanisms behind lifespan-extension by dietary restriction.

Figure 1.9. Schematic Representation of the steps occurring during translation initiation in eukaryotes.

Figure 1.10. Schematic representation of the elongation phase of translation in eukaryotes.

Figure 1.11. Diagram of the termination phase of translation in eukaryotes.

Figure 1.12. The eukaryotic ribosomal subunits based on Tetrahymena thermophila.

Figure 1.13. Main pathways that regulate protein translation in response to different stimuli such as stress, growth factors and hormones or endoplasmic ER stress.

Figure 1.14. Schematic representation of aminoacylation and ribosome decoding: their proofreading mechanisms and the possible mistranslation events.

Figure 1.15. Interactions of streptomycin and paromomycin with the decoding centre on prokaryotic ribosomes.

Figure 1.16. Representation of the position of ram or restrictive mutations in the ribosome.

Figure 1.17. Schematic representation of the PhD thesis outline.

Chapter 2.

Figure 2.1. Gene-Switch system in Drosophila melanogaster. Example shown of the S1106 driver.

Figure 2.2. Schematic representation of the negative geotaxis or climbing assay using Drosofilppers.

Figure 2.3. Dual luciferase reporter constructs to measure translation fidelity.

Figure 2.4. Dual luciferase constructs cloned into the final vector.

Figure 2.5. Representative image of the gel for the PCR products of the screen for positive S1106, dual luciferase reporters double mutants.

Figure 2.6. Image of the T7 assay to test the efficiency of different gRNAs.

Figure 2.7. RPS2 and RPS23 sequences and mutations introduced by CRISPR.

Figure 2.8. Map of pCFD3 with gRNA for RPS2 as an example.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 2.9. Representative images of the gel pictures of the PCR products of the RPS23 screen.

Figure 2.10. Dual luciferase constructs cloned into their final vectors.

Chapter 3.

Figure 3.1. Representative survival curves of ActGS>eIF4G RNAi (CG10811), ActGS>eEF2 RNAi (CG2238), ActGS>eIF3-S9 RNAi (CG4878), ActGS>EFTuD2 RNAi (CG4849), ActGS>RPS2 RNAi (CG5920), ActGS>eIF3-S10 RNAi (CG9805), ActGS>RPS26 RNAi (CG10305), ActGS>eIF4A RNAi (CG7483) and ActGS>RPS11 RNAi (CG8857).

Figure 3.2. Representative survival curves of S1106>eIF1 RNAi (CG17737), S1106>eIF3-S8 RNAi (CG4954), S1106>RPS23-mit RNAi (CG1842) and S1106>myc RNAi (CG10798).

Figure 3.3. Representative survival curves of the control, S1106>eIF4E RNAi (CG4035), S1106>eEF2 RNAi (CG2238), S1106>RPS23 RNAi (CG8415) and S1106>eIF4G RNAi (CG10811).

Figure 3.4. Relative abundance of mRNA in the S1106>UAS-RNAi lines.

Figure 3.5. Survival of the S1106>UAS-RNAi flies exposed to the ER stressor tunicamycin.

Figure 3.6. The resistance of the S1106>UAS-RNAi flies to heat shock.

Figure 3.7. Fertility assessment of the S1106>UAS-RNAi flies.

Figure 3.8. Assessment of the senescence of negative geotaxis in the S1106>UAS-RNAi flies.

Figure 3.9. Levels of phosphorylation of eIF2α (Ser51), pS6K (Thr389) and 4EBP (Thr 37/42) in the gut and fat body of the S1106>UAS-RNAi flies.

Figure 3.10. Sample preparation optimization for the puromycin-based assay to measure de novo protein synthesis.

Figure 3.11. Relative levels of de novo protein synthesis in the fat bodies of flies with downregulated translation-associated proteins and their relative controls.

Figure 3.12. Relative abundance of RNA encoding for each translation-associated protein in S2R+ cells treated with decreasing amounts of dsRNA downregulating eIF4E, eIF4G, eEF2 or RPS23.

Figure 3.13. Relative levels of de novo protein synthesis in S2 cells treated with several concentrations of dsRNA against the translation-associated proteins, eIF4E, eIF4G, eEF2 and RPS23.

Figure 3.14. Optimisation of the conditions required for the paromomycin epistasis experiment.

Figure 3.15. Effect of paromomycin on the survival of S1106>UAS-RNAi flies.

Figure 3.16. Effect of paromomycin on the fertility of S1106>UAS-RNAi flies.

Chapter 4.

Figure 4.1. Dual luciferase assay optimisation.

Figure 4.2. Drugs known to affect lifespan in model organisms, translation or both, can also alter the level of stop codon readthrough or amino acid misincorporation.

Figure 4.3. Effect on lifespan of rapamycin, Torin 1, Torin 2, diazaborine, cycloheximide and salubrinal.

Chapter 5.

Figure 5.1. A single point mutation in an evolutionarily conserved region of RPS23 can alter translation.

Figure 5.2. Highly conserved region across all living organisms in the homologues of Drosophila’s RPS23.

Figure 5.3. The RPS23 K60R mutation leads to decreased stop codon readthrough in young and old flies.
Figure 5.4. The fertility and different stages of development of the RPS23 K60R flies are affected by several environmental conditions.

Figure 5.5. RPS23 K60R adult flies emerged later than the controls under all the conditions tested.

Figure 5.6. Effect of RPS23 K60R mutation on lifespan under two different temperatures.

Figure 5.7. Effect of RPS23 K60R mutation on lifespan fed 0.5 SYA, 1 SYA and 2 SYA.

Figure 5.8. Assessment of the senescence of negative geotaxis in the RPS23 K60R flies.

Figure 5.9. Effect of paromomycin on the lifespan of RPS23 K60R flies.

Figure 5.10. Schematic representation of the accuracy requirements for the best fitness depending on the translation load.

**Chapter 6.**

Figure 6.1. There is an inherent trade-off between speed and accuracy, and this has to be balanced depending on the physiological requirements.
List of tables

Chapter 1.
Table 1.1 Percentage of total population by broad age group, both sexes (per 100 total populations) in more developed regions and less developed regions.
Table 1.2. Proteins involved in eukaryotic translation initiation.

Chapter 2.
Table 2.1. Ingredients and their measurements necessary to cook 1 L sugar yeast agar medium.
Table 2.2. Ingredients for one litre of holidic medium.
Table 2.3. List of RNAi lines and their Computer Generated numbers (CG).
Table 2.4. Drugs used in lifespans.
Table 2.5. List of primers and their sequence.
Table 2.6. Primers and their sequences.
Table 2.7. List of primers and their sequences.
Table 2.8. List of primers and their sequences.
Table 2.9. List of primers and sequences.
Table 2.10. List of primers and their sequences.
Table 2.11. List of primers, their sequence and annealing temperature.
Table 2.12. Table of the primers and sequences.
Table 2.13. Drugs used in the in vitro dual luciferase and their concentrations.

Chapter 3.
Table 3.1. Median and maximum lifespan data from RU486-induced ActGS>RNAi flies.
Table 3.2. Median and maximum lifespan data from RU486-induced S1106>UAS-RNAi flies.
List of symbols and abbreviations

/+: Over wild type
♀: Virgin female
♀: Female
♂: Male
4EBP: Eukaryotic translation initiation factor 4E-binding protein 1
5': 5 Prime
5mC: 5-methylcytosine
A site: Aminoacyl site
aa: Amino acid
aaRSs: Aminoacyl-tRNA synthetases
ABCE1: ATP binding cassette subfamily E member 1
Act5c: Actin 5C
AGE: Advanced glycation end-products
AMP: Adenosine monophosphate
AMPK: 5' AMP-activated protein kinase
Aop: Anterior open
ATM: Ataxia telangiectasia mutated
ATR: Ataxia telangiectasia and Rad3-related
ATP: Adenosine triphosphate
BCA: Bicinchoninic acid
BDSC: Bloomington Drosophila Stock Centre
BSA: Bovine serum albumin
Cas9: CRISPR associated 9
cDNA: Complementary DNA
CG: Computer generated
CIAP: Calf intestinal phosphatase
CR: Caloric restriction
CRISPR: Clustered regularly interspaced short palindromic repeats
CrV: Cricket paralysis virus
CTDK-1: Carboxyl-terminal domain kinase 1
Cyo: Curly
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

ddH₂O: Double-distilled water
DENR: Density release protein
dlnR: *Drosophila* insulin-like receptor
DLR: Dual luciferase reporter
DMSO: Dimethyl sulfoxide
DNA Pol: DNA polymerase
DNA: Deoxyribonucleic acid
dNTPs: Deoxyribonucleotide triphosphate
DPP1: Diacylglycerol diphosphate phosphatase
DR: Dietary restriction
dsRNA: Double stranded RNA
DTT: Dithiothreitol
ECL: Enhanced chemiluminescence
EDTA: Ethylenediaminetetraacetic acid
eEF: Eukaryotic elongation factor
eEF2K: eEF2 kinase
EFG: Elongation factor G
EFTu: Elongation Factor Tu
eIF: Eukaryotic initiation factor
ER: Endoplasmic reticulum
eRF: Eukaryotic release factor
ERK1/2: Extracellular Signal-Regulated Kinases 1 and 2
E-site: Exit site
EtOH: Ethanol
ETS (E-twenty-six)
eukaryotic initiation factor 4E-binding protein (4EBP)
F1 (F2, F3, etc): Filial generation 1, etc.
FBP: Fructose-1,6-biphosphate
FBS: Foetal bovine serum
FOX: Forkhead box
FOXO: Forkhead box O
GAAC: General amino acid control
GAL4: Galactose-induced gene 4
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GCN2 and 4: General control nonderepressible 2 and 4
GDP: Guanosine diphosphate
GFP: Green fluorescence protein
GHR: Growth hormone receptor
gRNA: Guiding RNA
GTP: Guanosine-5'-triphosphate
GTPase: GTP hydrolase
GTPBP2: GTP binding protein 2
H27: Helix 27
H3K27me3: Histone methylation occurring on the amino (N) terminal tail of the core histone H3
H44: Helix 44
HATs: Histone acetyltransferases
HCV: Hepatitis C virus
HDACs: Histone deacetylases
Ifs: Initiation factors
IGF: Insulin-like growth factor
IIS: Insulin/IGF-like pathway
InR: Insulin receptor
IRES: Internal Ribosomal Entry Sites
IRS: Insulin receptor substrate
LAR II: Luciferase Assay Buffer II
LB media: Lysogeny broth media
LEP: Life energy potential
m^7G: Methylated on the position 7
MAPK: Mitogen-activated protein kinases
MCTS1: Multiple copies in T-cell lymphoma-1
MEK1 and 2: MAPK/ERK Kinase 1 and 2
Met-tRNAi: Initiator methionyl-trNA
Mitoribosomes: Mitochondrial ribosomes
MPN: Mpr1/Pad1 N-terminal
mRNA: Messenger RNA
mtDNA: Mitochondrial DNA
mTOR: Mechanistic/mammalian target of rapamycin
NAD\(^+\): nicotinamide adenine dinucleotide
NMR: Naked mole rat
Non-phospho: Non-phosphorylated
NOS: Reactive nitrogen species
nos-Cas9 flies: Nanos promoter-Cas9 flies
NSUN5: NOP2/Sun RNA methyltransferase family member 5
ORF: Open reading frame
Oxidative phosphorylation
P site: Peptidyl site
p70S6K: p70 ribosomal protein S6 kinase -1
PABP: Poly (A) binding protein
PAM sequence: Protospacer adjacent motif sequence
pAW: Actin promoter
PBS: Phosphate-buffered saline
PCI: Proteasome/CSN/eIF3
PERK: PKR-like ER kinase
pHW: Heat shock promoter
PI3K: phospho-inositol 3 kinase
PKB: Protein kinase B
PKR: Protein kinase
PLB: Passive lysis buffer
pMT: Metallothionein promoter
poly (A) cap: 5’ Polyadenylated cap
poly(U): 3’ Polyuridylation tail
(p)ppGpp: Guanosine pentaphosphate or tetraphosphate
PP1: Protein phosphatase 1
PP1R15a: PP1 regulatory subunit 15A region
PRAS40: Proline-rich Akt substrate of 40 kDa
pre-tRNAs: precursor tRNA molecules
Proteostasis: Protein homeostasis
Ram: Ribosomal ambiguity mutation
Rapalog: Rapamycin analogue
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Res: restrictive phenotype mutation
RelA: Ribosome-associated protein A
Ribosomopathies: Diseases caused by ribosomal abnormalities
RL: Replicative lifespan
RNA: Ribonucleic acid
Pol I: DNA polymerase I
Pol II or III: RNA polymerase II or III
RNAi: RNA interference
RNAse free-DNAse
ROS: Reactive oxygen species
RPL: Ribosome protein of the large subunit
RPS: Ribosome protein of the small subunit
rRNA: ribosomal RNA
RT-qPCR: Reverse transcriptase-quantitative polymerase chain reaction
RU486: Mifepristone
S106: Scer\GAL4Switch1.106
S2R+ cells: Schneider 2 cells
S6K: p70-S6 kinase
SAM: Senescence-accelerated mice
SASP: Senescence-associated secretory phenotype
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM: Scanning electron microscope
Sirtuin: Silent mating type information regulation 2 homolog 1
ssODN: single-stranded oligodeoxynucleotides
SDS: Sodium Dodecyl Sulphate
Sre1: Sterol regulatory element 1
ssODN: Single-stranded oligodeoxynucleotides
SLEs: Standardized laboratory environments
SmR: Streptomycin-resistant
SmD: Streptomycin-dependent
SmS: Streptomycin-sensitive
SunRISE: SUnSET-based Ribosome Speed of Elongation
SUnSET: Surface sensing of translation
SOD: Superoxide dismutase
SYA: Sugar Yeast Agar
TBST: Tris-buffered saline (TBS) and Polysorbate 20 (also known as Tween 20).
TCER-1: Transcription elongation regulator homolog
NSAIDs: Nonsteroidal anti-inflammatory drugs
TISU: Translation Initiator of Short 5’ UTR
TORC1 or 2: mTOR complex 1 or 2
TFEB: transcription factor EB
TSAA: Translation state array analysis
tRNA: transfer RNA
TSC2: Tuberous sclerosis protein 2
2D electrophoresis: Two-dimensional (2D) gel electrophoresis
UAS: Upstream activation system
UN DESA: United Nations Department of Economic and Social Affairs
ULK: Unc-51 like autophagy activating kinase
RelA: Ribosome-associated protein A
UPR: Unfolded protein response
uORFs: Untranslated open reading frames
UTR: Untranslated region
UTX-1: Ubiquitously transcribed TPR on X
VDRC: Viena Drosophila Stock Centre
W1118: white 1118
wDah: white Dahomey
WT: Wild-type
X→Y: X to Y substitution
YT: yeast and tryptone
Chapter 1. Introduction
1.1. Introduction to the biology of ageing

In this chapter, I will give a brief introduction about the history of biogerontology, followed by an explanation of some of the most relevant evolutionary and mechanistic theories of ageing, whilst briefly touching upon some possible anti-ageing interventions.

1.1.1. Introduction to ageing

Ageing is defined as “the process of growing old” by the Oxford Dictionary so if we consider about ageing in this context, we will usually think from an anthropomorphic perspective about how, with time, we wrinkle, our movements become slower, our hair falls out or greys and we acquire more wisdom and/or lose memory (Oxford-Dictionary, 2018). However, ageing is a process that affects from single-cell to multicellular organisms, with many shared characteristics but also some unique ones. The main shared characteristics usually involve the biologically-caused decline of certain properties, such as impaired metabolism or cognitive ability. Therefore, biogerontology (i.e. the study of the biology of ageing) focuses on senescence, which is defined as the deterioration of adult organisms that prompts pathologies and death (Janac et al., 2017).

The study of longevity originates in the 19th century, and is either linked to medicine and what could affect human longevity or linked to zoology and the differences in lifespan among animals. Already some studies of mid-19th century explored how diet and exercise affected ageing, but with a certain bias against what ‘civilized’ people should be eating and doing to live longer and healthier (Thackrah, 1831, Bell, 1844). Nonetheless, zoologists started studying longevity in animals with particular interest at the end of the 19th century. In the beginning of the 20th century, for example C. Mitchell, in 1911, published a laborious compendium of mammalian and avian lifespans and tried to explain why there was such a variation among species (Mitchell, 1911).

Ageing manipulations started at the beginning of the 20th century, when using Drosophila several groups studied what conditions make these insects live longer (Loeb and Northrop, 1917, Loeb, 1916, Pearl, 1921). Progressing to the late 1920s beginning of the 1930s, scientists were worried about the food shortage during the Great Depression, and two important studies opened the field of biogerontology: the first, a study in Drosophila found that alternate fasting extended the flies’ lifespan, the second, using laboratory rats, found that dietary restriction (DR) could also extend lifespan (McCay et al., 1935, Kopec, 1928, Alpatov, 1930). Several decades later, in the 1980s and 1990s, when mutagenesis techniques were
improved in *C. elegans*, it was shown that ageing was a malleable process and that mutations in the IIS [insulin/IGF (insulin-like growth factor)-like] pathway could extend the lifespan of this organism (Friedman and Johnson, 1988, Kenyon et al., 1993, Klass, 1983, Morris et al., 1996). These mechanisms behind ageing have later been found to be evolutionary conserved across many model organisms, particularly yeast, flies and mice (Bodkin et al., 2003, Colman et al., 2009, Miller et al., 2005, Grandison et al., 2009, Clancy et al., 2001, Tatar et al., 2001, Bluher et al., 2003, Holzenberger et al., 2003).

1.1.1.1. Relevance of anti-ageing interventions to societies

Thanks to medical developments and improvements in lifestyle and sanitation, human life expectancy has rapidly increased since the 19th century (Oeppen and Vaupel, 2002). This has meant an increment in the population proportion of elderly. In both less and more developed countries the number of people over 65 years old has been increasing over the last decades and it is forecasted to continue on the rise (Table 1.1) (United Nations, 2017). This increase in lifespan is obviously encouraging, but it has a worrying socioeconomic impact too. Ageing is the major risk factor for cancer, cardiovascular, respiratory, metabolic and neurodegenerative diseases, which are the leading causes of death in upper-middle income countries (Partridge, 2014, WHO, 2018). Therefore, it is crucial to tackle these diverse ageing-related conditions and diseases and, critically, it has been shown that it is possible to improve health span and prevent age-related disease in model organisms with anti-ageing interventions (Partridge, 2014, Castillo-Quan et al., 2015).

<table>
<thead>
<tr>
<th>Year</th>
<th>Age</th>
<th>1950</th>
<th>1980</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65+</td>
<td>75+</td>
<td>85+</td>
<td>65+</td>
</tr>
<tr>
<td>More developed countries (%)</td>
<td>7.7</td>
<td>2.4</td>
<td>11.7</td>
<td>4.3</td>
</tr>
<tr>
<td>Less developed countries (%)</td>
<td>3.8</td>
<td>1</td>
<td>4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year</th>
<th>Age</th>
<th>2020</th>
<th>2030</th>
<th>2050</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65+</td>
<td>75+</td>
<td>85+</td>
<td>65+</td>
</tr>
<tr>
<td>More developed countries (%)</td>
<td>19.4</td>
<td>8.7</td>
<td>2.5</td>
<td>22.9</td>
</tr>
<tr>
<td>Less developed countries (%)</td>
<td>7.4</td>
<td>2.5</td>
<td>0.5</td>
<td>9.7</td>
</tr>
</tbody>
</table>

1.1.2. Biology of ageing

Biology is a very broad science, it comprises from basic genetics to physiology or evolution; therefore, it is not surprising that biogerontology tries to answer a wide range of
questions about ageing. Two of the main questions that can be asked are, why do living organisms age and how does this happen? The first one will be reviewed here mainly under the ‘1.1.2.1. Evolutionary theories’ section; the second question will be discussed under the ‘1.1.2.2. Mechanistic theories’ and the ‘1.1.2.3. Molecular mechanisms behind ageing’ sections.

1.1.2.1. Evolutionary theories

One of the main questions that we have to ask about ageing if we want to understand why it happens is: is it an evolved trait or is it just inevitable? Zoology research answers this question quite clearly, ageing can be avoided, as there are organisms that do not senesce over time, such as *Hydra* or anemones (Martinez, 1998) (Fig 1.1). Nevertheless, although mortality rates in nature are mainly caused by external hazards or accidents, senescence has been recorded in many different wild animals (Nussey et al., 2013), so the question of why would senescence, such a deleterious traits, evolve in so many organisms arises logically. Thus, some of the main evolutionary theories of ageing will be discussed.
Figure 1.1. Mortality, fertility and survivorship in several organisms ranging from vertebrate to invertebrate, plants and algae. In some organisms such as modern humans or predators like lions, survivorship (grey line) and mortality (red line) are tightly linked. However, in other organisms such as red abalones or collared flycatchers, even though mortality is almost flat, extrinsic conditions affect survivorship and make it rapidly decline. Fertility (blue line) in most organisms remains fairly constant across the lifespan, but in other organisms, like humans or bdelloid rotifers, fertility peaks at younger ages to then decrease in adults. Figure from (Jones et al., 2014).
1.1.2.1.1. Ageing as an evolved trait

Quoting T. Dobzhansky “nothing in biology makes sense except in the light of evolution” so ageing also needs to be explained in the context of evolution (Dobzhansky, 1973). This is problematic to study because senescence occurs, in the cases that it does, in very different ways across taxa. Thus, a valid evolutionary theory of ageing should take into account these discrepancies. Here, some of the most famous and relevant theories will be explained.

1.1.2.1.2. Ageing to benefit the species

One of the earliest evolutionary theories argued that ageing has evolved as a trait that even though it is detrimental for the individual, it confers an advantage to the group (Wallace, 1889). This could be done through a removal of the individuals that have reproduced, so they no longer consume resources that can be used by the younger and fitter progeny (Wallace, 1889). Therefore, in order to successfully adapt to the environment, the prosperity of the species is more important than that of the individual; this means that it is advantageous to remove ‘worn out’ adults to have the majority of the group formed by healthy individuals (Weismann, 1889).

This theory is contradicted by the fact that there are organisms that do not exhibit reproductive senescence such as African elephants (Loxodonta africana), damselflies (Coenagrion puella) or the flowering plant Hypericum cumulicolae or other organisms that have prolonged post-reproductive lifespans in the wild, such as humans, Asian elephants (Elephas maximus), short-finned pilot whales (Globicephala macrorhynchus), resident killer whales (Orcinus orca) or the aphid Quadrartus yoshinomiya (Hassall et al., 2015, Croft et al., 2015, Ackermann et al., 2007, Jones et al., 2014). Moreover, this theory does not take into account that resources would become available for genetically distinct individuals that would not inherit the ageing-causing genetic information (Ackermann et al., 2007). Another major drawback of this theory is that it does not explain why aged animals would exist in the first place. Thus, ageing is not selectively favoured in nature as it is not advantageous neither for the group nor the individual, so it must have evolved as a side-effect.

1.1.2.1.3. Extrinsic hazards and natural selection

Death is part of what defines life, and even ageing-free organisms die from extrinsic hazards such as disease, predators and accidents. In wild populations, this is shown by survival curves, which are very dependent on the environment; for example, in humans survival curves have become rectangular since the 16th century as extrinsic hazards and diseases have lowered
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

(Kertzer and Laslett, 1995). Nevertheless, there are more individuals that survive only the early stages of their life and natural selection is higher at this time, especially coupled to the age of first breeding. An example of this force of natural selection is shown by the recurrence of Huntington’s disease; a genetic neurodegenerative disease caused by a dominant mutation which onsets at 35.5 years average. Due to the post-reproductive onset of this mutation, and that pre-modern humans had shorter lifespans (usually not reaching beyond their 40s), this mutation has not been selected against throughout evolution (Fig. 1.2) (Haldane, 1941).

Moreover, because natural selection acts on younger individuals that have reproductive potential, any trait that is beneficial only at an advanced age will not be selected, and in the same way, if a mutation is beneficial earlier in life but has a detrimental side-effect later, it will still be selected (Medawar, 1946). These concepts led to the formulation of the ‘Mutation accumulation’ theory of ageing by P. Medawar and the ‘Antagonistic Pleiotropy’ theory by G. C. Williams.

Figure 1.2. Diagram of the reasoning behind the theories supporting that ageing evolved as a consequence of the force of natural selection declining and mutations accumulating with age. Extrinsic hazards greatly affect the survival of a species in the wild, so there are more individuals at younger ages in any population. Natural selection will favour any mutation that has a beneficial effect before or during reproductive maturity, whereas mutations that have beneficial effects at more advanced ages will not be “seen” at early reproduction and hence will stay in a “selection shadow” and will not expand through population. In addition, if the naturally selected mutations have a detrimental side-effect later in life they will not be eliminated from the genetic pool (antagonistic pleiotropy).
1.1.2.1.4. Mutation accumulation theory

P. B. Medawar theorized that, if there are no natural selection forces acting in advanced ages, the mutation pressure in older individuals is also lower, so detrimental mutations can accumulate in the genome without being eliminated. Moreover, these negative effects could hide in a ‘selection shadow’ and would not be selected against, as they are not apparent until already passed on the progeny (Fig 1.3) (Medawar, 1946). There are plenty of studies that show that de novo mutations accumulate with age (Dolle et al., 2000, Lee et al., 1994, Milholland et al., 2015, Ono et al., 2000).

However, this hypothesis implies that spontaneous mutations will affect older individuals more than younger, or if not, they would be eliminated from the genetic pool over time. Nevertheless, it has been shown that new mutations impair survival and fecundity, that they affect younger and older individuals and that they are not correlated with mortality rates at older ages (Pletcher et al., 1998, Pletcher et al., 1999). Furthermore, mathematical models have concluded that although mutation accumulation can have an effect in the mortality shapes of extremely old individuals, it does not explain ageing in adults and neither its evolution across wild populations (Danko et al., 2012, Moorad and Promislow, 2009). Therefore, the mutation accumulation theory is likely to be important in organismal senescence, but it cannot fully explain why ageing has evolved.
Figure 1.3. Model of the modified mutation accumulation theory. In this theory survival depends on extrinsic conditions, so under higher extrinsic hazards (blue line), there is a rapid decline in the number of survivors over age in a population so mutations that have positive pleiotropic effects (i.e. they are deleterious at both young and old ages, but the phenotypes are more pronounced with age) are not selected against (red line). However, when there are low extrinsic hazards (green line) these detrimental mutations are removed from the population (yellow zone). This selection stops once reproduction ceases (dashed red line). In the species that exhibit prolonged reproductive senescence and they continue reproducing at older ages, this ‘removal by selection stage will be even longer (chequered-yellow zone).

1.1.2.1.5. Antagonistic pleiotropy theory

This theory was proposed by G. C. Williams in 1957 and it expanded on the previously explained mutation accumulation theory. Those mutations that are beneficial in the youth but have a pleiotropic (i.e. an apparently unrelated phenotypic trait to the original one) and detrimental effects in advanced ages will be incorporated in the genetic pool; therefore, ageing is just a side-effect of antagonistic pleiotropy (Fig 1.3.) (Williams, 1957). This occurs as a trade-off between current and future fitness. There is evidence that some traits that benefit the youth, especially if they confer an advantage in growth or reproduction, have negative effects at older ages (Everman and Morgan, 2018, Rodriguez et al., 2017, Sgro and Partridge, 1999, Luckinbill et al., 1984). For example, Luckinbill et al. (1984) did a classic inbreeding selection experiment in which they selected for longer-lived flies and they saw that this longevity effect was coupled with later ages of reproduction. However, in a more recent study,
Khazaeli and Curtsinger (2013), took these inbred longer-lived flies and showed that the seemingly ‘antagonistic pleiotropic’ effect disappeared when they produced recombinant ‘superflies’ that had both extended lifespans and early fecundity (Khazaeli and Curtsinger, 2013). In addition, a recent study also in Drosophila showed that the same alleles that conferred higher reproduction and fitness in young flies were the ones that improved lifespan at advanced ages (Kimber and Chippindale, 2013). This suggests that antagonistic pleiotropy most likely happens in nature leading to ageing phenotypes, but it is not likely to be the sole cause of the evolution of ageing.

1.1.2.1.6. The disposable soma theory

This theory of the evolution of ageing was coined by T. B. Kirkwood in 1977. It suggests that there is a difference between germ and somatic cells, which are in competition, so the individual has to allocate fewer resources to the maintenance of somatic tissues in order to keep the demanding process of reproduction active leading to a progressive damage of the somatic tissues (Kirkwood, 1977). This theory has been seen as an explanation of antagonistic pleiotropy in a physiological context; however, it assumes that there is a dynamic allocation of resources to maximize fitness across the whole lifespan (Reichard, 2017). The individual invests in the maintenance and repair of somatic cells as long as it confers a higher chance of survival in the wild, but always taking into account that a proportion of the metabolic resources will go to maintain reproduction/replication and the germline; this means that across chronological time, somatic cells can accumulate damage and other functional problems that cause ageing.

It is important to note that T. B. Kirkwood states in his theory that ageing only evolves in organisms that have a differentiated soma and germline (Kirkwood and Austad, 2000). Observations of different organisms have shown that, for example, Hydra that can swap from asexual reproduction to sexual reproduction but still do not age; similarly, two oligochaete species that reproduce by symmetrical fission do not age; however, other species (e.g. rotifers, ostracod or cladoceran crustacean) that reproduce asexually, but by producing eggs, do age (Bell, 1984, Martinez, 1998, Nishimiya-Fujisawa and Kobayashi, 2012). Furthermore, recent empirical observations have found that there is a trade-off between reproduction and lifespan (Lemaitre et al., 2015, Lemaitre and Gaillard, 2017, Tidiere et al., 2017, Boonekamp et al., 2014). Selection and genetic experiments, have shown that delaying or reducing reproduction in Drosophila extends the lifespan of these flies (Zwaan et al., 1995, Stearns et al., 2000, Sgro and Partridge, 1999, Luckinbill et al., 1984)
Nonetheless, reproduction and lifespan extensions can also be uncoupled, as it has been shown in several studies using worms and flies (Grandison et al., 2009, Partridge et al., 2005, Kimber and Chippindale, 2013, Kenyon et al., 1993). Moreover, the disposable soma theory cannot be applied to some organisms such as flowering plants that age but have germline cells that differentiate from somatic cells (Reichard, 2017). It would neither explain ageing in unicellular organisms, some reproduce by asymmetric replication, leaving the older cell to age and others, such as *E. coli* have apparent symmetric divisions but also age when they inherit the old pole in replication (Stewart et al., 2005).

### 1.1.2.1.7. Modified Mutation Accumulation (MMA) theory

If a mutation has a detrimental effect at young ages that is small enough not to be eliminated, it can progressively become stronger at older ages and eventually accumulate in the genome (Fig. 1.3.) (Maklakov et al., 2015). This theory accommodates the presence in nature of positive pleiotropic alleles (i.e. deleterious at both early and late-life, but with a stronger phenotype at later stages) and of the dynamics of survival that are condition-dependent or independent (Reichard, 2017). Under this theory, mutations that have a mild detrimental effect on youth accumulate during times of high extrinsic hazards, when mortality is higher and ageing more rapid. However, in times where the extrinsic hazards are lower, these mutations that conferred slightly lower fitness in youth are removed and ageing evolves to be slower (Maklakov et al., 2015). Moreover, these positive pleiotropic alleles might be maintained in the genome because of epistasis (i.e. the interaction between two alleles, one masking the other) in an age or condition-dependent context (Reichard, 2017, Houle et al., 1994).

It should be noted that positive pleiotropy does not always result in a deleterious phenotype, some beneficial phenotypes can also become stronger with age (Kimber and Chippindale, 2013).

### 1.1.2.2. Mechanistic theories

Biogerontologists also try to answer how do we age, and several theories have been proposed. Nonetheless, this does not come without a problem, since organisms show many differences in their senescence patterns. Some of these differences will be because ‘private’ mechanisms (i.e. characteristic of certain evolutionary lineages) that cannot be applied to global theories. Nonetheless, mounting evidence shows that there are ‘public’ (i.e. in common across remotely related evolutionary lineages) mechanisms behind ageing, especially those
involving evolutionary conserved molecular pathways (Partridge and Gems, 2002). Here, some of the main mechanistic theories with more scientific support will be discussed.

1.1.2.2.1. Wear and tear theory

This theory by A. Weismann (1882) supports that ageing occurs as a consequence of irremediable mechanical damage accumulation (Weismann, 1889). This damage can result from thermal denaturation, glycation or oxidation. Elevated temperatures denature proteins, and even though there are repair systems in place, if this damage is considerably greater than what the repair machinery can handle, or it directly affects heat-shock factors and chaperones, then these non-functional proteins can accumulate and lead to ageing. Although several studies have supported this detrimental effect of heat on longevity (Alpatov and Pearl, 1929, Loeb and Northrop, 1917, Miquel et al., 1976), heat cannot be held responsible for ageing, as there are many organisms that are never exposed to temperatures warm enough to cause denaturation and they still age.

Glycation, or non-enzymatic glycosylation, is the process by which a sugar molecule is covalently linked to a protein or lipid. The products of glycation can undergo further modifications producing what is termed as advanced glycation end-products (AGE), and these were thought to be damaging for the cell (Cerami, 1986). In older humans, elevated serum glucose has been detected, which could make glycation more readily available (Kalyani and Egan, 2013). However, more research has shown that AGE formation only affects certain proteins that have lower turnover rates, especially eye lens crystallins, elastin and collagen (Gkogkolou and Bohm, 2012, Luthra and Balasubramanian, 1993). Therefore AGE products have an effect in human ageing, AGE-related damage of these proteins accumulates in humans over time and it causes age-related health problems, especially in the skin (Gkogkolou and Bohm, 2012).

Furthermore, oxidative damage and its accumulation were the centre of attention in the ageing field a few decades ago, and much research focused on this topic (Gems and Doonan 2009). In fact, it gave rise to the ‘Oxidative damage theory’ which will be discussed in section 1.2.2.6.

In conclusion, even if the ‘wear and tear’ theory seems plausible and logical, it explicitly requires cellular repair systems not being able to work indefinitely in their required capacity, which is not the case in immortal cell lines, the already discussed Hydra or germline cells (Hayflick, 1977, Martinez, 1998, Weismann, 1893).
1.1.2.2. Somatic mutations theory

This theory was proposed by L. Szilard, a former nuclear physicist that switched to biology after working in the Manhattan project; in 1959, he proposed that ageing is caused by the accumulation of random mutations in somatic cells, which leads to eventual non-functional proteins (Szilard, 1959). As previously discussed, the disposable soma evolutionary theory of ageing explains that organisms invest energy repairing their somatic cells if it does not prevent maintenance of the germline; therefore, somatic mutations could accumulate more easily than in the germline mutations. In fact, germline mutation rates have been found to be significantly lower than somatic mutation rates (Lynch, 2010, Milholland et al., 2017a). This also makes sense from an evolutionary perspective, as mutations in the germline present a substantial risk for the fitness of the species (Kennedy et al., 2012).

Experiments using senescence-accelerated mice (SAM) mice, which are a progeroid model (i.e. progeria is a syndrome in which the individual has accelerated ageing and usually stunted lifespan) have shown that these have higher mutation frequencies, whereas long-lived Ames dwarf mice and mice under caloric restriction have decreased accumulation of mutations (Garcia et al., 2008, Odagiri et al., 1998). Moreover, human patients suffering from progeroid pathologies such as Werner or Cockayne syndromes also have defects in the DNA repair machinery (Kennedy et al., 2012). It should be noted that eukaryotic cells have also a mitochondrial genome that can be damaged. It has been suggested that mitochondrial DNA (mtDNA) mutations contribute to ageing and studies in both aged mice and human tissues have found increased levels of damaged mtDNA compared to samples from younger individuals (Brossas et al., 1994, Cao et al., 2001, Kraytsberg et al., 2006, Trifunovic et al., 2004).

Nevertheless, low doses of DNA-damaging radiation can also extend the lifespan of rodents and Drosophila, which is sometimes explained by hormesis, which is the theory explaining how low doses of a damaging agent, like radiation, promote activation of repair mechanisms that can protect against other cellular insults (Carlson et al., 1957, Sacher, 1963, Luckey, 2006).

Szilard already stated the limitation of his theory as it worked mainly for mammals but no other species; however, his theory was a great contribution to the ageing field because it regarded ageing as the result of the accumulation of cellular molecular damage. Therefore, even though there is mounting evidence that the accumulation of DNA damage and mutations do happen during chronological ageing, it has still not been proven if it directly causes ageing.
1.1.2.2.3. **The error catastrophe theory**

In 1963, following and expanding, on the somatic mutation theory, L. Orgel proposed 'The error catastrophe theory' of ageing. He suggested that errors in the protein synthesis apparatus could contribute to ageing (Orgel, 1963). A cell inherits the DNA from its mother, but also enzymes such as ribosomes; thus, if ribosomes are defective the cell’s fitness could be compromised. Moreover, cells could become more erroneous with time, either through the accumulation of DNA or RNA mutations or protein errors. If this happened, there are two possible scenarios: a) the defective protein is an enzyme taking part in any process but not in protein synthesis or b) the defective enzymes are part of the protein synthesis machinery (Fig. 1.4. A). Under the first scenario, the cell would be affected due to the impairment of the average turnover, specificity or control of said enzyme. In the case that the defectiveness came from a faulty mRNA, once this was degraded the problem would be solved. Under the second scenario, the ‘error catastrophe’ could occur; if the defective enzyme is, for example, a tRNA (transfer RNA) synthetase, and the error is in the active site of the enzyme, the proteins that tRNA is helping to translate would be affected and an accumulation of error frequency would occur (Fig. 1.4. B) (Orgel, 1963). One of the major draw-backs of this theory is that even if there is an error in an enzyme it might not affect its specificity or activity, and that there are plenty of other factors in the cell that could cope with translation non-erroneously.
Figure 1.4. Orgel’s error catastrophe theory. When there is a non-functional protein this can accumulate throughout chronological time and be passed to the progeny. A) When the non-functional protein takes part of any cellular process that is not protein synthesis, this protein will eventually be removed from the proteome as mRNA degrades so it will not be passed into daughter cells. B) If the protein forms part of the protein synthesis machinery, it will produce a cascade of erroneous proteins which will accumulate throughout chronological time and that can also be passed into daughter cells.

However, this theory was quite attractive, and there were plenty of experiments trying to test it. An error catastrophe was attempted in *D. suboscura* by giving the flies, p-fluoro phenylalanine, an amino acid analogue that could be incorrectly incorporated in polypeptide chains, but no shortening of lifespan was observed (Dingley and Smith, 1969). Later, *E. coli* was used in several experiments to test this theory; in two experiments bacteria were supplemented with the error-inducing antibiotic streptomycin, and the level of misincorporated cysteine was tracked, however, the mistranslation levels after a steep increase reached stability (Edelmann and Gallant, 1977, Gallant and Palmer, 1979). However, in other experiments, three with *E. coli* and one with *Neurospora*, cultures were supplemented with sub-lethal concentrations of streptomycin which resulted in translation errors and after a few generations, usually around 15, the cells reached an unstable point and died (Branscomb and Galas, 1975, Fast et al., 1987, Rosenberger, 1982, Lewis and Holliday, 1970).
In the 1970s and 1980s, a lot of effort was put into measuring the level of translation errors in young and old cells, either from ex vivo cultures or from animal samples. By utilizing one of the most commonly used techniques of that time, two-dimensional (2D) gel electrophoresis—proteins are separated by charge and size, so misincorporated amino acids result in ‘stuttering’—the levels of protein ‘stuttering’ were measured in samples from young and old C. elegans or Drosophila and no difference was found (Parker et al., 1981, Vanfleteren and De Vreese, 1994). The 2D electrophoresis footprints of early and late passages of human derived fibroblasts had no difference either (Harley et al., 1980). Nevertheless the heated debate was not over, in 1996, R. Holliday wrote an article explaining why the error catastrophe theory had still not been proven wrong (Holliday, 1996). He proposed that cells have proof-reading mechanisms to ensure optimal translation accuracy, but that these are energetically expensive, so the cell has to balance between the need for accuracy and the energy sources that can be invested in this accuracy system. Therefore, increasing translation fidelity beyond certain level does not confer an evolutionary advantage, but it was still not known how far this upper limit set by evolution is, and where aging cells stand. In addition, he criticized that all the experiments that had been done trying to measure the levels of translation fidelity were too artificial because a) they used amino acid analogues that can interfere with normal translation (for example, the histidine amino acid histidol, used in Harley et al. 1980 experiments, is also an inhibitor of protein synthesis), b) they had to purify the proteins through very complicated processes that could mask the effects of a real cell environment, c) sometimes they were not sensitive enough and had to use error-inducing antibiotics, d) they were based on enzymes that were heat-labile or d) they used virus-derived RNA that could be translated differently in higher eukaryotes.

This was answered in another set of letters by J. Gallant, C. Kurland and J. Parker (Gallant et al., 1997). They proposed that the fact that increasing errors led to shorter lifespans could just be a correlation and that cells under normal physiological conditions had stable levels of errors that did not lead to any positive feedback or catastrophe. In fact, Galland and Kurland proposed that errors in translation are not problematic when they form part of ribosomal proteins but if the abrogated proteins that form part of membranes, because these could prompt changes in the intracellular ionic conditions, leading to further mistranslation and therefore an even more damaged cell. R. Rosenberg, also took part of the debate and argued that the 2D gel electrophoresis were not sensitive enough, as they only detect amino acid substitutions that lead to charge changes, which only account for 32% of all substitutions (Rosenberger, 1997). In addition, he proposed that a very small number of mistranslated
proteins are the ones that would account for a positive error feedback, namely ribosomal proteins in the decoding centre, ribosomal RNA (rRNA) polymerases, etc. so a low level of detected translational errors could still be detrimental. Moreover, R. Rosenberg also argued in his letter that *E. coli* might be able to sustain higher error frequencies than more complex organisms. After all, they agreed that a loss of different homeostasis pathways could lead to less accurate translation and that the term “error catastrophe” was maybe too overdramatic, as the experiments of the time did not show this was happening in any of the organisms tested.

Nowadays, one of the hallmarks of ageing is loss of proteostasis, and recent studies with more powerful techniques, such as dual luciferase assays, have shown that translation accuracy can be linked to ageing. Experiments in *S. cerevisiae* have shown that errors in both transcription and translation can shorten yeast cellular lifespan (Vermulst et al., 2015, von der Haar et al., 2017, Schosserer et al., 2015). Furthermore, a mutation in a tRNA synthetase that increased the level of translation errors also led to shortened lifespan in *Drosophila* (Lu et al., 2014). Studies in rodents have shown that maximum lifespan has co-evolved with translation fidelity and that the longest-lived rodent, the naked mole rat has shown to have the most accurate translation compared to the rest of rodents tested (Azpurua et al., 2013, Ke et al., 2017). Mutations in polymerase I (Poll, the polymerase (I) that transcribes rRNA) that affect translation fidelity have been found to cause the progeroid Cockayne syndrome too (Alupei et al., 2018).

It is also interesting how ‘The somatic mutation catastrophe theory’ has recently proposed merging ideas from both Szilard and Orgel’s theories (Milholland et al., 2017b). Under this theory, mutations that arise from DNA replication, DNA damage or defective repair systems will accumulate during the time in the soma resulting in dysregulation of gene expression and altered protein synthesis eventually leading to senescence.

**1.1.2.2.4. Replicative theory of ageing**

In 1961, Hayflick and Moorehead published a breakthrough but controversial finding, cultured fibroblasts eventually lose their replicative capacity after a certain number of passages (Hayflick and Moorhead, 1961). This phenomenon is called the ‘Hayflick limit’ and it is produced by what is known as replicative or cellular senescence. One of the molecular mechanisms causing replicative senescence is believed to be telomere shortening. In DNA replication, it is impossible to replicate the end of the lagging strand, so most organisms in order to protect the end of chromosomes have evolved telomeres, which are repetitive
sequences of DNA (TTAGGG)<sub>n</sub> (Harley et al., 1990). Importantly, telomeres have been found to shorten with each division providing a ‘molecular clock’ that can limit replicative lifespan (Harley et al., 1990). Nevertheless, the enzyme telomerase can synthesize (TTAGGG)<sub>n</sub> at the end of chromosomes and it can be found in cells from many organisms, for example in the immortal Tetrahymena, but also in human embryonic cells, specific germ line cells, or proliferative cells of renewal tissues (Greider and Blackburn, 1985, Liu et al., 2007, Shay and Wright, 2005). In human somatic proliferative cells, the levels of telomerase are so low that they do not prevent senescence. In fact, when telomerase is expressed in normal human somatic cells replicative senescence is prevented and immortality achieved (Bodnar et al., 1998). So, if telomere shortening can be avoided, why do only some cells express telomerase? Telomere-based replicative senescence has been proposed to have evolved to protect against tumour formation in eutherians (i.e. placental mammals) (Gomes et al., 2010). This evolutionary branch may have evolved replicative senescence by repressing the expression of telomerase in the soma in order to avoid tumour formation, which is enhanced by the increase in body temperature (Gomes et al., 2010). In fact, invertebrate and some vertebrate such as fish, amphibians and reptiles that have lower body temperatures and lower tumour incidence also have active telomerase that results in higher regenerative potential.

Another question to answer is how replicative senescence affects organismal ageing. Although in the short term, replicative senescence is cancer protective as it stops cells with mutations from proliferating, in the long run, accumulation of senescent cells has been found to, promote cancer formation by secreting chemokines and growth factors, called senescence-associated secretory phenotype (SASP) cells that alter the cellular environment (Krtolica et al., 2001, Campisi, 2013). Nevertheless, senescent cells also contribute to wound healing and SASP are a chemical cue for immune clearance (Munoz-Espin and Serrano, 2014). Thus, this could be seen as an example of antagonistic pleiotropy: replicative senescence was evolved to prevent tumour formation in younger individuals, but in the older, the accumulation of these cells can lead to more tumours. In conclusion, their role in ageing is complicated and more studies are needed to reach any conclusion.

1.1.2.2.5. Rate of living (ROL) theory

As early as 1889, A. Weismann explained that animals with extremely active metabolisms have shorter lifespans than slower-living ones and that this is also affected by the rates of personal and reproductive expenditure (Weismann, 1889). However, he also concluded that this did not explain the variability of lifespans and that there was not a
constant correlation (Mitchell, 1911). Nonetheless, four decades later in 1928 R. Pearl took some of these notions applied them to contemporary experiments and proposed the ‘Rate of living theory’ of ageing (Pearl, 1928). Based on his research on the effect of diet, population density and temperature on the lifespan of Drosophila he suggested that duration of life is inversely related to the rate of energy expenditure, and hence rate of growth is negatively correlated to lifespan too. Pearl also coined the term inherent vitality to explain the total capacity of an organism to carry out vital activities; this concept was later explored by H. Atlan and colleagues also in Drosophila; they saw that flies reared at temperatures from 18°C to 30°C had reduced lifespans as temperature increased but very similar lifetime oxygen consumption, ergo, at lower temperatures the flies lived ‘slower’ and used their life energy potential (LEP) for longer (Atlan et al., 1976, Miquel et al., 1976). Later research has had controversial results, as correlating body sizes and temperatures to maximum lifespan only works in certain organisms, such as most mammals, but not marsupials and bats nor birds (Austad, 2005).

1.1.2.6. The free radical/oxidative damage theory of ageing

Still today, this is one of the most influential and controversial theories of ageing. In 1956, D. Harman stated that ageing was caused by free radicals damaging the cell’s molecular content (Harman, 1956). Free radicals are species containing an unpaired electron and they are usually highly reactive, especially with DNA, lipids and proteins (Harman, 1956, Beckman and Ames, 1998). This theory was later expanded and termed ‘Oxidative damage theory’ as other metabolic by-products such as reactive oxygen species (ROS) and reactive nitrogen species (NOS) also produce molecular damage in an analogous way to free radicals. ROS, NOS and free radicals occur naturally in cells as a consequence of metabolic or Fenton reactions and the levels of oxidized/damaged proteins, lipids and DNA have been shown to accumulate with age (Beckman and Ames, 1998, DeGroot, 2004). Therefore, this theory is tightly linked to the previously discussed ‘Rate of living’ and ‘Wear and tear’ theories of ageing, as higher metabolic rates could link to increased levels of ROS/NOS-resultant molecular damage.

Nevertheless, cells have a very complex antioxidant defence system, comprised of several non-enzymatic and enzymatic antioxidants. An important enzymatic antioxidant is superoxide dismutase (SOD). Overexpressing SOD in Drosophila extended the life of these flies (Parkes et al., 1998, Sun et al., 2002, Sun and Tower, 1999); however, overexpression of SOD or catalase (an H₂O₂ clearing enzyme) in mice did not affect lifespan (Huang et al., 2000, Perez et al., 2009b). The superoxide radical O₂⁻ is a by-product of mitochondrial respiration and it has been tightly linked to ageing (Beckman and Ames, 1998, Sohal and Weindruch, 1996). In mice
overexpressing mitochondrial-targeted catalase can extend lifespan (Schriner et al., 2005). Experiments with *C. elegans* have been contradictory, supplementing worms with non-enzymatic antioxidants such as vitamin E or troxol extended lifespan in some studies (Benedetti et al., 2008, Harrington and Harley, 1988, Ishii et al., 2004) but not others (Adachi and Ishii, 2000, Schulz et al., 2007). Similarly, SOD mimetic experiments have shown that increasing SOD activity can extend worm lifespan (Melov et al., 2000) but not in other studies (Keaney and Gems, 2003, Keaney et al., 2004, Kim et al., 2008, Van Raamsdonk and Hekimi, 2012). One of the explanations of these contradictions could be due to dosage differences. Another explanation is that the longevity effects could be a result of stress-response induction rather than lowering of ROS/free radicals because when lifespan was extended, the heat shock resistance also improved (Gems and Doonan, 2009). Moreover, loss of certain cytosolic SOD isoforms in *C. elegans* can slightly shorten lifespan, whereas overexpression of some isoforms can extend it, but altering mitochondrial SOD does not affect longevity (Chavez et al., 2007, Doonan et al., 2008, Honda et al., 2008, Van Raamsdonk and Hekimi, 2009, Yang et al., 2007, Yen et al., 2009).

It is important to note that ROS such as O$_2^-$ and H$_2$O$_2$ are also signalling messengers (Gems and Doonan, 2009). In fact, administration of antioxidants to *C. elegans* or *E. coli* can shorten their lifespan in a dose-dependent fashion (Keaney and Gems, 2003, Keaney et al., 2004, Kim et al., 2008, Mattheyssens et al., 2008). Veritably, the lifespan extension seen in some of the studies might be due to changes in other signalling pathways, since most of these studies have not been able to directly measure cellular O$_2^-$ and H$_2$O$_2$ (Gems and Doonan, 2009).

To conclude, the oxidative damage theory might hold some truth, as free radicals, ROS and NOS can both directly damage other biomolecules when the antioxidant systems fail, and they can also alter longevity-related signalling pathways, but there is not enough evidence to consider it the sole cause of ageing. To test the levels of these agents has proven challenging; especially there are some ROS which are difficult to accurately measure in vivo. In addition, sometimes antioxidants are not targeted to the site of ROS production neither they are potent enough to decrease production of all ROS generated. Furthermore, some ROS act as signalling molecules that are important for organismal functions (Gough and Cotter, 2011).

1.1.2.2.7. The green theory of ageing

Another theory of ageing encompassing some of the notions of the oxidative-damage theory is the ‘Green theory of ageing’ by D. Gems and J. McElwee (2005). They argued that cells produce metabolic by-products and xenobiotics that can gradually accumulate and cause
ageing. Using an analogy with human industry, ‘green’ environmentalists recommend that there is more investment in clean waste disposal, reduced pollution and better recycling; however, since this is expensive, it does not always happen and waste gathers, thereby damaging the environment (Gems and McElwee, 2005). In a similar way, the cell has a ‘rubbish’ coping systems, especially the smooth ER that moves and excretes toxins preventing molecular damage, but they are energetically expensive (Gems and McElwee, 2005). Notably, IIS long-lived C. elegans or rats and flies under dietary restriction have improved detoxification and chaperonin systems and reduced amounts of damaged compounds (Gems and McElwee, 2005, Iqbal et al., 2009, Wen et al., 2013).

1.1.2.2.8. Quasi-programmed and hyper-functional ageing theory.

This more recent theory of ageing is tightly linked to the ‘Antagonistic pleiotropy evolutionary’ theory and the ‘Green mechanistic’ theory. In 2006, M. Blagosklonny proposed that ageing and ageing-related disease are a consequence of hyper-functional and damaging development (Blagosklonny, 2006). He explained this with a simple analogy: if to warm a glass of water you put a pot of water to boil in the stove (the program) and once your water is warm, you leave the pot with the rest of the water and the stove on, the pot will eventually be damaged (the quasi-program). The program, to boil water, did not include damaging the pot, but consequently it was indeed damaged, the quasi-program. This would be avoidable by switching off the stove, but it would require investing extra energy.

This means that the development process has not evolved to be switched off once it is no longer required since most of the population dies of accidental/external causes before the effects of hyper-function are shown (Fig. 1.5. A). Therefore, ageing can be regarded as a quasi-program of the developmental program. Supporting this theory, nutrient sensing pathways that are related to growth such as TOR and IIS when reduced, extend lifespan (Fig. 1.5. B) (Kenyon, 2010b, Gems and Partridge, 2013b, Castillo-Quan et al., 2015). Moreover, age-related pathologies such as neurodegeneration, cancer, inflammation or hypertension can be explained as hyper-functional consequences of metabolism and growth; thus, studying not only lifespan but age-related pathologies can be a better way to test of this hypothesis (Wang et al., 2018).
Figure 1.5. Ageing as a quasi-program of development. A) Once development is completed and there is no natural selection pressure (t₁: external death), development-related pathways continue as a hyper-function, leading to ageing-related diseases and damage that eventually lead to death (t₂: maximum lifespan). B) The mTOR pathway is a pro-ageing pathway that leads to ‘hyper-function’ but when it is switched off it can extend lifespan. Figure from (Blagosklonny, 2006, Blagosklonny, 2008).
1.2.3. Model organisms used in ageing research

To understand how and why ageing happens, plenty of model organisms have been used and new ones keep being proposed. The traditional or standard model organisms are *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* and *Mus Musculus* (Cohen, 2018). These have shed very important insights about ageing, such as the fact that ageing is a malleable process and also that there are several evolutionary conserved signalling pathways involved in the ageing of very distant organisms (Gems and Partridge, 2013b). These four model organisms have several common characteristics that have led to their frequent use in laboratories: they have been carefully genetically studied, there are plenty of techniques and reagents readily available, the ortholog and homolog genes with humans are well known and their lifespans are relatively short (Partridge and Gems, 2002).

The yeast *S. cerevisiae*, also known as Brewer’s, Baker’s or budding yeast, is a unicellular organism mitotically active that undergoes both replicative and chronological lifespan. Replicative lifespan (RL) is defined as the number of daughters a mother cell can give before it becomes senescent and it lasts around 3 weeks (Mortimer and Johnston, 1959). It has a rapid doubling time and it has been shown that there are conserved genetic modulators for RL in yeast and the higher eukaryotic model organisms (Spivey and Finkelstein, 2014). Chronological lifespan is measured by the time a non-dividing cell lives and it usually lasts around 10 days in wild types (Longo and Fabrizio, 2012). Usually this is achieved by growing a yeast culture in a postdiauxic phase (i.e. the period after inoculation in which the extracellular glucose gets depleted and the cells switch from mitochondrial respiration to fermentation) until the cells exit the cell cycle (Longo and Fabrizio, 2012, Longo et al., 1996). Both assays are important and although they are controlled by different mechanisms, many of them, for example reduction of mTOR and IIS, reduced translation or enhanced autophagy, have been shown to be relevant for the rest of model organisms (Longo and Fabrizio, 2012).

*C. elegans* is a free-living nematode that lives in the soil of temperate environments feeding on several types of bacteria (Felix and Braendle, 2010). They live around three weeks in laboratories, they are very easy to culture and to manipulate genetically, especially by RNA interference (RNAi) so they are a very popular organism used in biogerontology (Conte et al., 2015). To study their lifespan, age-synchronized adults are grown on bacterial agar lawns and every few days they are tapped to score their motility (Spivey and Finkelstein, 2014). Remarkably, the first genes involved in ageing (i.e. *age-1, daf-2*) were discovered in this organism (Friedman and Johnson, 1988, Morris et al., 1996, Kenyon et al., 1993, Klass, 1983).
D. melanogaster, also known fruit or vinegar fly, is a dipteran insect that was first described in 1830 by J. Meigen and they have been a crucial instrument in the study of genetics but also in ageing studies (Fig. 1.6.) (Meigen and Hoffmannsegg, 1830, Piper and Partridge, 2018). Since 1921, R. Pearl and S. Parker, worked with Drosophila and they found several environmental requirements for the flies’ survival, including effects of anesthetics, inbreeding, population densities and temperature changes (Pearl, 1921). Since then, fruit flies have become a very useful model organisms in biogerontology due to their inexpensive housing requirements, small size and relatively short lifespan (approximately 70 days median and 90 days maximum when reared at 25°C), which makes it possible to generate and study large populations (Ziehm et al., 2013, Ziehm and Thornton, 2013). In addition, fruit flies display ageing phenotypes in metabolism (e.g. reduced resting rate, decreased protein and fat synthesis), in behaviour (e.g. reduced feeding, courtship and exploration and a decline of negative geotaxis, voluntary walking and flying), in fertility (reduced spermatogenesis and oogenesis) and impaired learning and memory (Gargano et al., 2005, Grotewiel et al., 2005, Iliadi et al., 2012, Tamura et al., 2003); this makes possible the study of health span apart from lifespan. Furthermore, fly tissues are equivalent to many found in mammals so ageing can be studied at the tissue-level too (Piper and Partridge, 2018).
Figure 1.6. Illustration of the different eye colour phenotypes associated to mutations in the third chromosome. This illustration published in 1923 in an example of how *Drosophila melanogaster* has been used in biology to study the effects of different mutations and their phenotypes since an early age. Illustration by E. M. Wallace Pink and published in (Bridges et al., 1923).

*M. musculus*, or house mice, live around three years in laboratories, so they are used for having one of the shortest lifespans within rodents and because of their similarities to humans at the gene level (Miller et al., 2002). Cancer is the major cause of death in these mice, which makes them a good model for cancer biology too (Harper et al., 2006). There are several long lived mouse models such as the Snell dwarf, GHR (growth hormone receptor) knockout mice and the longer-lived mice under caloric restriction or treated with rapamycin (Briga and Verhulst, 2015). However, one of the major drawbacks of mice is that they come from very homogeneous backgrounds due to inbreeding (Briga and Verhulst, 2015).

Importantly, traditional model organisms are kept at standardized laboratory environments (SLEs), such as a constant climate, lack of pathogens, sometimes less
opportunities to reproduce than in the wild and ad libitum food. These SLEs reduce the level of external variability and they allow the animals to reach their maximum lifespan potentials; however, the effects seen in longevity might not relate to the wild (Briga and Verhulst, 2015). Another implication of keeping model organisms under SLEs, is that the mechanisms that extend lifespan under SLEs are probably not the same that can explain the high variation of lifespans across taxa —there is a 150,000 fold difference between the shortest and the longest living organism (Austad, 2010).

For this reason, other non-standard model organisms are increasingly studied to understand ageing. One example of these models is Arctica islandica, the longest-lived non-colonial animal, a cold water clam known to have reached 507 years and that has recently shown to have exceptional proteome stability and increased stress resistance (Gruber et al., 2015, Munro and Blier, 2012, Treaster et al., 2014). Heterocephalus glaber, commonly known as the naked mole rat (NMR), occurs naturally in hot arid regions of Africa, lives subterranean and notably is the longest-lived rodent (Ma and Gladyshev, 2017). NMR do not show increased age-associated mortality risk and they maintain long health spans for approx. 85% of their maximum lifespan (Lagunas-Rangel and Chavez-Valencia, 2017). Furthermore, NMRs are the only eusocial mammal, but unlike eusocial insects, in which the queens have much longer lifespans than the workers, NMRs queens and workers live more than 30 years (Austad, 2010). They are also an interesting model because although they have high levels oxidative damage, they do not display cancer; in addition, they have improved proteasome activity, protein stability and translation fidelity (Andziak et al., 2006, Azpurua et al., 2013, Perez et al., 2009a).

Other interesting types of model organism are those considered to be immortal. The most well-known is the Hydra, a member of the Cnidaria phylum, a fresh water polyp (Martinez, 1998). It does not present senescence, neither a rise in mortality nor a decrease in budding reproduction with age. One of the possible reasons behind this apparent immortality is its high and continuous rates of division to select against damaged cells (Danko et al., 2015). Other examples of this type of model organisms are the Planaria, such as Schmidtea mediterranea. These are free-living nonparasitic invertebrates with one of the simplest body plans (Lagunas-Rangel and Chavez-Valencia, 2017). They are interesting for biogerontology because of their remarkable regenerative capacity with tissues being renewed constantly leading to a technical immortality too (Cohen, 2018).

Finally, it is also important to study organisms with uniquely short-lifespans, and one that has come up as a promising model for ageing is the African turquoise killifish
(Nothobranchius furzeri) (Valenzano et al., 2015). These fish live in African ponds that are only present for 4 to 6 months, hence they have adapted to survive in diapause in the dry season; however, in captivity they live 4 to 6 months (Reichard et al., 2015). They have been recently used to study the genomic variances between strains with different lifespans too (Valenzano et al., 2015).

Therefore, it is important to study both standard and non-standard model organisms in ageing to elucidate the private and public mechanisms that govern longevity across taxa (Austad 2010 from cell paper killifish). In addition, lifespan might be just one phenotype of ageing, and thus it is essential to also monitor health span in any longer-lived organisms—either with naturally long lifespans or treated with anti-ageing interventions—, as this might be one of the main clues to help in tackling age-associated diseases in humans (Briga and Verhulst, 2015).

1.1.3. Molecular mechanisms behind ageing

One of the milestones of ageing research was the finding that this process is malleable (Klass, 1983, Kenyon et al., 1993, Friedman and Johnson, 1988). Since then, several signalling pathways and molecular mechanisms have been shown to affect lifespan and postpone age-related diseases. Most of these longevity-related genes take part in nutrient sensing pathways or stress responses. This means that when conditions are optimal (there is enough food and low-stress levels) these genes act to promote the organism to grow, develop and reproduce. On the contrary, when there are environmental stresses and harder external conditions, the organism experiences a physiological change that promotes cell maintenance and protection, guarding the organism and extending its lifespan. Here, the most relevant mechanisms and pathways involved in ageing will be discussed.

1.1.3.1. Insulin/IGF (insulin-like growth factor)-like signalling (IIS)

The ageing field was revolutionized when it was found that single mutations could lead to lifespan extension in C. elegans. In 1983, M. Klass found a mutation that increased lifespan of the roundworm but he concluded that this lifespan extension was due to impaired feeding and hence the already known caloric restriction (Klass, 1983); nevertheless, further characterization of the mutated gene led to the identification of age-1, the worm homologue of PI3K (phospho-inositol 3 kinase) (Friedman and Johnson, 1988). Later in 1993, C. Kenyon opened the ageing research field by investigating in more detail lifespan-extending mutations, and she found that daf-2 mutants, the homologue of the IGF-1 receptor, had doubled lifespan
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

without losing reproductive capacity and with improved health compared to controls (Kenyon et al., 1993). All these mutants have reduced IIS signaling, and interestingly this effect is evolutionary conserved, as reducing this pathway has been shown to improve lifespan and healthspan in Drosophila and mice (Tatar et al., 2001, Clancy et al., 2001, Holzenberger et al., 2003, Selman et al., 2008, Bluher et al., 2003, Broughton et al., 2005, Grönke et al., 2010).

The IIS pathway is a nutrient sensing network that has evolved in multicellular organisms to coordinate nutritional cues with growth, metabolism and reproduction (Gems and Partridge, 2013a). IIS is activated by insulin-like peptides (ILPS) that are secreted in response to food or its perception and acts through a kinase signalling cascaded through PI3K/Akt (protein kinase B) (Mathew et al., 2017) (Fig. 1.7.). Activation of these protein kinases leads to phosphorylation and hence sequestration in the cytoplasm of the transcription factor forkhead box O (FOXO) (DAF-16 in C. elegans). In the non-phosphorylated and active form, FOXO proteins bind to a conserved DNA binding domain, the forkhead box (FOX) found in genes that promote stress resistance, damage repair and cell cycle arrest (Hansen et al., 2007). Therefore, activation of FOXO is a signal that promotes cell survival when nutrient levels are low, delivering a metabolic shift from glucose to lipid oxidation, suppressing inflammation, enhancing mitochondrial biogenesis and autophagy (van Heemst, 2010). Importantly, FOXO can act non-autonomously and regulate insulin gene expression in other tissues. In worms, expressing daf-16 in one tissue (intestinal or adipose) increases the activity of DAF-16 in other tissues, especially in heads (Murphy et al., 2007, Kappeler et al., 2008).

In humans, IIS also plays a role in longevity. Studies of very long-lived populations such as Ashkenazi Jews and Japanese, or centenarians across many countries, have shown that these have special variants of Akt or FOXO3A (Anselmi et al., 2009, Flachsbart et al., 2009, Kojima et al., 2004, Li et al., 2009, Pawlikowska et al., 2009, Suh et al., 2008, Willcox et al., 2008, Lunetta et al., 2007, Morris, 2018). In addition, FOXO activation has been linked to prevention of age-related diseases such as cancer, neurodegeneration and diabetes in model organisms and humans (Demontis and Perrimon, 2010, Soerensen et al., 2015, Sun et al., 2015, Zeng et al., 2016).

Apart from FOXO, IIS interacts with members of other mechanisms and pathways. The interaction of IIS and mechanistic/mammalian target of rapamycin (mTOR) is quite remarkable and it can be considered a nutrient-sensing network (Fig. 1.7.) (Bjedov and Partridge, 2011). mTORC2 phosphorylates Akt, this activates it and hence it inhibits proline-rich Akt substrate of 40 kDa (PRAS40) and tuberous sclerosis protein 2 (TSC2), leading to upregulation of mTOR.
signalling (Bjedov and Partridge, 2011). mTORC1, however, inhibits IIS by the insulin receptor substrate (IRS) via ribosomal p70 S6 kinase (SK6) mediated negative feedback. Additionally, IIS can also alter epigenetic marks that are associated with ageing; which will be further discussed under the ‘1.1.3.7. Epigenetics section’.

Figure 1.7. Interactions between the mTOR and IIS pathways and stress-sensing mechanisms. The IIS pathway (blue) responds to nutrients and growth factors sequestering FOXO in the cytoplasm and preventing the transcription of stress-related genes, but also inhibiting mTORC1 and the TSC complex. mTOR responds to high amino acid levels leading to protein synthesis, ribosomal biogenesis, transcription and metabolism, and inhibit autophagy. Interestingly, mTOR inhibits the IIS pathway through the insulin receptor substrate (IRS) and it activates Akt leading to further up-regulation of mTOR itself. Low levels of oxygen or energy and oxidative and genotoxic stress also regulate mTOR/IIS through the TSC complex. Figure from (Bjedov and Partridge, 2011).
1.1.3.2. Dietary restriction

Dietary restriction (DR) is a reduction of nutrients, either partial or total, that does not lead to malnutrition; some examples include caloric restriction (CR) (reduction of the total level of nutrients provided), intermittent fasting (periodic reduction of nutrients), and the elimination or reduction of proteins, lipids or carbohydrates. DR is one of the best-known lifespan-extending manipulations, and it has been shown to affect from yeast to primates (Bodkin et al., 2003, Colman et al., 2009, Mattison et al., 2012). For long, DR was thought to extend lifespan by reducing the level of by-products formed in metabolism; however, now it has been shown that DR affects IIS, mTOR, 5′ adenosine monophosphate-activated protein kinase (AMPK), sirtuins, histone modifications and DNA methylation levels (Fig. 1.8) (Gems and Partridge, 2013a, Mathew et al., 2017). Long-lived IIS mutants in C. elegans, Drosophila and mice respond to DR as if the two pathways were overlapping to some extent (Arum et al., 2009, Honjoh et al., 2009, Clancy et al., 2002, Grandison et al., 2009).

![Figure 1.8: Mechanisms behind lifespan-extension by dietary restriction](image)

DR mainly delays the onset of age-related diseases and improves longevity by downregulating the nutrient sensing pathways IIS and mTOR (thick arrows) but also some more recent studies have shown how DR can alter epigenetic marks (thinner arrows). DR acts through mTOR and IIS to upregulate autophagy, downregulate translation and increase FOXO has been shown to be evolutionarily conserved from yeast to mammals. The effect of DR on SIRT1 levels and the relationship between SIRT1 and longevity are still controversial (dashed arrow) Figure based on (Kenyon, 2010b) (Mathew et al., 2017).

1.1.3.2. Mechanistic/mammalian target-of-Rapamycin (mTOR) signaling

The mTOR pathway senses when nutrients and amino-acids levels are copious, so it can stimulate growth. This pathway is tightly linked to ageing, its downregulation by genetic
and pharmacological manipulation has been shown to extend lifespan in an evolutionary conserved way, from yeast to mammals (Johnson et al., 2013, Kapahi et al., 2004, Lam et al., 2013, Laplante and Sabatini, 2012, Pyo et al., 2013, Selman et al., 2009, Simonsen et al., 2008).

mTOR is formed of two complexes, mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin, and it phosphorylates and activates p70-S6 kinase (S6K) and it also phosphorylates but inhibits eukaryotic initiation factor 4E-binding protein (4E BP) (Fenton and Gout, 2011, Wolfson and Sabatini, 2017, Saxton and Sabatini, 2017). mTOR (specifically through mTORC1) is the master regulator of translation, so when nutrient levels are high, translation is high too and conversely, when nutrients are low, translation is shut down. By inhibitory phosphorylation of 4EBP, this protein can no longer sequester eIF4E, which is then free to form the eIF4F complex and take part in translation initiation. Activation of S6K allows it to phosphorylate eIF4B and possibly eIF4A enhancing translation initiation too (Wolfson and Sabatini, 2017). On the contrary, mTORC2 is insensitive to rapamycin in acute doses and it phosphorylates several proteins of the AGC (PKA/PKG/PKC) family involved in proliferation and cell survival (Saxton and Sabatini, 2017). Some members of the PKC family are known substrates of mTORC2 and are involved in cytoskeleton remodelling (Saxton and Sabatini, 2017). Importantly, it can also phosphorylate and activate Akt, a significant effector of the IIS pathway (Sarbassov et al., 2005).

mTOR also inhibits autophagy when nutrients are plentiful by phosphorylating and inactivating Unc-51 like autophagy activating kinase (ULK) (this prevents its binding with AMPK, which will be discussed later) and by phosphorylating and sequestering the transcription factor EB (TFEB), which regulates the expression of lysosomal biogenesis and autophagy genes (Saxton and Sabatini, 2017). This process itself has also been linked to longevity; activating autophagy can extend lifespan of worms, *Drosophila* and mice (Pyo et al., 2013, Simonsen et al., 2008, Ulgherait et al., 2014, Hansen et al., 2008, Bjedov et al., 2010, Toth et al., 2008)

As already mentioned, DR is closely linked to mTOR (Fig. 1.8). DR cannot further increase the lifespan of long-lived mTOR-inhibited organisms, and the physiological effects of both manipulations are analogous (Hansen et al., 2007, Kaeberlein et al., 2005, Kapahi et al., 2004, Bjedov et al., 2010). In fact, the gene expression patterns of mice with life-extending mutations in S6K or under DR are actually comparable (Selman et al., 2009).

Importantly, decreasing mTOR not only extends lifespan but it can also improve healthspan. In female mice, knockout of S6K improves age-related locomotor senescence, loss
of bone mass and metabolism (Selman et al., 2009). In addition, giving elderly humans a rapalog (rapamycin analogue) has been shown to improve their immune response to vaccination system (Mannick et al., 2014)

1.1.3.3. AMP kinase

Another nutrient and energy sensor associated with ageing is AMPK. AMPK can detect low AMP/ATP ratio levels to activate catabolism and inactivate anabolism (Kenyon, 2010b). AMPK inhibits protein synthesis and promotes autophagy through inhibition of mTORC1 and downregulation of pathways involved in rRNA biosynthesis (Templeman and Murphy, 2018). In C. elegans, reducing the activity of AAK-2 (the worm homologue of AMPK) by mutating its catalytic subunits reduces lifespan, whereas overexpression of AAK-2 or its truncated versions extends it (Apfeld et al., 2004, Greer et al., 2007, Mair et al., 2011). In Drosophila, RNAi-mediated knockdown of the catalytic subunit of AMPK can reduce lifespan in a tissue-specific manner (Tohyama and Yamaguchi, 2010). Overexpression of AMPK can extend flies and worms lifespan (Ulgherait et al., 2014). AMPK activation decreases with age in rat and mice tissues; nonetheless, the effect of directly modifying AMPK has not been tested in mammals yet (Templeman and Murphy, 2018).

1.1.3.4. Sirtuins

Sirtuins are a family of NAD⁺ (nicotinamide adenine dinucleotide) - dependent protein deacetylases that have been reported to affect lifespan in model organisms. In yeast, C. elegans and Drosophila overexpressing Sirtuin 1 (SIRT1) (silent mating type information regulation 2 homolog 1) can extend lifespan (Schmeisser et al., 2013, Kaeberlein et al., 1999, Whitaker et al., 2013, Tissenbaum and Guarente, 2001, Rogina and Helfand, 2004). Nevertheless, this lifespan-extending effect has been contested in another experiment, when C. elegans and Drosophila were extensively backcrossed, the longevity effect of SIRT1 was suppressed (Burnett et al., 2011). In mice, ubiquitous overexpression of SIRT1 does not extend lifespan but overexpression restricted to neurons does (Satoh et al., 2013). Overexpressing SIRT2 can extend the lifespan of mice too (Kanfi et al., 2012).

Resveratrol is a plant stress-response biomolecule, which was found to be an activator of SIRT1 on an in vitro screen (Wood 2004). Nevertheless, other studies both in vitro and in vivo have failed to show SIRT1 activation by resveratrol (Kennedy and Pennypacker, 2014). In the same controversial fashion, some studies found that resveratrol could extend the lifespan of C. elegans (Wood et al., 2004) but in a SIRT1-independent manner (Bass et al., 2007b). In
flies and mice resveratrol does not affect lifespan either (Miller et al., 2011, Pearson et al., 2008, Wang et al., 2013). However, this molecule has beneficial effects on high-fat diets including in humans (Novelle et al., 2015, Park and Pezzuto, 2015).

1.1.3.6. Signals from the reproductive system

As it has been already discussed under the ‘1.2. Mechanistic theories of ageing’ section, it has been proposed that organisms decide whether to allocate their energy resources to soma maintenance or reproduction. Although there is some evidence for a trade-off between longevity and reproduction, it is possible to uncouple these two processes. Strong mutations in IIS that extend lifespan decrease reproduction, whereas weaker mutations in IIS that still exhibit longer lifespans do not affect reproduction (Kenyon, 2010b).

However, there are signals from the reproductive system that do affect longevity. Removing the germline, but not the somatic cells, from the reproductive system of C. elegans extends its lifespan (Hsin and Kenyon, 1999, Kenyon, 2010a). Interestingly when the germline is lost, FOXO is activated by TCER-1 (transcription elongation regulator homolog) and KRI-1 in an IIS-independent manner (Kenyon, 2010a). Moreover, this intervention has a non-autonomous effect that activates the overexpression of TCER-1 in other tissues, and in fact, overexpressing TCER-1 on fully fertile worms has been also shown to extend lifespan. In adult Drosophila, making germline stem cells differentiate and subsequently eliminating them prolongs lifespan (Flatt et al., 2008). Furthermore, a recent study in flies has shown that mated males live shorter than virgin males and this is accompanied by gene expression changes: testis-specific genes are overexpressed whereas metabolic and mitochondrial genes are repressed (Branco et al., 2017). In mice, when ovaries from young females were transplanted into older recipients this led to an extension of lifespan (Mason et al., 2011, Cargill et al., 2003). Therefore, although germline manipulations such as removing it or transplanting it from younger individuals can be beneficial (Grandison et al., 2009), ageing and reproduction can still be uncoupled.

1.1.3.7. Epigenetics

Epigenetic alterations are considered one of the hallmarks of ageing (López-Otín et al., 2013). These are characterized by changes in DNA methylation patterns, histone modifications and chromatin remodelling. Therefore, there is an ample array of proteins involved in keeping these epigenetic marks under control and some of them have been shown to alter longevity.
DNA methylation changes with age mainly in two ways: a) 5-methylcytosine (5mC) varies its distribution across the genome decreasing global gene expression; b) some promoters become hypermethylated, therefore decreasing the level of activation of their gene (Waki et al., 2003). These patterns have been well characterized in mammals, especially in humans, in which DNA methylation ‘clocks’ that can predict the donor’s age, health status and even mortality have been developed (Chen et al., 2016, Hannum et al., 2013, Horvath, 2013). Dietary restriction has been shown to prevent cancers by increasing methylation levels of the ras proto-oncogene, transcription factor RUNX3 and the tumour suppressor p16INK4a (Hass et al., 1993, Kim et al., 2004, Lichtenberg, 2011). Moreover, progeroid patients and mice models show DNA methylation patterns that overlap the patterns seen in normal ageing (Osorio et al., 2010, Shumaker et al., 2006).

Directly modifying DNA methylation in model organisms is not easy, since neither S. cerevisiae, C. elegans nor Drosophila have them. In flies, overexpression of dDnmt2 (DNA methyltransferase encoding gene) gene was shown to extend lifespan and knockdown decreases it (Lin et al., 2005). Nevertheless, a more recent study showed no changes in the methylation of these mutants, so the lifespan extension could be due to other pleiotropic effects of the dDnmt2 gene (Benayoun et al., 2015). Thus, more research is necessary, because at the moment DNA methylation could be regarded as a private mechanism of mammalian ageing or just a hallmark of the ageing process.

Histone modifications by histone acetyltransferases (HATs), deacetylases (HDACs), methyltransferases and demethylases also control gene expression and have been associated with ageing in a public fashion (Mathew et al., 2017). Altering HATs and HDACs activity by genetic manipulation or by supplementation of certain chemicals and drugs can extend lifespan in an evolutionarily conserved way. Spermidine, a natural polyamine that inhibits HATs that deacetylate histone H3, led to upregulation of autophagy and extended lifespan in yeast, flies, worms and human immune cells (Eisenberg et al., 2009). Sirtuins are class III HDACs, that although have other non-histone substrates, as it has been discussed, they have been linked to lifespan extension through their deacetylation activity (Benayoun et al., 2015). For example, dietary restriction has been shown to stimulate SIRT1 and HDCA1, which leads to deacetylation of foxo, p53, ku70 and p16INK4a gene loci (Li et al., 2011). Moreover, the IIS pathway has been shown to be epigenetically regulated by the demethylase UTX-1 (Ubiquitously transcribed TPR on X), that targets histone methylation occurring on the amino (N) terminal tail of the core histone H3 (H3K27me3) (Mathew et al., 2017). Research using C. elegans revealed that UTX-1
levels increase with age and that therefore when the H3K27me3 mark is repressed in *daf-2, akt-1* and *akt-2* genes are transcribed more (Jin et al., 2011).

### 1.1.3.9. Involvement of translation

One of the underlying mechanisms behind lifespan extension by nutrient and stress sensing pathways is downregulation of translation and in addition, directly downregulating translation has been shown to promote longevity in an evolutionarily conserved way. The link between translation and ageing is the focus of this thesis, and hence it will be discussed in more under the ‘1.2.2.8. Translation and ageing’ section once the translational machinery is explained.

### 1.1.4. Pharmacological treatments to ameliorate ageing

As already mentioned, human life expectancy has increased in the last few decades and it is predicted to continue increasing; however, this has not been totally paralleled by better health in the elderly; meaning there is a rise in age-associated diseases that pose a great burden to societies in both less and more developed countries (Hung et al., 2011). Therefore, based on our knowledge from longer-lived model organisms with improved health spans, it has been proposed to tackle these diseases with anti-ageing drugs (Longo et al., 2015).

The best known intervention that extends lifespan and decreases morbidity in model organisms, and even in humans is chronic DR (Fontana et al., 2010, Mattison et al., 2012, Colman et al., 2009, Colman et al., 2014). However, this intervention must be severe to obtain longevity benefits, thus other easier to take treatments, including DR Mimetics, have been studied. Intermittent fasting (IF) has shown to extend lifespan of *E. coli, C. elegans* and mice and although it has several benefits in human health, it could have detrimental effects in more frail and old patients (Harvie et al., 2011, Heilbronn et al., 2005, Longo and Mattson, 2014). Other kind of dietary intervention is protein or selective amino acid restriction. Amino acid levels are controlled by mTOR and general control nonderepressible 2 (GCN2), both evolutionary conserved and related to longevity in model organisms as already discussed. Nevertheless, there are still not many studies following on the effects of reducing protein intake in humans (Longo et al., 2015).

One group of pharmacological interventions that mimic DR are the inhibitors of the TOR pathway. Rapamycin is a very well-known mTORC1 inhibitor in acute doses and both mTORC1 and mTORC2 when administered chronically (Sarbassov et al., 2006). Although
rapamycin is approved to be used in clinical conditions, it has side-effects that make it unsuitable for anti-ageing purposes in humans, and hence other rapalogs, and ATP-competitive inhibitors, such as the Torin family might be considered in future research (Longo et al., 2015). Another group of pharmaceuticals that mimic DR are the inhibitors of glycolysis (Minor et al., 2010). An example is mannoheptulose, a component naturally occurring in unripen avocados, which increased the lifespan of nematodes and mice (Minor et al., 2010). Nevertheless, it has to be noted that elderly patients very often present malnutrition and hence glycolysis inhibitors could have unwanted side-effects. Inhibitors of the GH/IGF-1 axis are also considered DR mimicking-substances; an example is pegvisomant, an approved drug that blocks GHR and it is planned to be studied in the future (Trainer et al., 2000). The last group of DR mimicking-pharmaceuticals are the activators of sirtuins pathways (STACs), such as resveratrol (Hubbard and Sinclair, 2014). Resveratrol has been shown to contribute to the treatment of age-related diseases such as cancer, type 2 diabetes or cardiovascular diseases among others in preclinical models (Hubbard and Sinclair, 2014). In humans it has had also some positive outcomes in obese humans but not in normal patients (Timmers et al., 2011, Yoshino et al., 2012).

AMPK activators, such as metformin, can lead to insulin-sensitizing effects that result in better metabolism and longer lifespan in C. elegans, rats and mice (Anisimov, 2010, Cabreiro et al., 2013, Martin-Montalvo et al., 2013). Importantly, this approved drug has shown to decrease the risk of cancer and cardiovascular incidence and mortality (UKDS, 1998, Ng et al., 2014). Nevertheless, due to its tight link to metabolism, it is important to monitor the effects of metformin under different human diets (Longo et al., 2015).

Finally, there are other drugs that are being considered for anti-ageing interventions in humans. Inflammation is associated with a plethora of age-related disease and conditions, so inhibitors such as the NSAIDs (nonsteroidal anti-inflammatory drugs) are promising anti-ageing treatments ( Francescchi and Campisi, 2014). Epigenetic modulators, like spermidine, a naturally occurring histone acetyltransferases inhibitor, can extend the lifespan of yeast, C. elegans and Drosophila (Eisenberg et al., 2009). Finally, β-blockers, e.g. metoprolol and nebivolol, which extended the lifespan of Drosophila and male mice are also being studied (Spindler et al., 2013).

In conclusion, our knowledge from decades of studies in model organisms and cells have paved the ground for possible anti-aging interventions, but we still have to wait to see what the clinical trials show about their effects in humans.
1.2. Introduction: Translation and translational fidelity

In this section I will explain the process of translation initiation, elongation and termination. I will briefly talk about the ribosomes and the other factors that take part in translation. I will also explain the signaling pathways involved in the regulation and protein synthesis. Finally, I will focus on translation fidelity and especially, how both protein synthesis and translation fidelity link to ageing.

1.2.1. Translation.

Translation is the cellular mechanism by which proteins are synthesised based on the information carried by RNA. This is a complex process because it facilitates the translation of the nucleic acid language of RNA to the amino acid language of proteins. Although many protein factors take part in this process, the most important is the ribosome, a macromolecular machine that coordinates and catalyses the union of the specific amino acids carried by transfer RNAs (tRNAs) based on the nucleotide sequence of a messenger RNA (mRNA). Due to the complexity of translating two completely different alphabets, the error potential is great; however, the *E. coli* has an error frequency as low as $10^{-3}$, while in yeast error per amino acid residue is $10^{-5}$, which makes possible to make accurate proteins at a fast rate to meet the cell’s needs (Berg et al., 2002, Gingold and Pilpel, 2011). Different translation errors in this complex translation machinery have been implicated in many pathologies and ageing. I will further discuss the three main steps of translation, the involved translation factors, how translation fidelity is achieved, and how it can affect cells and organisms.

1.2.1.1. tRNA charging

One of the main players in protein synthesis is tRNA, which delivers amino acids to the translation machinery. tRNAs are produced in a complex process, fairly conserved in eukaryotes; they are transcribed by RNA polymerase III (PolIII) as precursor molecules (pre-tRNAs) and then they experience a set of post-transcriptional changes to become functional (Huang and Hopper, 2016). The 5’ and 3’ end sequences are removed and 3’ terminal CCA sequence is added, introns have to be removed, and other modifications (more than 100 are known) are added in order to give specificity and functionality (Czerwoniec et al., 2009, Reuven and Deutscher, 1993, Zhu and Deutscher, 1987). The process of pre-tRNA charging with their cognate amino acids is carried out by aminoacyl-tRNA synthetases (aaRSs). Each aaRS has specificity for a single amino acid and it also recognizes the cognate tRNA because of its identity elements (Pang et al., 2014b). Aminoacylation occurs in a two-step process that
allows for proofreading: 1) the aaRS activates an amino acid \((aa)\) via an adenylate intermediate \((aa-AMP)\), 2) it covalently links this intermediate to the 3’ end of the tRNA:

\[
ATP + aa \leftrightarrow aa – AMP + PP_i \quad (1)
\]

\[
aa – AMP + tRNA^{aa} \rightarrow aa – tRNA^{aa} + AMP \quad (2)
\]

Although the selection process is fairly accurate, sometimes non-cognate amino acids or tRNAs can be bound, so this process is proofread by the aaRS themselves through their hydrolytic editing domain that can get rid of wrong aa-tRNAs (Fig. 1.9) (Mascarenhas and Martinis, 2008). Nevertheless, due to the similarities between the near-cognate amino acid functional groups, this editing by tRNAs is not the only mechanism to control misaminoacylation; one example is alanine -tRNA synthetase (Ala-RS) which can accommodate and hence misactivate serine and glycine at very high levels (Guo and Schimmel, 2012). Due to this problem, all kingdoms of life also possess AlaXp’s, which are free-standing homologues of the editing site of Ala-RS, that help with hydrolysing any misaminoacylated serine or glycine (Guo and Schimmel, 2012).

**1.2.1.2. Initiation**

Although translation is well conserved throughout evolution, there are distinct aspects between prokaryotes and eukaryotes. One of the main distinctions in the spatial separation between transcription (in the nucleus) and translation (in the cytoplasm) in eukaryotes and the other is the process of initiation. In prokaryotes, there are only three Initiation factors (IFs) and mRNAs are polycistronic (i.e. they encode more than one protein) whereas eukaryotes have many initiation factors (eIFs; summarized in Table 1.2) and monocistronic mRNAs (Hinnebusch et al., 2016). Initiation is the first step in eukaryotic translation and it is considered to be the most highly regulated (Sonenberg and Hinnebusch, 2009). The classical process of initiation, also known as cap-dependent, starts with the formation of the 43S preinitiation complex: this occurs by joining of the 40S ribosomal subunit with the eukaryotic initiation factors eIF1 eIF1A, eIF3 and eIF5 and the ternary complex (eIF2-GTP and initiator methionyl-tRNA (Met-tRNA)) (Guca and Hashem, 2018) (Fig. 1.9). Once the 43S complex is formed, it is ready to bind activated mRNA. This mRNA in eukaryotes has to be capped in the 5’ end, consisting of a 5’ to 5’ triphosphate linkage with a guanosine that gets methylated on the position 7 (m7G) (if an uncapped mRNA reaches the cytoplasm, it is rapidly degraded) and have a 3’ poly(A) tail (Labno et al., 2016, Arribas-Layton et al., 2013). eIF4E binds the methylguanosine cap in the 5’ end and then the scaffold protein eIF4G; after this, the capped mRNA can be activated through
an ATP-dependent reaction facilitated by eIF4A, which together with eIF4G and eIF4E can form the eIF4F complex. Then, poly (A)-binding protein (PABP) binds to the 3’ end of the mRNA circularizing it. eIF1, homologue of prokaryotic IF3 and eIF1A, homologue of IF1, have conserved functions: eIF1 destabilizes codon-anticodon interactions and eIF1A stays in the A site preventing early elongation (Shirokikh and Preiss, 2018).

Once the pre-initiation complex has the mRNA loaded, it can start scanning the strand looking for a start codon; this needs hydrolysis of ATP by the helicase activity of eIF4A and DHX29 and by the binding of eIF4B and eIF4H. When this is found, eIF1 is ejected which, with the help of eIF5, partially hydrolyses eIF2•GTP. This triggers the closure of the pre-initiation complex, so mRNA is stabilized, and the scanning process arrested. At this point, eIF2•GDP, eIF3 and 5 are released, so eIF5B•GTP (which is partially homologous to prokaryotic IF2) together with eIF1A can facilitate the assembly of the 60S ribosomal subunit. Once the 40S and the 60S ribosomal subunits join, eIF5B is hydrolysed and released along with eIF1A and hence the 80S ribosome is formed and ready to enter the elongation phase of translation (Fig. 1.9) (Sonenberg and Hinnebusch, 2009, Aitken and Lorsch, 2012).

Table 2.2. Proteins involved in eukaryotic translation initiation. Main factors involved in initiation.

<table>
<thead>
<tr>
<th>Initiation Factor</th>
<th>Yeast name</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF1A</td>
<td>SUI1</td>
<td>Destabilization of codon-anticodon interactions.</td>
</tr>
<tr>
<td>eIF1</td>
<td>TIF11</td>
<td>Scanning and start codon recognition.</td>
</tr>
<tr>
<td>eIF2 (composite)</td>
<td></td>
<td>The positioning of Met-tRNAMet in the P site.</td>
</tr>
<tr>
<td>eIF2α</td>
<td>SUI2</td>
<td>Control of initiation by its phosphorylation in Ser51, Recognition of downstream start codon context.</td>
</tr>
<tr>
<td>eIF2β</td>
<td>SUI3</td>
<td>Binding to Met-tRNA; and eIF5</td>
</tr>
<tr>
<td>eIFγ</td>
<td>GCD11</td>
<td>GTP/GDP binding and GTP hydrolysis</td>
</tr>
<tr>
<td>eIF2A</td>
<td>YGR054W</td>
<td>Start codon recognition, including noncanonical CUG and UUG</td>
</tr>
<tr>
<td>eIF2D</td>
<td>TMA64</td>
<td>GTP-independent Met-tRNA; binding and start codon recognition</td>
</tr>
<tr>
<td>eIF3 (composite)</td>
<td></td>
<td>Part of Proteasome</td>
</tr>
<tr>
<td>eIF3a</td>
<td>RPG1</td>
<td>Interaction with mRNA, MPN/PCI subunit</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>eIF3b</td>
<td>PRT1</td>
<td>Interaction with mRNA, MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF3c</td>
<td>N1P1</td>
<td>MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF3d</td>
<td></td>
<td>Binding to the cap in RNA</td>
</tr>
<tr>
<td>eIF3e</td>
<td></td>
<td>MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF3f</td>
<td></td>
<td>MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF3g</td>
<td>TIF35</td>
<td>Interaction with mRNA</td>
</tr>
<tr>
<td>eIF3h</td>
<td></td>
<td>MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF3i</td>
<td>TIF34</td>
<td>Exterior subunit</td>
</tr>
<tr>
<td>eIF3j</td>
<td>HCR1</td>
<td>Exterior subunit</td>
</tr>
<tr>
<td>eIF3k</td>
<td></td>
<td>MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF3l</td>
<td></td>
<td>MPN/PCI subunit. Possibly binding to the cap.</td>
</tr>
<tr>
<td>eIF3m</td>
<td></td>
<td>MPN/PCI subunit</td>
</tr>
<tr>
<td>eIF4F (composite)</td>
<td></td>
<td>Formation of mRNA closed loop, loading of mRNA to the ribosomal small subunit, mRNA unwinding and possibly checking directionality</td>
</tr>
<tr>
<td>eIF4A</td>
<td>TIF1/TIF2</td>
<td>ATP-dependent RNA helicase, possibly coupling of ATP hydrolysis with scanning</td>
</tr>
<tr>
<td>eIF4E</td>
<td>CDC33</td>
<td>Binding to the cap, interaction with eIF4G. Regulation of translation by being prevented from binding to eIF4G.</td>
</tr>
<tr>
<td>eIF4G</td>
<td>TIF4631/TIF4632</td>
<td>Facilitation of mRNA attachment to eIF3 (mammals) or the small ribosomal subunit (yeast). Possibly coupling of ATP hydrolysis by eIF4A with scanning</td>
</tr>
</tbody>
</table>
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

<table>
<thead>
<tr>
<th>eIF4B</th>
<th>TIF3</th>
<th>Binding single-stranded RNA. Possibly contribution to checking for directionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4H</td>
<td></td>
<td>Binding to single-stranded RNA</td>
</tr>
<tr>
<td>eIF5A</td>
<td>HYP2/ANB1</td>
<td>Stimulates peptide elongation and peptide release during elongation</td>
</tr>
<tr>
<td>eIF5B</td>
<td>FUN12</td>
<td>GTP-dependent positioning of Met-tRNA ribosomal subunits association</td>
</tr>
<tr>
<td>eIF6</td>
<td>TIF6</td>
<td>Prevention of ribosomal subunits from associating, microRNA-mediated silencing</td>
</tr>
<tr>
<td>PABP</td>
<td>PAB1</td>
<td>Binding to poly(A) tail, interaction with eIF4G, formation of mRNA closed loop. Stimulation of translation and possibly promotion of ribosomal recycling</td>
</tr>
<tr>
<td>DAP5</td>
<td></td>
<td>Functional analogue of eIF4G in cap-independent translation</td>
</tr>
<tr>
<td>MCT-1</td>
<td>TMA20</td>
<td>GTP-independent Met-tRNA delivery on particular mRNAs and in reinitiation</td>
</tr>
<tr>
<td>DENR</td>
<td>TMA22</td>
<td>GTP-independent Met-tRNA delivery on particular mRNAs and in reinitiation</td>
</tr>
<tr>
<td>DHX29</td>
<td>DED</td>
<td>ATP-dependent RNA helicase, scanning of 5’ UTRs (untranslated region) with highly stable hairpins</td>
</tr>
</tbody>
</table>

New evidence has emerged showing that some of the initiation factors that are composed by several subunits, such as eIF3 and eIF4F, can change their composition in response to cellular signals (Genuth and Barna, 2018). These factors have several isoforms that are regulated by genetic or enzymatic regulation and that serve different functions within translation; for example, eIF4G3 is specialized in translating certain mRNAs, amongst them a chaperone critical for spermatogenesis in mice, so mutating just this isoform leads to loss of spermatogenesis (Sun et al., 2010).
The role of decreased protein synthesis in delaying ageing  
Martínez Miguel, V.E.

Figure 1.9. Schematic Representation of the steps occurring during translation initiation in eukaryotes. Initiation starts when several initiation factors join the small ribosomal subunit forming the 43S complex. This is then ready to attach to an activated mRNA and start scanning in an ATP-dependent process. Once the start codon is recognized, the large 60S subunit can join, the initiation factors leave and elongation start. Phosphate groups are shown as yellow and GTPs as green circles. Figure based on (Sonenberg and Hinnebusch, 2009).
Other less common forms of initiation are the cap-independent or noncanonical mechanisms of translation, which are quite diverse. These alternative forms of translation do not depend on most eIFs for the recruitment of the ribosomal subunits; some of them are based on the use of internal ribosomal entry sites (IRES), others use a tRNA-like structure to promote ribosome biogenesis or they just “shunt” over mRNA before reaching the start codon (Kwan and Thompson, 2018). Cells adopt cap-independent pathways depending on several physiological conditions; for example under some viral infections, like hepatitis C virus (HCV), the cell host translates the viral IRES mRNAs but also in situations of cellular stress when cap-dependent translation is halted (Liwak et al., 2012). Although the mechanisms behind cap-independent ways of translation are not fully understood yet, it is known that these alternative translation processes are very varied and they act to maintain synthesis of certain proteins required for stress responses by recognizing particular elements in their coding and non-coding mRNAs (Spriggs et al., 2008).

Furthermore, recent studies have demonstrated another type of non-classical initiation that is cap-dependent but it does not use most eIFs, nor the normal process of scanning - it mostly relies on eIF4G1 and eIF1 (Sinvani et al., 2015). This allows efficient translation of mRNAs that have short 5’UTR mRNAs and it is governed by the presence of TISU elements (translation initiator of short 5’UTR) near the 5’ end, which allow these mRNAs to make direct contacts with the ribosomal proteins S3 and S10 (Haimov et al., 2017). TISU elements are found in approximately 4% of mammalian genes (mainly “housekeeping” genes) and this TISU-driven initiation provides resistance to the stress-induced inhibition of global protein synthesis (Sinvani et al., 2015).

1.2.1.3. Elongation

The elongation phase is well conserved from prokaryotes to eukaryotes and hence most studies have been done using bacteria (Rodnina and Wintermeyer, 2009). This phase initiates with the start codon of the mRNA base-paired to the anticodon of the met-tRNA in the P (peptidyl) site of the 80S (70S in prokaryotes) ribosomal complex (Fig. 1.10.). After the P site, there is an A (aminoacyl) site which has the second codon of the open reading frame (ORF) waiting for the eukaryotic elongation factor 1A (eEF1A) (EFTu in prokaryotes) to bind and direct the cognate aminoacyl-tRNA (Dever and Green, 2012). When the codon is recognized by the aminoacyl-tRNA, GTP is hydrolyzed by eEF1A which triggers its release. After the eEF1A release, a peptide bond is rapidly formed between the tRNAs in the A and P sites; this is catalysed by ribosomal RNAs (rRNAs) in the large ribosomal subunit (Klinge et al., 2011). After
this, the tRNAs are prompted to move to the hybrid P/E and A/P states in which the ends of the mentioned tRNAs are in the E (exit) and P sites and the remaining anticodon locks in the P and A sites. This translocation is mediated by eEF2 (EFG in prokaryotes) that, by hydrolysing GTP, elicits a conformational change that allows movement of tRNA and mRNA and prevents backward movement of the tRNA. The release of the phosphate and the eEF2 lock the subunits in the post-translocation state, in which a deacylated tRNA can occupy the E site so the peptidyl-tRNA can be in the P site. The vacant A site is then ready for the next eEF1A and aminoacyl-tRNA and hence more amino acids to be added to the polypeptide chain (Fig. 1.10.) (Dever and Green, 2012).

**Figure 1.10.** Schematic representation of the elongation phase of translation in eukaryotes. This phase starts with the start codon (AUG) recognition (top of the figure) and paring on the met-tRNA. eEF1A helps in recruiting the next corresponding aminoacyl-tRNA (depicted in purple). eEF2•GTP triggers peptide formation and the translocation of the tRNAs to the E and P sites. The tRNA in the E site does no longer have an aminoacyl group (depicted in grey), so it is released from the ribosome and another circle can start with the next corresponding aminoacyl-tRNA. Phosphate groups are shown as yellow and GTPs as green circles. Figure based on (Schneider-Poetsch et al., 2010b) and (Dever and Green, 2012).

### 1.2.1.4. Termination and ribosome recycling

The termination phase of translation occurs when a stop codon in the mRNA sequence reaches the A site. The canonical stop codons are UAA, UAG and UGA, but there are some organisms that either only use UAA and UAG (UGA is a sense codon), others in which UGA is the only stop codon (UAA and UAG are sense codons) or UAA and UGA are stop codons (and
UAG is a sense codon (Panek et al., 2017, Keeling, 2016). In eukaryotes, eukaryotic Release Factor 1 (eRF1) is a tRNA shaped protein that recognizes all three stop codons and hydrolyses the peptidyl-tRNA in order to release the polypeptide chain (Dever and Green, 2012). In addition, eRF3 is a GTPase that, although not fully necessary for peptide release (eRF1 can induce it alone), it facilitates this release and increases termination efficiency with its GTPase activity (Eyler and Green, 2011, Alkalaeva et al., 2006) (Fig. 1.11). Interestingly, eRF1 increases the association of eRF3 to GTP by forming a stable ternary complex formed by eRF1:eRF3•GTP (Mitkevich et al., 2006, Pisareva et al., 2006).

Recycling of the ribosome is initiated by the ABCE1 (ATP binding cassette subfamily E member 1) protein, and it starts when the polypeptide chain is released, but the mRNA, the deacetylated-mRNA and eRF1 are still bound (Hellen, 2018). The process of recycling in eukaryotes is quite different than in bacteria and it is not fully understood yet. However, it is suggested that ABCE1 protein mediates the splitting of the ribosome by an ATP-dependent reaction and conformational changes that disrupt the inter-subunit bridges and drive the separation of the two ribosomal subunits (Pisarev et al., 2010, Barthelme et al., 2011, Becker et al., 2012, Franckenberg et al., 2012). Furthermore, ABCE1 directly enhances the rate of peptide release by eRF1:eRF3, GTP dissociation and subsequently their dissociation from the ribosome, at least in S. cerevisiae (Shoemaker and Green, 2011) (Fig. 1.11). Finally, the release of deacetylated tRNA and mRNA from the small ribosomal subunit is enabled by elf1, elf1A and elf3 initiation factors (Hellen, 2018). The detached subunits are then ready to be recruited by the elfs for another round of initiation.

This full dissociation only happens right after termination in some cases, whereas in others there is a partial dissociation called re-initiation. This process is important for control of gene expression in eukaryotes and it can happen through different mechanisms. One of the mechanisms is based on the presence of short untranslated open reading frames (uORFs) that can lead to re-initiation when mRNA is still associated to the 40S subunit (Luukkonen et al., 1995, Kozak, 2001); which is dependent on the presence of elf1, elf1A, elf2, elf3 and Met-tRNA_Met, and also elf4F, which provides the 5’ to 3’ directionality (Poyry et al., 2004, Skabkin et al., 2013). Other mechanisms of re-initiation are mediated by reinitiation factors such as ligatin (elf2D), density release protein (DENR) and multiple copies in T-cell lymphoma-1 (MCTS1), they depend on uORF length and they happen at least in human cells and in Drosophila (Janich et al., 2015, Skabkin et al., 2013, Schleich et al., 2017, Schleich et al., 2014).
Therefore, termination and ribosomal recycling are also complex steps of translation that can be regulated at several points to adapt to different conditions.

Figure 1.11. Diagram of the termination phase of translation in eukaryotes. The polypeptide chain is attached to the tRNA in the P site until a stop codon is recognized by the eRF1:eRF3•GTP complex in the A site. ABCE1 binds and facilitates the peptide release and subunit dissociation. In addition, ABCE1 enhances GTP hydrolysis of the eRF1. GTP depicted as green and free phosphate as yellow circles. Figure based on (Dever and Green, 2012).

1.2.1.5. Ribosomal subunits

The ribosome is a complex structure comprised of two subunits, in eukaryotes, this is formed by more than 5500 nucleotides of rRNA, including the termed expansion segments (ES) and 80 ribosomal proteins (RPs) (79 in yeast) with extra r-protein extensions. In eukaryotes, 35 out of the 80 RPs (79 RPs in yeast) have been found to have homologs in bacteria/archaea, 32 only have archaeal homologs and hence only 13 (12 in yeast) ribosomal proteins are eukaryotic specific (Fig. 1.12.) (Wilson and Cate, 2012). Furthermore, the protein: RNA ratio in the large subunit of eukaryotes is 1:1 whereas in bacteria it is 1:2; most of this additional protein mass is in the back of the structure and it forms a network that interacts with other conserved proteins and rRNA (Fig. 1.12.). Moreover, the mRNA exit site in eukaryotes has two extra proteins, RPS26 and RPS28 which might be implicated in the interaction with eIF3 (Srivastava et al., 1992, Siridechadiilok et al., 2005). The A, P and E sites are composed principally of ribosomal RNA (r-RNA) conserved in archaea and eukaryotes; however, in the latter, there are also ribosomal proteins that play important roles in decoding, accommodating and stabilizing the tRNAs in these sites (Wilson and Cate, 2012). Furthermore, the eukaryotic ribosome has a tunnel through which the nascent peptide passes and it is thought to regulate translation rate, enhance early folding events and gather translation factors in the exit site (Wilson and
Beckmann, 2011). It has to be noted that based on recent evidence, nowadays the ribosome is not seen as a fixed entity, but a more heterogeneous machine that can vary its RNA and protein composition in response to the environment, during development or diseases (Sauert et al., 2015, Guimaraes and Zavolan, 2016, Sloan et al., 2017).

Another concept to consider is the presence of specialized ribosomes within cells and organisms. New evidence has shown that there is heterogenic compositions of rRNA, ribosomal proteins or protein modifications that can result in the ribosome having different functions (Xue and Barna, 2012). Although this is still a fairly new field, it has been shown that for example, deletion of Rpl38 does not alter global protein synthesis but only the translation of specific mRNAs (Kondrashov et al., 2011). Many other ribosomal proteins have specialized...
functions; RPS11, RPS19 and RPS25 promote IRES translation, RPS26 promotes translation from start codons in the Kozak sequence and RPS13a inhibits translation of specific mRNAs (Genuth and Barna, 2018) Nevertheless, how this heterogeneity is regulated and how it originates is still not clearly understood.

1.2.1.6. Mitochondrial translation

Mitochondria are special organelles that evolved from an endosymbiotic bacterium, so even though they transferred most of their genetic information to the nucleus of the cell, they still have some vestigial ‘own’ genetic system (Ott et al., 2016). Most of their proteins are synthesized by the cytosolic host system, but a subset of oxidative phosphorylation (OXPHOS) complexes is still synthesized by the mitochondrial translation machinery. The loss of genetic information occurred independently through evolution, so the mitochondrial genomes are very varied across organisms. However, the mitochondrial-encoded translational machinery is quite incomplete and it requires the import of certain factors, importantly all the ribosomal proteins are imported from the host cytosol.

These evolutionary forces have favored mitochondrial translation to be very different from the bacterial one. For example, they use a different genetic code from the universal one and they have less mt-tRNAs (Anderson et al., 1981, Barrell et al., 1979). In addition, mitochondrial ribosomes (mitoribosomes) are very varied between organisms and very different from bacterial ones. One of the striking differences is that many mitoribosomes have recruited and changed the function of proteins that were not naturally ribosomal, such as yeast mit-RPL50 which originally was a lipid binding protein and human mRPL43 which was an oxidoreductase (Amunts et al., 2014, Amunts et al., 2015, Greber et al., 2015). However, the decoding center and the peptidyl transferase catalytic core are still highly conserved and not specific to mitochondria, which causes them to be off-targets of certain antibiotics such as the aminoglycoside family (Zhang et al., 2005). Not surprisingly, there are also differences in the translation steps too, but in all organisms, mitochondria need to use factors imported from the cytosol, some homologous to bacterial ones and some adapted from the host. In addition, a recent study has shown that mitochondrial translation fidelity can alter cytosolic translation fidelity too (Suhm et al., 2018).

1.2.1.7. Signalling pathways involved in translation

Translation is the most energetically expensive cellular process, so it is tightly regulated by growth factors, hormones, internal and external stressors. Different signalling
pathways integrate a variety of stimuli to appropriately adjust translation levels (Fig. 1.13.). Growth factors, hormones and cytokines act on receptor tyrosine kinases that can activate the Ras pathway or the mTOR pathway through insulin receptor substrate 1 (IRS1). IRS1 activates mTORC1 through PI3K and Akt, which results in inhibition of tuberous sclerosis 2 (TSC2) hence activating cap-dependent translation and promoting cellular growth and proliferation (Saxton and Sabatini, 2017). This results from two main downstream effectors of mTORC1: a) inhibitory phosphorylation of 4E-BP which then cannot sequester eIF4E thereby allowing eIF4F complex formation and b) activating phosphorylation of S6K, which then phosphorylates and activates eIF4B, and possibly eIF4A, increasing in this way the activity of translation initiation. Akt also phosphorylates and inhibits PRAS40 facilitating its release from mTORC1 and therefore alleviating the inhibition of mTORC1 (Nascimento et al., 2010, Sancak et al., 2007, Vander Haar et al., 2007). Although this is an important link between IIS and mTOR, in Drosophila PRAS40 regulates growth only in ovaries but not in somatic tissues (Pallares-Cartes et al., 2012). Amino acids at the surface of the lysosome through a set of Rags activate mTORC1 (Wolfson and Sabatini, 2017). Also, at the surface of the lysosome, AMPK can be switched on when ATP or fructose-1,6-biphosphate (FBP; a glycolytic intermediate) are low or when AMP is high (Lin and Hardie, 2018, Ben-Sahra and Manning, 2017). Activated AMPK directly inhibits mTORC1 and it also phosphorylates eEF2 kinase (eEF2K), activating it and hence slowing down elongation (Browne et al., 2004, Johanns et al., 2017).

Moreover, the transcriptional regulator Myc has been implicated as a direct activator of eIF4E in an mTOR-independent manner promoting translation (Matsumoto et al., 2015, Pourdehnad et al., 2013). Furthermore, S6K phosphorylates eEF2K, inactivating it, and subsequently activating elongation (Dunlop and Tee, 2009). The Ras pathway also results in inhibition of TSC2 and TSC1, phosphorylation of eIF4B and activation of Mnk1 and Mnk2 that promote translation through phosphorylation of eIF4E and eIF4B (Mendoza et al., 2011). Likewise, stress signals can promote the activity of p38 through the MAPK pathway resulting in activation of Mnk1 (Blagden and Willis, 2011). In contrast, endoplasmic reticulum (ER) stress promotes unfolded protein response (UPR) pathways that lead to activation of double-stranded RNA-activated protein kinase (PKR), PKR-like ER kinase (PERK) and GCN2 which phosphorylate Ser52 in the α-subunit of eIF2 causing repression of global translation but paradoxically upregulated translation of mRNAs with IRES and uORFs that are involved in cellular adaptation to stress (Do et al., 2009).
Figure 1.13. Main pathways that regulate protein translation in response to different stimuli such as stress, growth factors and hormones or endoplasmic ER stress. Growth factors, hormones and cytokines upregulate translation through the Ras/Raf/MEK pathway (light blue) or PI3K/Akt/mTOR pathway (light green). At the lysosomal surface, nutrient and energy levels are sensed by Rags (orange) and AMPK (burgundy). In addition, stresses can downregulate or upregulate translation through UPRs (purple) or p38 (dark green). All these act directly or indirectly on eIFs (light pink) or eEFs (dark pink). Figure based on (Blagden and Willis, 2011) and (Silvera et al., 2010).

1.2.2. Translational fidelity

One of the fundamentals of biology is the expression and conservation of the genome. Although this happens with notable accuracy, both in vivo and in vitro experiments have shown that gene expression is more erroneous than theoretical calculations. As gene expression involves transcription and translation, both their error rates can compromise its fidelity; however, DNA replication has been shown to be exceptionally accurate, with error rates ranging from $10^{-8}$ to $10^{-10}$ in prokaryotes and eukaryotes respectively, which means most of the inaccuracy comes from translation (Kunkel and Bebenek, 2000). In fact, translation error rates in vivo have been calculated to be several orders of magnitude higher, from $10^{-3}$ to $10^{-4}$ (Kramer and Farabaugh, 2007).
Errors in translation are a result of 1) aminoacyl-tRNA synthetases adding an incorrect aminoacyl to tRNAs, 2) imbalances in the tRNA pool and low abundance of certain tRNAs, 3) the ribosome selecting a tRNA with the wrong codon or 4) frameshifts during elongation (Fig. 1.14.) (Mohler and Ibba, 2017, Ogle and Ramakrishnan, 2005). Aminoacyl-tRNA synthetases have been shown to be very accurate enzymes that uses a “double sieve” editing mechanism: this mechanism is based on the fact that non-cognate amino acids are too large to fit the active site (isosteric first sieve) and that the editing site of aaRSs hydrolyses any mischarged amino acids (editing second sieve) (Nureki et al., 1998, Fersht, 1977, Guo and Schimmel, 2012). This proofreading ability by aaRSs is especially important when near-cognate amino acids are too similar in topology or size, as is the case of AlaRS, which without the editing site would misactivate Ser and Gly at much higher levels (Guo et al., 2009). Despite this high accuracy of aaRSs, they can still misactivate tRNAs when the tRNA pool is altered or imbalanced, for example by nutritional or environmental stresses (Mohler and Ibba, 2017, Nawrot et al., 2011).

This means, that the decoding process by the ribosome is the main contributor to the high error rates of translation. This decoding had historically been proposed to be mainly determined by the affinity of base pairing between the mRNA codon and the trinucleotide anticodon of the tRNA (Loftfield, 1963); however, more in-depth knowledge of enzymology showed that hydrolysis of GTP to GDP when a catalytic step follows the binding of the substrate increases the selectivity of said enzyme, which in translation means that when EF-Tu (eEF1)-mediated hydrolysis of GTP enhances the discrimination of tRNA selection (Ogle and Ramakrishnan, 2005). Therefore, the current kinetic model of ribosomal decoding explains that there are two main steps in this process that ensure fidelity (Fig 1.14.). The ‘initial selection’ step is comprised of the initial binding, which is codon-independent and it is driven by the interactions between the ternary complex and the ribosome, totally driven by EF-Tu biding (Diaconu et al., 2005, Rodnina et al., 1994) and it is followed by a codon-dependent step that it is stabilized by the ribosome (Gromadski and Rodnina, 2004a). Finally, the initial binding step involves EF-Tu undergoing a rate-limiting conformational change in its active site that facilitates GTP hydrolysis and that is dependent on codon-anticodon interactions in the decoding helix (Gromadski et al., 2006). It is important to note, that this is the exploited step by hyperaccurate mutants, which will be further explained later (Bilgin et al., 1992). The second step of decoding that ensures translation fidelity is the proofreading step and it totally depends on codon-anticodon interactions; here, the tRNA can either move to the A-site to continue into the peptidyl transfer, or it can be rejected leading to its dissociation (Zaher and
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Green, 2009). This proofreading step is the one exploited by antibiotics that increase the level of errors, which will also be explained in more detail later.

Figure 1.14. Schematic representation of aminoaacylation and ribosome decoding: their proofreading mechanisms and the possible mistranslation events. Aminoacyl-tRNA synthetases activate cognate amino acids by adding adenosine monophosphate (AMP) with high fidelity by using kinetic and structural selection mechanisms. Nonetheless, a non-cognate amino acid can be misactivated, in which case it can either be hydrolysed, or it can be mischarged to a tRNA, which can further proofread and resample it. In addition, an activated cognate amino acid can be charged to a non-cognate tRNA, which can be caught by the aaRS proofreading leading to hydrolysis, or it can escape this mechanism and interact with an elongation factor. Moreover, elongation factors can also select for correct aatRNAs through thermodynamic interactions; however, if these two bind, they can be initially selected by the ribosome. However, the ribosome itself has a proofreading mechanism that can also avoid mistranslation by being separated in two main steps, initial selection and proof-reading. Figure adapted from (Mohler and Ibba, 2017, Zaher and Green, 2010).

1.2.2.1. Mutations and antibiotics affecting fidelity

For a long time, it was believed that the ribosome acted to just stabilize the codon-anticodon interactions, but its important role in enhancing specificity for cognate tRNAs was not clear (McLaughlin et al., 1966). This all changed with the discovery of ribosomal mutations and antibiotics that changed the fidelity of translation (Ogle and Ramakrishnan, 2005).

1.2.2.1.1. Streptomycin, ram and restrictive mutants

The work of Gorini and colleagues in the 1960s showed that the use of streptomycin, an aminoglycoside antibiotic that binds the small subunit of the ribosome, could induce errors in translation (Anderson et al., 1965, Davies et al., 1964, Gorini and Kataja, 1964a, Gorini and
Kataja, 1964b). These experiments used auxotrophic variants of *E. coli* that had premature stop codons in certain essential metabolic enzymes, so they could only grow when all the metabolites were given, or the media was supplemented with streptomycin (termed SmD for their streptomycin dependency). This was further supported by *in vitro* studies showing that wrong amino acids were misincorporated in polypeptides in the presence of streptomycin (Davies et al., 1964), which eventually led to the discovery that this antibiotic increased the level of stop codon read-through and also that the ribosome could recognize and influence the codon-anticodon interaction.

In addition, new ribosomal mutants were isolated, either showing streptomycin-resistance (SmR) (also termed restrictive, as they restrict the action of streptomycin) or having a revertant phenotype that suppressed streptomycin-dependency (termed ram for ribosomal ambiguity) and that were error-prone. These SmR mutations were found in the gene *rpsL* encoding RPS12 and the ram mutations affected RPS4 and RPS5 (*rpsD* and *rpsE* genes) in bacteria (Ozaki et al., 1969, Hasenbank et al., 1973, Piepersberg et al., 1975). The mutations in RPS12 that confer resistance do so by either preventing the binding of the antibiotic to the ribosome, as is the case of RPS12 Lys42Arg (K42R) or by both preventing binding and also conferring hyperaccuracy that reverts the streptomycin-induced inaccuracy Lys42 → Ala, Thr or Gln (K42A, K42T or K42Q) (Funatsu and Wittmann, 1972, Sharma et al., 2007, Vila-Sanjurjo et al., 2007).

### 1.2.2.1.2. Paromomycin

It should be noted that not only streptomycin but also the rest of antibiotics in the aminoglycoside group, e.g. neomycin, kanamycin or paromomycin, can increase the level of translational errors, but in different degrees. Therefore, to further understand the decoding process, this group of antibiotics has been widely used in structural studies to see how they interfere with the ribosome and how this might affect translation fidelity. For example, paromomycin induces conformational changes of the helix 44 (H44) of 16S rRNA to a position where it can engage the minor-groove of the codon-anticodon in the A-site (Ogle et al., 2001) (Fig 1.15.). This leads to a reduction in the rate of near-cognate tRNA dissociation from the A-site and it accelerates the reaction rates of tRNA selection, switching the ribosome into a ‘hyperactive’ state (Pape et al., 2000). In contrast, streptomycin binds between RPS12 and helix 27 (H27) of 16S rRNA, reducing the reaction rates of GTPase activation for cognate tRNA, inducing a state of intermediate activation and stabilizing the closed domain form (Gromadski and Rodnina, 2004b).
Figure 1.15. Interactions of streptomycin and paromomycin with the decoding centre on prokaryotic ribosomes. **A)** Streptomycin (brown molecule) binds h44 (helix 44 from 16S rRNA) (light blue) on the outside and it also interacts with h27 and h18 (helices 27 and 18 from 16S rRNA) (both grey) and importantly RPS12 (u12, light green). **B)** Paromomycin (purple), conversely, binds h44 on the inside inducing a flipped-out conformation of A1492 and A1493 in this helix. Figure adapted from (Lin et al., 2018).

Accuracy mutants can also be either resistant or sensitive to paromomycin. Strains of bacteria with mutations in 16S rRNA are more susceptible to this antibiotic, and the same mutations that confer resistance to streptomycin (K42R, K42A, K42T and K42Q) also confer it to paromomycin (Kalapala et al., 2010). Eukaryotes are also susceptible to aminoglycosides, including paromomycin, and in yeast, mutations in 18S rRNA (homologous to bacterial S16) also increased the susceptibility to this antibiotic (Kalapala et al., 2010, Prokhorova et al., 2017). In the same fashion, there are mutations in *S. cerevisiae* that confer resistance against paromomycin; these are mutations in the highly conserved region of RPS23 (formerly known as RPS28), which is the equivalent to bacterial RPS12 K42, and they are the same substitutions K62R, K62A, K62T and K62Q (Alksne et al., 1993).

### 1.2.2.2. Ribosomal structures affecting decoding and fidelity

Several biochemical studies showed that the *E. coli* ribosomes with mutations disrupting certain lysines (e.g. K42R, K42N, K42Q and K42T) of S12 were hyperaccurate, having reduced frameshifts and mistranslation errors, which makes the SmR mutants gain their resistance to streptomycin not by preventing the binding of the antibiotic, but, generally, by compensating its error-inducing effects (Chakrabarti and Gorini, 1975, Davies et al., 1964,
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Bouadloun et al., 1983, Funatsu and Wittmann, 1972, Finken et al., 1993, Ogle and Ramakrishnan, 2005). The mechanism by which restrictive mutations increase translation accuracy has long been studied and high-resolution crystal structures of ribosomes have shed light on how this might occur: when cognate aminoacyl-tRNAs bind the decoding center, three highly conserved nucleotides, A1492, A1493 and G530 rearrange their conformation in the minor groove of the codon-anticodon helix (Ogle et al., 2002, Carter et al., 2001, Wimberly et al., 2000); in addition, RPS12 has several lysine residues that form hydrogen bonds with backbone phosphate groups from 16S rRNA’s h13 and h44, which enables the closed conformation of the small subunit and enhances cognate tRNA to be in contact with the codon-anticodon helix in the A site (Fig 1.16. A) (Carter et al., 2001, Tran et al., 2011).

Therefore, until recently it was assumed that mutations in RPS12 that disrupt these interactions can lead to a more loose closed form of the ribosome, costing more energy to be formed (Ogle and Ramakrishnan, 2005). However, a new biochemical study by Zaher and Green has shown that RPS12 mutants are hard "proofreaders", which means that their hyperaccuracy comes from enhancing the accommodation of cognate and rejecting near non-cognate, tRNAs in the A site (Zaher and Green, 2010).

On the contrary, ram mutants are hypoaccurate and reverse the effect of restrictive mutations (Brownstein and Lewandowski, 1967, Rosset and Gorini, 1969). These mutants show
a “leaky” phenotype; they enhance mistranslation; they are additive to the effect of streptomycin and antagonize the effect of S12 mutations that can restore mistranslation to background levels (Biswas and Gorini, 1972). Structural studies have revealed that RPS4 and RPS5 have polar interactions that are in the opposite side of the RPS12/h44/h27 interface and that are disrupted in the closed domain form (Ogle and Ramakrishnan, 2005). Until recently, this was thought to be due to the salt bridges between S4 and S5 being disrupted, which could lower the energetic cost of tRNA selection and accommodation, allowing near-cognate tRNAs to induce domain closure more easily and therefore lower accuracy (Ogle et al., 2002) (Fig 1.16. B). However, now it has been proposed that the salt bridges between S4 and S5 are broken during the initial codon recognition step before the S12/h44/h13 interface forms, which allows EFTu to be activated and hence GTP to be hydrolyzed (Zaher and Green, 2010). Thus, ram mutations that reduce these interactions, reduce the free energy necessary to activate EFTu, and their effect on initial selection step results in increased errors.

It should be noted that different mutations in different helices of 16S RNA, can both increase and decrease accuracy. In addition, these mutations can confer either resistance or sensitivity to streptomycin. Several mutations of h13 and h44 in E. coli led to decreased translation fidelity, whereas different substitutions in h27 led to increased accuracy and streptomycin resistance in Thermus thermophilus (Gregory and Dahlberg, 2009, Tran et al., 2011).

1.2.2.3. Translation fidelity studies in eukaryotes

As it has been already mentioned, the first studies exploring translation fidelity were carried out using bacteria, usually E. coli. However, in 1964 two mutants affecting translation were found in S. cerevisiae, sup1 and sup2 (Inge-Vechtomov and Kozhin, 1964, Smirnov et al., 1974). The name sup comes from suppressor, which refers to a mutant that suppresses the phenotype of another mutation in a different gene, in this case, sup1 and sup2 reverse the effect of nonsense mutations that led to premature peptide termination. These suppressor of nonsense mutations are located in genes SUP45 and SUP35 encoding release factors eRF1 and eRF3 respectively and have been shown to be hypoaccurate by having higher levels of stop codon read-through (Zhouravleva et al., 1995, Stansfield et al., 1995, Frolova et al., 1994).

Although this nonsense suppression (SUP) could be intuitively attributed to the reduction in the availability of release factors that could lead to termination, further research showed that there is a complex interaction between the ribosomes and translation fidelity in eukaryotes too (Nizhnikov et al., 2014). After this, many other nonsense suppressors were
found by mutating other proteins. Particularly interesting is the finding of two suppressors of nonsense mutations, \textit{SUP44} and \textit{SUP46}, which are mutants of the genes encoding RPS2 and RPS9, the homologues of bacterial RPS4 and RPS5 cause \textit{ram} phenotypes (Eustice et al., 1986, Ono et al., 1981, All-Robyn et al., 1990, Vincent and Liebman, 1992).

Furthermore, translation accuracy in yeast can also be affected by mutating RPS23 (formerly known as RPS28) the homologue of bacterial RPS12. Bacterial S12 mutants show hyperaccurate phenotypes, similarly, in \textit{S. cerevisiae}, several mutations in the lysines of RPS23 (e.g. RPS23 K62Q, K62N and K62T) can enhance translation fidelity, while the K62R mutation surprisingly behaves as antisuppressors and decrease fidelity (Alksne et al., 1993, Anthony and Liebman, 1995). The lysine at position 62 forms two hydrogen bonds with paromomycin, and also a salt bridge with 18S rRNA; the K62R substitution results in the hydrogen bonds to be broken, but not the salt bridge, which results in paromomycin resistance but not increased accuracy (Kalapala et al., 2010). In the case of the other lysine substitutions, the salt bridge is also disrupted so they are both hyperaccurate and resistant to paromomycin. One of the important implications of these studies was the realization that the ribosomal decoding center is highly conserved from bacteria to yeast.

In addition, suppressors of nonsense mutations were found to be produced by mutations in rRNAs of the small and large subunits. Several of these mutations were isolated in 18S rRNA, which is located in the decoding center of the small subunit (Chernoff et al., 1996, Chernoff et al., 1994, Velichutina et al., 2001). The essential role of 18S rRNA in translation accuracy was proved by having both suppressor and antisuppressors phenotypes depending on the mutation (i.e. being hypo and hyperaccurate). Other suppressors of nonsense mutations are due to mutations in 25S and 5S rRNA, evolutionarily conserved rRNAs found in the large subunit (Baxter-Roshek et al., 2007, Smith et al., 2001, Panopoulos et al., 2004). Furthermore, mutations in elongation factors can also affect translation fidelity making yeast become suppressors or antisuppressors. Mutations in \textit{TEF2}, one of the two genes encoding eEF1A, can have either SNM or antisuppressor phenotypes (Sandbaken and Culbertson, 1988). When the \textit{TEF2} mutation leaves its product not functional, there is an overall reduction of eEF1A, which can improve the competition for termination factors and hence have antisuppressor i.e. hyperaccurate effects (Silar and Picard, 1994). More recently, a lysine-specific methyltransferase has been found to mutate an evolutionary conserved lysine in eEF2 altering translation fidelity, and it has been proposed this might be due to altered contact with RPS23 (Davydova et al., 2014).
As already mentioned, mitochondria have their own translational machinery, and its accuracy has been shown to affect cytoplasmic proteostasis. In a recent study ram or restrictive mutations were introduced in the highly conserved mitochondrial RPS12 (mit-RPS12) (Suhm et al., 2018). The hypoaccurate mutation, P50R increased the level of cytosolic ROS and protein aggregates whereas the hyperaccurate mutation K71T had the opposite effect, decreasing the levels of ROS and protein aggregation. Therefore, it is also interesting how cytoplasmic translation can be tuned even by mitochondrial translation.

1.2.2.4. Stress and translation fidelity

Different environmental or endogenous cues can affect or be affected by transition fidelity, either by directly altering the translational machinery or through signaling pathways. For example, mistranslation is usually seen in bacteria as a response to antibiotics that target the ribosome as it has been discussed above (Tuite and McLaughlin, 1984, Londei et al., 1988). In addition, imbalances in the tRNA pool can lead to misincorporation errors. In normal translation, the ribosome will encounter mainly non-cognate tRNAs so if there is limited availability, for example, due to amino acid starvation, this can terminate the peptide, stall the ribosome or misincorporated non-cognate amino acids (Pang et al., 2014a, Lovmar and Ehrenberg, 2006, Fluitt et al., 2007).

Furthermore, ROS arising as byproducts of cellular processes or being internalized from the environment can also affect translation fidelity. ROS are known to damage carbohydrates, lipids, DNA, proteins and RNA; in fact, oxidation of tRNAs or aaRSs has been shown to promote misincorporation (Gu et al., 2014).

Signaling pathways responding to stress usually trigger a global downregulation of translation, but sometimes they can also affect translation fidelity. In the case of amino acid starvation, uncharged deacylated tRNAs in the cytoplasm are monitored by GCN2 in eukaryotes, which dampens translation; however, sometimes deacylated tRNAs can enter the A site of the ribosome, which triggers its pausing (Hinnebusch, 2005). However, it has been shown that if there are misaminoacylated tRNAs, these can mask amino acid starvation and reduce the response of the nutrient-sensing pathways (Mohler et al., 2017). Similarly, in bacteria, when the ribosome is paused due to a deacylated tRNA, it associates with ribosome-associated protein A (RelA) which starts the stringent response by synthesizing (p)ppGpp, and as in yeast, this pathway can be inhibited if there is misaminoacylation (Brown et al., 2016, Loveland et al., 2016, Starosta et al., 2014, Bullwinkle and Ibba, 2016).
1.2.2.5. Harmful outcomes of mistranslation

Changes in the amino acid sequence of a protein can alter its chemical properties, especially its conformation, which can have detrimental effects on cells and organisms. Not only mistranslation is a waste of valuable nutrients and energy for the cell, but also non-functional misfolded proteins can lead to a build-up of toxic aggregates if the clearing and quality control systems do not work efficiently or they are oversaturated (Reynolds et al., 2010). A basic example of the detrimental effect of mistranslation is in the antibacterial effect of aminoglycosides, which target the decoding center of bacterial ribosomes. The hypoaccuracy triggered by these antibiotics causes misfolded membrane proteins that increase the levels of oxidative stress leading to cell death. (Kohanski et al., 2008).

It has been shown that mistranslation can lead to protein aggregation having severe effects on cells and even organisms. When *E. coli* was mutated to have aaRSs with defects in the editing sites, the increased misacylation led to an upregulation of the heat shock response (HSR) and aggregation (Ruan et al., 2008). In mice, a mutation in AlaRS that induces misacylation to serine, led to increased ubiquitination, aggregation and upregulation of the HSR causing the mice with this mutation to have neurodegeneration and hair loss (Lee et al., 2006). A follow-up experiment in which they tested mouse fibroblast with a mutation in the editing domain of ValRS, the autoimmune response was induced leading to apoptosis (Nangle et al., 2006).

Mistranslation is also involved in some human diseases. Erroneous translation can lead to protein aggregation which is especially detrimental in neurons, being the cause of several neurodegenerative diseases. Interestingly, when the editing site of AlaRS was defective, a greater proportion of tRNAs were misacylated with tRNA$^{\text{Ser}}$, resulting in protein misfolding and eventual neuronal loss in mice (Lee et al., 2006). This mutation was first seen in mice models; however, it has also been found in patients with microcephaly, hypomyelination and epilepsy (Nakayama et al., 2017). In addition, some patients with epilepsy, ataxia, autism or intellectual disabilities have missense mutations in eEF1A2 that in yeast produce frameshift and nonsense suppression (Inui et al., 2016, Lam et al., 2016, Lopes et al., 2016, Meriin et al., 2012b, Nakajima et al., 2015, Veeramah et al., 2013, Sandbaken and Culbertson, 1988, Belin et al., 2009).

Furthermore, some ribosomopathies (i.e. diseases caused by ribosomal abnormalities) are due to the haploinsufficiency of ribosomal RNA or proteins, interestingly 18S rRNA, which is important in decoding, is altered in Treacher Collins syndrome, UTP14-type male infertility
and Diamond Blackfan anemia (Narla and Ebert, 2010, Idol et al., 2007). Although it has not been studied the level of translational fidelity in patients with these syndromes, another ribosomopathy has been directly linked to hypoaccuracy. A mutation in the highly conserved decoding region of RPS23 results in microcephaly, hearing loss, dysmorphia and reduced intellectual capabilities (Paolini et al., 2017). The mutation is a substitution of Arg69 to a lysine, which is in close proximity to the already discussed Lys62 in yeast (Lys60 in humans), and that induces high levels of missense and nonsense translation. Nevertheless, it should be noted that, to date, none of the restrictive/hyperaccurate mutations have been found in humans.

Translation deregulation is associated with some cancers either due to disruptions in the signalling pathways that converge with translation or to translation proteins being directly oncogenic. However, the effect of this disturbance in translation fidelity is not well characterized. Nevertheless, it has been shown that aggressive breast cancer cells that had normal levels of global translation had less efficient IRES-mediated initiation and less accurate translation (Silvera et al., 2009). Moreover, in p53-inactivated cancer cells, a study showed that these cells had modified levels of rRNA methylation that led to higher codon read-through and misincorporation (Marcel et al., 2013). Recently, examination of ovarian carcinomas was found to have different mutations in eIF1A, which are predicted to lead to translation initiation errors (Etemadmoghadam et al., 2017).

### 1.2.2.6. Beneficial effects of translation inaccuracy

Errors in translation can lead to beneficial outcomes for cells and organisms, by giving an evolutionary advantage under environmental, nutritional or immunological stress (Wang and Pan, 2015, Ling and Soll, 2010). In unicellular organism having a more varied proteome can confer resistance against different environmental stresses that eventually lead to an evolutionary advantage. For example, *Candida albicans* tRNA<sup>Ser</sup> (CAG) can also read CUG codons that encode leucine, especially under lower pH, higher temperature or in the presence of H<sub>2</sub>O<sub>2</sub>, thus increasing its phenotypic variability under stress (Gomes et al., 2007). This fungal parasite can also benefit from mistranslation as this enhances its antigenic diversity helping it to evade the host immune system (Miranda et al., 2013, Sarkany et al., 2014). Another example of a unicellular organism benefitting from mistranslation is the *sup35* yeast mutants. As already discussed, *sup35* mutants have higher stop codon readthrough, which during stress can lead to the formation of proteins with new C-terminal domains that can enrich their phenotypic diversity (True and Lindquist, 2000). Importantly, prokaryotes can develop antibiotic resistance through mistranslation. An example of this has been seen in recent
studies with *Mycobacteria tuberculosis*; the antibiotic-resistant strains were found to have mutations leading to tRNA misacylation and therefore mistranslation (Javid et al., 2014, Su et al., 2016).

As discussed above, oxidative stress can directly alter tRNA aminoacylation. In *E. coli*, ThrRS has a cysteine in its editing domain that is sensitive to ROS, and upon oxidation, it can mischarged serine instead of threonine (Ling and Soll, 2010). Misincorporation of serine can lead to misfolding, but also it adds new phosphorylation sites that have been proposed to act as a signaling molecule to protect the cell (Ling and Soll, 2010). Another mechanism that acts to protect mammalian cells in an innate immune response against ROS-induced damage is Met-misacylation, which is triggered in cells that have been exposed to non-infectious viruses, toll-like receptor ligands, tunicamycin or MG132 (all increase the level of ROS) (Netzer et al., 2009).

1.2.3. Translation and ageing

As discussed in ‘1.1. Biology of ageing section’, ageing is influenced by evolutionary conserved alterations of nutrient-sensing pathways, for instance, IIS, mTOR and MAPK signalling pathways (Gems and Partridge, 2013b). Interestingly, one of the common mechanisms behind lifespan extension by these nutrient-sensing pathways is decreased protein translation.

mTOR can be regarded as an evolutionarily conserved regulator of longevity and translation (Gonskikh and Polacek, 2017). When mTOR is active under plentiful nutrient conditions, 4E-BP gets phosphorylated and it cannot bind eIF4E, which is free to join eIF4G initiating translation; thus mTOR affects what is considered one of the most crucial steps of translation initiation (Gebauer and Hentze, 2004). mTOR also regulates an essential step of elongation: it activates eEF2. mTOR leads to higher activity of eEF2 by inhibition of eEF2K, a highly specific kinase that inhibits its substrate by phosphorylation (Wang and Proud, 2006). Besides, mTOR activates S6K, which in turn is a kinase and activator of several translation factors, importantly ribosomal protein S6 (RPS6), eIF4B and eEF2 (Showkat et al., 2014). IIS forms a nutrient sensing network together with mTOR that affects translation. IIS activates AKT which in turn activates mTOR; additionally, IIS activates ERK1 and ERK2 that stimulate S6K (Tavernarakis, 2008). Consequently, lifespan-extending reduced levels of mTOR or IIS, either through pharmacological or genetic manipulations lead to a global decrease of translation both at initiation and elongation (Kaeberlein and Kennedy, 2008, Stanfel et al., 2009).
p38 protein kinases belong to the MAPK family which, as it has been discussed, are energy sensors largely involved in longevity. p38 activates mitogen-activated protein kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2). Mnk1 phosphorylates elf4E at Ser209, which has a conflicting effect: it has been shown to both stabilize the binding of elf4E and elf4G (leading to higher cap-dependent translation) but also to promote IRES-dependent translation (D’Abronzo et al., 2017). Thus, stress-mediated MAPK activation leads to translational changes that can affect ageing.

Another component linked to translation is the transcription factor Myc, which has been demonstrated to extend lifespan when downregulated in C. elegans, Drosophila and mice (Johnson et al., 2014, Greer et al., 2013, Hofmann et al., 2015). Most of the genes upregulated by myc are directly involved in translation and in fact, in the long-lived Myc+/− mice, there was also a decrease in the levels of ribosomal proteins (van Riggelen et al., 2010, Hofmann et al., 2015).

A direct role of translation in ageing has been found in several studies that genetically or pharmacologically modify levels of ribosomal proteins or translation factors. In yeast, lifespan extension has been achieved through a decrease in several ribosomal proteins, S6K or initiation factors (Kaeberlein et al., 2005, Smith et al., 2008, Chiocchetti et al., 2007, Steffen et al., 2012), also by genetically or pharmacologically depleting 60S ribosomal subunit biogenesis (Steffen et al., 2008a). Moreover, a study by the Kennedy and Kaeberlein labs, in which they screened 4698 single-gene deletions, showed that the long-lived strains fell mostly in the functional category of cytosolic ribosome mutants and were conserved in the nematode (McCormick et al., 2015). In C. elegans, decreasing the levels of several initiation factors or ribosomal proteins by RNAi or genetic depletion most robustly increases lifespan (Curran and Ruvkun, 2007, Hansen et al., 2007, Pan et al., 2007, Rogers et al., 2011, Syntichaki et al., 2007b, Dalton and Curran, 2018). In Drosophila, overexpression of 4EBP (i.e. repression of elf4E activity) extends lifespan (Zid et al., 2009). In mouse embryonic fibroblasts and B cells, overexpression of elf4E induces senescence (Ruggiero et al., 2004). Furthermore, a study found that the most striking hallmark of ageing in human-derived samples was a decrease in the expression of genes coding for proteins involved in translation and ribosomal function and that this could be a compensatory response to extend lifespan (Jung et al., 2015).

Despite a clear link between translation and ageing, the mechanism behind it remains largely unknown. Current hypotheses suggest that downregulation of translation extends lifespan by 1) differential translation of longevity-related genes, 2) improved protein
homeostasis and reduced proteotoxicity, 3) ameliorated cellular energy balance, 4) increased accuracy of protein synthesis.

One of the proposed mechanisms behind the lifespan-extending effects of reduced translation is that a subset of longevity-related genes is differentially expressed under this condition. In a study by the Kennedy and Kaeberlein laboratories, long-lived yeast cells with decreased levels of 60S ribosomal subunits had an overall reduction of mRNA levels but increased translation of Gcn4 mRNAs (Steffen et al., 2008a). Similarly, flies under dietary restriction had reduced cap-dependent translation but increased translation of mRNA with smaller 5'UTRs (Zid et al., 2009). Nevertheless, in C. elegans and yeast mutants with reduced eIF4G levels, there was not increased translation of any particular mRNA but there was enhanced translation efficiency of certain mRNAs compared to wild-type organisms (Rogers et al., 2011, Park et al., 2011). These more efficiently translated genes corresponded to proteins such as oxidative stress protectors and activating transcription factor 4 (Atf4), which interestingly is also translationally-upregulated in mice under dietary restriction or rapamycin treatment (Li et al., 2014).

Protein homeostasis, also known as proteostasis, has to be tightly regulated in cells to prevent aggregation of damaged proteins or their toxicity. Although evolution has helped develop very efficient protein control mechanisms, older organisms tend to accumulate altered and toxic proteins, and this is considered one of the hallmarks of ageing (López-Otín et al., 2013). Thus, if there is a decrease in protein synthesis, the protein control system has an increased capacity to eliminate the erroneous, oxidized and ubiquitinated proteins averting the ageing phenotype (Hipkiss, 2007). For example, in mammalian cells, inhibiting translation has shown to decrease the accumulation of ubiquitinated species and protein aggregates (Meriin et al., 2012b). Interestingly, studies with long-lived Snell mice, Crowded-litter mice, rapamycin-treated or calorie restricted mice have shown to have an increase in the newly synthesised protein to DNA ratio compared to controls, suggesting that these new proteins are created in the existing cells to replace damaged ones and thus that these models have better proteostasis than the shorter-lived controls (Drake et al., 2014, Drake et al., 2015, Drake et al., 2013).

In addition, translation is one of the most energy-expensive processes in cells, so it has been proposed that when there is a downregulation of this process, the cell, or even the whole organism, can invest its energy in other processes to maintain a healthier system overall (Labbadia and Morimoto, 2015).
1.2.3.1. Translation fidelity and ageing.

One of the less discussed hypotheses is that decreasing protein synthesis, or its rate, increases accuracy in the translated proteins. When translation rate is high, as for example in optimal growth conditions, decreasing fidelity and promotion of amino acid misincorporation has been described (Thompson and Karim, 1982, Fluit et al., 2007, Zaher and Green, 2009). Although translation accuracy has not been shown to decrease with age, any basal errors occurring can pose a special threat to older cells and organisms with less efficient protein turnover and clearing systems, because mistranslated and aberrant proteins can aggregate or/and accumulate damaging cells and causing age-related diseases (Ke et al., 2018, von der Haar et al., 2017).

Moreover, different drugs known to affect longevity alter translational errors. Paromomycin, increases translation errors and it has been shown to decrease the chronological lifespan of yeast, to accelerate cellular ageing in fibroblasts and to induce more errors in senescent human fibroblasts compared to young fibroblasts and (Holliday and Rattan, 1983, Luce and Bunn, 1989, Salas-Marco and Bedwell, 2005, von der Haar et al., 2017). Rapamycin extends lifespan in several model organisms and decreases the level of translational errors (Conn and Qian, 2013, Bjedov and Partridge, 2011); similarly, erythromycin that increases the lifespan of yeasts and other fungi also improves translational accuracy (Holbrook and Menninger, 2002, Menninger et al., 1994).

Furthermore, the correlation between translational accuracy and longevity has been shown in two recent studies using mammalian tissues. By using luciferase assays, the naked mole rat, the longest-lived rodent with unusual resistance to cancer, revealed increased translational fidelity compared to normal mice with a decrease in misincorporation, frameshift and stop codon skipping errors (Azpurua et al., 2013). Another study that investigated 17 different rodent species proved that translation fidelity (at the first and second codon especially) correlates with species maximum lifespan (Ke et al., 2017).

Genetically modifying translation accuracy has also been shown to affect lifespan. Yeast *SUP38* mutants are hypoaccurate RPS2 mutants that have shorter lifespans (von der Haar et al., 2017). In another fungus, *Podospora anserina*, eEF1α overexpression increased both accuracy and lifespan (Silar and Picard, 1994). Importantly, the lifespan-extending effect of this mutation is evolutionary conserved, as *Drosophila* is also longer lived when this elongation factor is overexpressed (Shepherd et al., 1989), and in germline-deficient long-lived *C. elegans eef1* gene is enriched compared to controls, albeit translation accuracy was not
measured in these two organisms (Sinha and Rae, 2014). Interestingly, it has been recently reported that mitochondrial translation accuracy can also affect ageing in *S. cerevisiae* (Suhm et al., 2018). In this report, the mitochondrial orthologue of bacterial RPS12 (mit-RPS12) was mutated either to be hypoaccurate (P50R) or hyperaccurate (K71T); the hypoaccurate strain had shorter chronological lifespan, was sensitive to paromomycin, and it had greater protein aggregation whereas the hyperaccurate strain had extended chronological lifespan, it was resistant to paromomycin it exhibited less aggregation and improved refolding. This translation accuracy experiments in yeast, using hyperaccurate and hypoaccurate mutants, suggest accuracy could be important pro-longevity effector in multicellular organisms.
1.3. Thesis Outline

The main aim of this thesis is to study if the common denominator of anti-ageing interventions is an increase in translation accuracy. For this purpose, I have used *Drosophila melanogaster* to study the effects of translation hyperaccuracy and hypoaccuracy on ageing and health span. For this, I have taken advantage of *Drosophila’s in vitro and in vivo* techniques to examine the effects of pharmacological and genetic interventions related to translation (Fig. 1.17). I downregulated in *Drosophila* several translation factors that can extend the lifespan of yeast and *C. elegans* (Hansen et al., 2007, Steffen et al., 2008b), and found several translation factors that when downregulated by RNAi can also improve lifespan and health span in flies. In parallel, I optimised for S2R+ cells a translation fidelity dual luciferase assay and I tested several anti-ageing drugs to see if they changed the levels of translation accuracy (Salas-Marco and Bedwell, 2005). In addition, I have studied how a mutation in the evolutionary conserved region of RPS23 that directly alters translation accuracy has an impact in the flies development, lifespan and response to environmental conditions (Alksne et al., 1993)

![Project outline diagram](image)
1.4. Thesis aims and objectives

1. Study if Drosophila’s lifespan can be extended by downregulating translation-related factors. This will be done by conducting a UAS-RNAi screen and assessing the lifespan of these flies.
2. Any lifespan extending UAS-RNAi lines will have to be corroborated by RT-qPCR.
3. We want to know if any of these longer-lived flies have also improved healthspan and stress resilience. For this, negative geotaxis assays and heat shock assays will be performed.
4. One of the main objectives is to determine if lifespan-extending interventions alter translation accuracy. For this, it will be necessary to adapt for Drosophila-derived S2R+ cells different accuracy reporters based on a dual luciferase assay. In addition, this assay will have to be optimised by producing lines stably expressing the reporters in S2R+ cells and the effect on accuracy of different drugs will be evaluated.
5. In order to study if the lifespan-extending effect of downregulating translation-related factors by UAS-RNAi is due to altered translation accuracy, the dual luciferase assay will have to be adapted to work in Drosophila.
6. In addition, to study how directly altering translation accuracy affects ageing, we aim to design and produce Drosophila accurate mutants by mutating evolutionarily conserved ribosomal proteins using the CRISPR-Cas9 system.
7. When these accuracy mutants are produced, we will aim to characterize any phenotypes, their developmental behaviour and eventually the effect of translation accuracy on lifespan, healthspan and stress resilience.
Chapter 2: Materials and Methods.
2. 1. *Drosophila* stocks and maintenance

2.1.1. Fly maintenance

All stocks were kept at 18°C in a 12:12 light: dark cycle in standard 1.0 sugar/yeast (SYA; sugar 50 g/l, yeast 100 g/l, agar 15 g/l) medium and tipped to fresh food every 4 weeks (Table 2.1). Experimental flies were reared at 25°C 60% humidity in a 12:12 light: dark cycle in the indicated food and transferred to fresh food every two to three days.

For experimental crosses, approximately 100 virgin flies and 60 males were left to mate in grape juice (Young’s Brew #5017458018513) agar for 48 hours. Upon 8 to 12 hours egg laying period, the eggs were collected in phosphate-buffered saline (PBS) pH 7.4 (ThermoFisher Scientific #10010023) and 18 µl squirted into bottles of 1.0 SYA food to ensure equal densities between treatments.

For experimental virgin flies were collected every 2 hours during a period of 8 hours post-emergence by anaesthetising them over ice as adult flies younger than 3 hours are susceptible to CO₂ (Perron et al., 1972). To split 2 days-old mated experimental females from males, the flies were anaesthetised using CO₂ (Perron et al., 1972).

2.1.2. *Drosophila* medium preparation

For most of the experiments, flies were fed standard sugar yeast agar (SYA) medium. This medium was prepared by boiling water with agar and then mixing sucrose and yeast. Once this mixture reaches 60°C, the antifungals nipagin (100 g/l dissolved in ethanol) and propionic acid (3 ml/l) were added and the food was dispensed into either vials (around 5 ml) or bottles (around 60 ml). Once the food had set, it was covered and stored at 4°C.

Table 2.9. Ingredients and their measurements necessary to cook 1 L sugar yeast agar medium.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>0.0 SYA</th>
<th>0.5 SYA</th>
<th>1.0 SYA</th>
<th>2.0 SYA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O (ml)</td>
<td>700</td>
<td>700</td>
<td>700</td>
<td>700</td>
<td>-</td>
</tr>
<tr>
<td>Agar (g)</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>Sigma #102000476</td>
</tr>
<tr>
<td>Sucrose (g)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>Silver Spoon</td>
</tr>
<tr>
<td>Yeast (g)</td>
<td>0</td>
<td>50</td>
<td>100</td>
<td>200</td>
<td>MP Biomedicals</td>
</tr>
<tr>
<td>Additional ddH₂O (ml)</td>
<td>223</td>
<td>196</td>
<td>170</td>
<td>118</td>
<td>-</td>
</tr>
<tr>
<td>Nipagin (ml)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>Sigma #H5501</td>
</tr>
</tbody>
</table>
The role of decreased protein synthesis in delaying ageing  
Martínez Miguel, V.E.

| Propionic acid (ml) | 3 | 3 | 3 | 3 | Sigma #402907 |

For all the experiments in which GAL4 Gene Switch driver was used, mifepristone (RU486) (Cayman Chemicals #10006317) was used for its activation (Fig. 2.1.). For this, a 100 mM stock was prepared by dissolving 1 g of RU486 in 23 ml ethanol (EtOH). 2 ml of this 100 mM stock solution was added to each litre of food to have a final concentration of 200 μM.

For the experimental crosses, grape juice agar is used, as this stimulates their mating and it facilitates egg collection (Eisses, 1997). These agar plates were prepared by boiling 25 g of agar with 500 ml of water and 300 ml of grape juice. Then, 50 ml of extra water were added and once the mixture reached 60°C, 21 ml of nipagin were added. This mixture was then dispensed and left to set until it could be stored at 4°C.

For some specific lifespans, the holidic medium was used as it allows to use less amount of drug due to its improved bioavailability compared to SYA medium, for example the optimal concentration for lifespan extension of rapamycin is 1 μM or 5 μM in holidic medium instead of 50 μM or 200 μM in 1 SYA food. This is a synthetic, chemically defined fly medium optimised for adult fly fitness described in (Piper et al., 2014). It was prepared by mixing the ingredients in Table 2.2 in a glass bottle, which was autoclaved. Then, the rest of the ingredients were added and the food dispensed. Once the food set, it was covered and stored at 4°C.

Table 2.2. Ingredients for one litre of holidic medium.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Stock concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
<td>-</td>
<td>Sigma</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.58 g</td>
<td>-</td>
<td>Sigma #I2752</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.82 g</td>
<td>-</td>
<td>Sigma #L8912</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.42 g</td>
<td>-</td>
<td>Sigma #T375</td>
</tr>
<tr>
<td>Sucrose</td>
<td>17.12 g</td>
<td>-</td>
<td>Sigma #S1888</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15 ml</td>
<td>20 mg/ml in 100% EtOH</td>
<td>Sigma #C8667</td>
</tr>
<tr>
<td>Acetate Buffer</td>
<td>100 ml</td>
<td>30 ml of acetic acid</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 g of KH₂PO₄ and 10 g of NaHCO₃, topped with water up to 1 l</td>
<td>#A.0400.PB15</td>
</tr>
</tbody>
</table>

For all the experiments in which GAL4 Gene Switch driver was used, mifepristone (RU486) (Cayman Chemicals #10006317) was used for its activation (Fig. 2.1.). For this, a 100 mM stock was prepared by dissolving 1 g of RU486 in 23 ml ethanol (EtOH). 2 ml of this 100 mM stock solution was added to each litre of food to have a final concentration of 200 μM.

For the experimental crosses, grape juice agar is used, as this stimulates their mating and it facilitates egg collection (Eisses, 1997). These agar plates were prepared by boiling 25 g of agar with 500 ml of water and 300 ml of grape juice. Then, 50 ml of extra water were added and once the mixture reached 60°C, 21 ml of nipagin were added. This mixture was then dispensed and left to set until it could be stored at 4°C.

For some specific lifespans, the holidic medium was used as it allows to use less amount of drug due to its improved bioavailability compared to SYA medium, for example the optimal concentration for lifespan extension of rapamycin is 1 μM or 5 μM in holidic medium instead of 50 μM or 200 μM in 1 SYA food. This is a synthetic, chemically defined fly medium optimised for adult fly fitness described in (Piper et al., 2014). It was prepared by mixing the ingredients in Table 2.2 in a glass bottle, which was autoclaved. Then, the rest of the ingredients were added and the food dispensed. Once the food set, it was covered and stored at 4°C.

Table 2.2. Ingredients for one litre of holidic medium.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Stock concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Autoclave</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
<td>-</td>
<td>Sigma</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.58 g</td>
<td>-</td>
<td>Sigma #I2752</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.82 g</td>
<td>-</td>
<td>Sigma #L8912</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.42 g</td>
<td>-</td>
<td>Sigma #T375</td>
</tr>
<tr>
<td>Sucrose</td>
<td>17.12 g</td>
<td>-</td>
<td>Sigma #S1888</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15 ml</td>
<td>20 mg/ml in 100% EtOH</td>
<td>Sigma #C8667</td>
</tr>
<tr>
<td>Acetate Buffer</td>
<td>100 ml</td>
<td>30 ml of acetic acid</td>
<td>ThermoFisher Scientific</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 g of KH₂PO₄ and 10 g of NaHCO₃, topped with water up to 1 l</td>
<td>#A.0400.PB15</td>
</tr>
</tbody>
</table>
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

### Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
<th>stock concentration</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂•2H₂O</td>
<td>1 ml</td>
<td>250 g/l</td>
<td>Sigma</td>
<td>#P9791 #S8875</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1 ml</td>
<td>250 g/l</td>
<td>Sigma</td>
<td>#C7902</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>1 ml</td>
<td>2.5 g/l</td>
<td>Sigma</td>
<td>#C7631</td>
</tr>
<tr>
<td>FeSO₄•7H₂O</td>
<td>1 ml</td>
<td>2.5 g/l</td>
<td>Sigma</td>
<td>#F7002</td>
</tr>
<tr>
<td>MnCl₂•4H₂O</td>
<td>1 ml</td>
<td>1 g/l</td>
<td>Sigma</td>
<td>#M3634</td>
</tr>
<tr>
<td>ZnSO₄•7H₂O</td>
<td>1 ml</td>
<td>25 g/l</td>
<td>Sigma</td>
<td>#Z0251</td>
</tr>
</tbody>
</table>

**After autoclave**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Description</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleic acid and lipid solution</td>
<td>8 ml</td>
<td>6.25 g of choline chloride, 0.63 g myo-inositol, 8.13 g inosine and 7.5 g of uridine. Water up to 1 l</td>
<td>Sigma</td>
<td>#C1879 #I7508 #I4125 #U3750</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>20 g</td>
<td>20 mg/ml</td>
<td>Sigma</td>
<td>#C8667</td>
</tr>
<tr>
<td>Glutamate</td>
<td>9.11 ml</td>
<td>100 g/ml</td>
<td>Sigma</td>
<td>#G5889</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2.64 ml</td>
<td>50 g/ml</td>
<td>Sigma</td>
<td>#C127</td>
</tr>
<tr>
<td>Vitamin solution</td>
<td>21 ml</td>
<td>67 mg thiamine, 33 mg riboflavin, 399 mg nicotinic acid, 516 mg calcium D-pentothenate, 83 mg pyridoxine and 7 mg biotin and water up to 1 l</td>
<td>Sigma</td>
<td>#T4625 #R4500 #N4126 #P2250 #P9755 #B4501</td>
</tr>
<tr>
<td>Folic acid</td>
<td>1 ml</td>
<td>0.5 g/l</td>
<td>Sigma</td>
<td>#F7876</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>6 ml</td>
<td></td>
<td>Sigma</td>
<td>#P5561</td>
</tr>
<tr>
<td>Nipagin</td>
<td>15 ml</td>
<td>100 g/l methyl 4-hydroxybenzoate in 95% EtOH</td>
<td>Sigma</td>
<td>#H5501</td>
</tr>
<tr>
<td>Essential amino acid stock solution</td>
<td>30.255 ml</td>
<td>23.51 g arginine, 11.21 g histidine, 28.70 g lysine, 5.62 g methionine, 15.14 g phenylalanine, 21.39 g threonine, 7.27 g tryptophan, 22.12 g valine and water up to 1 l</td>
<td>Sigma</td>
<td>#A5131 #H8000 #L5626 #M9625 #P2126 #T8625 #T0254 #V0500</td>
</tr>
<tr>
<td>Non-essential amino acid stock solution</td>
<td>30.255 ml</td>
<td>26.25 g alanine, 13.89 g asparagine, 13.89 g aspartic acid, 30.09 g glutamine, 17.89 glycine, 9.32 g proline, 12.56 g serine and water up to 1 l</td>
<td>Sigma</td>
<td>#A7627 #A0884 #A6383 #G3126 #G7126 #P0380 #S4500</td>
</tr>
</tbody>
</table>
2.1.3. Stock list

To drive the downregulation of the genes of interest we used RNAi lines (from Viena Drosophila Research Centre (VDRC)), combined with the Gene-Switch System, which is activated in presence of the drug mifepristone (RU486) (Nicholson et al., 2008, Osterwalder et al., 2001b). This was achieved by crossing a virgin female fly that has the Gene-Switch driver gene with a male carrying the UAS-RNAi construct. The driver expressed a modified version of the transcription factor GAL4, Gene-Switch that is activated upon binding to RU486 (Fig. 2.1.). This activated Gene-Switch transcription factor then binds the upstream activated sequence (UAS) of the RNAi construct, thereby yielding expression of a specific hairpin structure, which silences expression of the target gene via RNA interference (RNAi). Different RNAi lines were used to downregulate either translational factors, ribosomal proteins or components of signalling pathways involved in translation (Table 2.3). The drivers were either S106, which drives expression in the fat body and intestine or the ubiquitous actin Gene Switch (ActGS) which drives the expression ubiquitously (Poirier et al., 2008). RU486 was administered in the fly food.

Table 10.3. List of RNAi lines and their Computer Generated numbers (CG). These were purchased from the Vienna Drosophila RNAi Centre (VDRC) or Bloomington Drosophila Stock Centre (BDSC) so their stock numbers are shown too.

<table>
<thead>
<tr>
<th>Protein</th>
<th>CG number</th>
<th>Stock number</th>
</tr>
</thead>
<tbody>
<tr>
<td>elf1</td>
<td>CG17737</td>
<td>105763</td>
</tr>
<tr>
<td>elf1α</td>
<td>CG1873</td>
<td>102736</td>
</tr>
<tr>
<td>elf2α</td>
<td>CG9946</td>
<td>104562</td>
</tr>
<tr>
<td>elf3-S8</td>
<td>CG4954</td>
<td>26667</td>
</tr>
<tr>
<td>elf3-S9</td>
<td>CG4878</td>
<td>26651</td>
</tr>
<tr>
<td>elf3-S9</td>
<td>CG4878</td>
<td>107829</td>
</tr>
<tr>
<td>elf3-S9</td>
<td>CG4880</td>
<td>27609</td>
</tr>
<tr>
<td>elf3-S10</td>
<td>CG9805</td>
<td>34353</td>
</tr>
<tr>
<td>elf4A3</td>
<td>CG7483</td>
<td>32444</td>
</tr>
<tr>
<td>elf4A</td>
<td>CG9075</td>
<td>33970</td>
</tr>
<tr>
<td>elf4E</td>
<td>CG4035</td>
<td>7800</td>
</tr>
<tr>
<td>elf4E</td>
<td>CG4035</td>
<td>34096</td>
</tr>
<tr>
<td>elf4E</td>
<td>CG4035</td>
<td>100722</td>
</tr>
<tr>
<td>elf4E</td>
<td>CG4035</td>
<td>7800</td>
</tr>
<tr>
<td>elf4G1</td>
<td>CG10811</td>
<td>33049</td>
</tr>
<tr>
<td>elf4G1</td>
<td>CG10811</td>
<td>17002</td>
</tr>
<tr>
<td>elf4G1</td>
<td>cg10811</td>
<td>17003</td>
</tr>
<tr>
<td>elf4G2</td>
<td>CG10192</td>
<td>18031</td>
</tr>
<tr>
<td>elf4G2</td>
<td>CG10192</td>
<td>41963</td>
</tr>
<tr>
<td>elf4G2</td>
<td>CG10192</td>
<td>42893</td>
</tr>
<tr>
<td>Gene</td>
<td>Accession</td>
<td>Position</td>
</tr>
<tr>
<td>--------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>eEF2</td>
<td>CG2238</td>
<td>107268</td>
</tr>
<tr>
<td>EFTuD2</td>
<td>CG4849</td>
<td>21963</td>
</tr>
<tr>
<td>EFTuD2</td>
<td>CG4849</td>
<td>108596</td>
</tr>
<tr>
<td>EFTuD2</td>
<td>CG4849</td>
<td>10828</td>
</tr>
<tr>
<td>EF-G2</td>
<td>CG31159</td>
<td>46144</td>
</tr>
<tr>
<td>EF-G2</td>
<td>CG31159</td>
<td>104187</td>
</tr>
<tr>
<td>eRF1</td>
<td>CG5605</td>
<td>45027</td>
</tr>
<tr>
<td>RpS2</td>
<td>CG5920</td>
<td>20963</td>
</tr>
<tr>
<td>RPS2</td>
<td>CG5921</td>
<td>100308</td>
</tr>
<tr>
<td>RPS3</td>
<td>CG6779</td>
<td>37742</td>
</tr>
<tr>
<td>RPS9</td>
<td>CG3395</td>
<td>100712</td>
</tr>
<tr>
<td>RPL9</td>
<td>CG6141</td>
<td>109647</td>
</tr>
<tr>
<td>RPS11</td>
<td>CG8857</td>
<td>23475</td>
</tr>
<tr>
<td>RPS15</td>
<td>CG8332</td>
<td>104439</td>
</tr>
<tr>
<td>RPS20</td>
<td>CG15693</td>
<td>105298</td>
</tr>
<tr>
<td>RpS23</td>
<td>CG8415</td>
<td>35421</td>
</tr>
<tr>
<td>RpS26</td>
<td>CG10305</td>
<td>33393</td>
</tr>
<tr>
<td>RpS26</td>
<td>CG10305</td>
<td>16012</td>
</tr>
<tr>
<td>RPL19</td>
<td>CG2746</td>
<td>41952</td>
</tr>
<tr>
<td>RPL19</td>
<td>CG10122</td>
<td>110680</td>
</tr>
<tr>
<td>mRPL4 RNAi</td>
<td>CG5818</td>
<td>101351</td>
</tr>
<tr>
<td>Gcn2</td>
<td>CG1609</td>
<td>103976</td>
</tr>
<tr>
<td>myc</td>
<td>CG10798</td>
<td>36123</td>
</tr>
<tr>
<td>myc</td>
<td>CG10798</td>
<td>2947</td>
</tr>
<tr>
<td>myc</td>
<td>CG10798</td>
<td>106066</td>
</tr>
<tr>
<td>raptor</td>
<td>CG4320</td>
<td>34814</td>
</tr>
<tr>
<td>raptor</td>
<td>CG4320</td>
<td>41912</td>
</tr>
<tr>
<td>raptor</td>
<td>CG4320</td>
<td>106491</td>
</tr>
<tr>
<td>rictor</td>
<td>CG8002</td>
<td>36699</td>
</tr>
</tbody>
</table>
The role of decreased protein synthesis in delaying ageing  
Martínez Miguel, V.E.

Driver flies were backcrossed at least 6 times into the outbred white *Dahomey* (*w*^Dah^). This is a stock that was produced by incorporating the white 1118 (*w*1118) mutation into the wild-type *Dahomey* background (Bass et al., 2007b).

### 2.1.4. Lifespan analysis

In the case of lifespan analysis of flies with downregulated translation by RNAi, crosses were obtained by mating approximately 100 female virgin flies (with the driver transgene) and 60 adult males (with the UAS-RNAi transgene) in cages with grape juice agar (see Table 2 for the lines used in each lifespan experiment). The eggs were squirted as described in ‘2.1.1 General fly maintenance section’. Once the adult flies hatched and mated for 48 hours, the...
males and females were split, selected and put on ethanol supplemented control food or 200 μM RU486 supplemented food.

To study the effect of different drugs on longevity, wDah female and male flies were crossed also in cages with grape juice agar plates and the 18 μl of eggs were squirted per bottle, thereby raising experimental flies under equal densities. Once the adult flies emerged, they were left to mate for 48 hours and the females were selected and placed into vials with food containing different drug concentration.

For the lifespan analysis of the translation fidelity mutation rps23-K60R, both heterozygotes and homozygotes were generated. For the heterozygous cross, around 100 female virgin wDah flies were crossed with either experimental or control males. For the homozygous cross, virgin female and male flies from the same genotype were crossed. In both cases, the crosses were left to mate and the eggs were squirted at equal densities. Because of the developmental delay of RPS23 K60R mutants, this cross was set and squirted 2 days prior to the control cross. Once the adults emerged, they were left to mate for 48 hours and females and males were split under CO₂ and put in vials with their corresponding media.

These flies were then reared at 25°C in a 12h: 12h light: dark cycle and constant 60% humidity with a density of 15 flies per vial. They were changed to fresh vials three times a week and deaths were scored.

### 2.1.5. Drugs used in lifespans

Different drugs were tested to see the effects on longevity. Some of them were dissolved in the standard 1.0 SYA food (Bass et al., 2007a), whereas others were dissolved in holidic medium, as this medium allows the drug to be more bioavailable and hence ten to hundred fold less amount is required (Table 2.4) (Piper et al., 2014).
### Table 2.4. Drugs used in lifespans.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Media used</th>
<th>Stock Concentration</th>
<th>Drug amount</th>
<th>Drug Solvent Volume</th>
<th>Vol. drug / Vol.</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>1 SYA</td>
<td>65 mM</td>
<td>7 mg</td>
<td>20 ml EtOH</td>
<td>15 ml/l</td>
<td>Sigma (C7698)</td>
</tr>
<tr>
<td>Diazaborine</td>
<td>Holidic</td>
<td>30 mM</td>
<td>7 mg</td>
<td>778 μl DMSO</td>
<td>1 ml/l</td>
<td>Calbiotech (5.30729.0001)</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>1 SYA</td>
<td>106.7 mM</td>
<td>0.55 g</td>
<td>5.625 ml H2O</td>
<td>7.5 ml/l</td>
<td>Sigma (P5057)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Holidic</td>
<td>20 mM</td>
<td>16.5 mg</td>
<td>902.5 μl EtOH</td>
<td>250 μl/l</td>
<td>LC Laboratories (R5000)</td>
</tr>
<tr>
<td>Salubrinal</td>
<td>Holidic</td>
<td>10 mM</td>
<td>3 mg</td>
<td>625 μl DMSO</td>
<td>1 ml/l</td>
<td>CruzChem (SC202332A)</td>
</tr>
<tr>
<td>Torin 1</td>
<td>Holidic</td>
<td>10 mM</td>
<td>9.1 mg</td>
<td>1.029 ml DMSO</td>
<td>1 ml/l</td>
<td>Adooq BioScience (A11587)</td>
</tr>
<tr>
<td>Torin 2</td>
<td>Holidic</td>
<td>4 mM</td>
<td>1 mg</td>
<td>587 μl DMSO</td>
<td>250 μl/l</td>
<td>LC Laboratories (T8448)</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>Holidic</td>
<td>10 mM</td>
<td>10 mg</td>
<td>1.184 ml H2O</td>
<td>1.5 ml/l</td>
<td>Cayman Chemical Co. (1145)</td>
</tr>
</tbody>
</table>

#### 2.2. Healthspan and behavioural analysis in *Drosophila*

##### 2.2.1. Negative geotaxis or climbing assay

Negative geotaxis is a reflex motor behaviour exhibited by flies. When the flies’ container is tapped, a climbing behaviour is induced which has been repeatedly shown to naturally decline with age (Jones and Grotewiel, 2011). For this purpose, 10 vials (without food) with 15 flies in each were kept in a Drosoflipper device with other 10 empty vials on top...
(Fig. 2.2.). These flies were left for at least 15 minutes to accommodate. Then, a lamp was placed above the Drosoflipper to encourage climbing towards the light source, and the vials in the Drosoflipper were tapped five times; the flies were left to climb up for one minute and a picture was taken. This was repeated three times for each genotype (with 10 vials in each Drosoflipper). The number of flies in the upper part and the lower part were scored afterwards and the average between the three times was determined. The performance index (PI) for each vial was then calculated as $\frac{1}{2} \left( (\text{total number of flies} + \text{flies at the top} - \text{flies at the bottom}) / \text{total number of flies} \right)$. This assay was performed once a week for the first 5 or 6 weeks. This assay is a standard assay to estimate fly health.

![Figure 2.2. Schematic representation of the negative geotaxis or climbing assay using Drosoflippers.](image)

2.2.2. Stress resistance assays

For the stress assays, flies were aged for 7-10 days on RU486 or control food before the assay was performed. For all the assays, deaths were scored around 3 times a day when the flies started dying.

In order to study the unfolded protein response of the flies, tunicamycin (Cayman Chemical Company #11445) the drug was supplemented in holidic medium as shown in Table 2.4.

A heat shock assay was performed to study the resistance of the experimental flies. For this purpose, 15 flies were transferred to empty vials. These vials were placed in a water bath at 39°C and the number of immobile flies was counted every five minutes.
2.2.3. Fertility assay

A parameter of the health of female flies is their fecundity (Partridge et al., 2005). To assess this, flies were in a vial with the corresponding food for approximately 24 hours and the eggs they laid were counted. The total number of eggs was divided by the number of flies and the exact time spent in the vial.

2.2.4. Developmental assay

Flies were let to lay eggs in the corresponding food for 24 hours and the number of eggs was counted. Then, the number of pupae was also counted. The adult flies emerging were also scored and their sex and phenotype recorded twice every day until no more flies emerged.

2.3. Gene expression analysis

To measure the level of gene silencing by the RNAi in the experimental flies or S2R+ cells treated with double stranded RNA (dsRNA), Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) was performed in the RU486 supplemented and control flies.

2.3.1. Tissue preparation

The flies studied by RT-qPCR were RNAi lines under the S1106 fat body and intestine driver; therefore, only these tissues were tested. From five flies per sample the fat body and intestine were dissected in cold 1x PBS and immediately placed in BioPulverizer Lysing Matrix D tubes (MP Biomedicals #16913100) on dry ice. The dissected samples were then stored at -80°C.

We also examined the RNA abundance of S2R+ cells that had been previously treated with dsRNA. For this, Celia Lujan seeded 100,000 cells/well in 48 well plates and treated with dsRNA as indicated in ‘2.9.2. dsRNA treatment of S2R+ cells’. After 72 hours treatment with the indicated dsRNA, 12 wells of each condition were pulled together to then extract their RNA.

2.3.2. RNA extraction

For RNA extraction, 1 ml of trizol (ThermoFisher Scientific #15596026) per sample was added to the frozen dissected fly fat body and intestines. These were then homogenised in a Ribolyser (FastPrep® Classic) (MP Biomedicals 116004500) at 6.5 m/s for 10 seconds. The
samples were incubated at room temperature for 5 minutes and 200 μl of chloroform (Sigma #C2432) was added and vortexed for 15 seconds. After 3 minutes at room temperature, the samples were centrifuged at 11000 rpm (12000 G) for 15 minutes at 4°C. An aqueous layer was formed in the upper part, which was transferred to a new microcentrifuge tube. One volume of isopropanol (Sigma #I9516) was added to each sample and incubated for 10 minutes at room temperature. After this incubation, the samples were spun at 11000 rpm for 10 minutes at 4°C. The pellets were then washed with 1 ml of 75% ethanol and again spun at 11000 rpm for 10 minutes at 4°C. Finally, the pellets are resuspended in 20 μl of nuclease-free water (ThermoFisher Scientific #AM9937). Concentration and purity was determined using NanoDrop OneC spectrophotometer (ThermoFisher Scientific). The RNA samples were stored at -80°C.

To extract the RNA of the cells that had been previously treated with dsRNA were centrifuged at 1200 g for 7 minutes and their media was removed. The pellets were then resuspended in 1 ml Trizol and left for 5 minutes at room temperature. After this incubation, they were stored at -80°C. These samples were then thawed at room temperature for 5 minutes and 200 μl chloroform added. These were shaken and incubated for 3 minutes to be then centrifuged at 12000 g for 15 minutes at 4°C. To recover the RNA, 500 μl isopropanol were added and incubated for 10 minutes at room temperature. These were then centrifuged for 10 minutes at 12000 g at 4°C and the pellets were washed twice with 75% ethanol and left to air dry. Once the pellets were dried, they were resuspended in 50 μl RNase-free water. The RNA concentrations were then measured by NanoDrop OneC spectrophotometer.

2.3.3. cDNA conversion

The RNA has to be converted into complementary DNA (cDNA). For this, 1 μg of RNA is diluted in 3.5 μl of nuclease-free water. Then, 0.5 μl of 10x DNA Buffer (Ambion DNase I kit) (ThermoFisher Scientific #AM2222). This was then incubated at 37°C for 30 minutes. After this incubation, 1 μl of 24 mM ethylenediaminetetraacetic acid (EDTA; 0.5 M, pH 8) (ThermoFisher Scientific #15575020) was added and the samples were incubated at 75°C for 5 minutes. The samples were then chilled on ice for 2 minutes, and 2 μl of Oligo d (T) 23 VN were added and followed by an incubation at 65°C for 5 minutes. After this, they were spun briefly and left on ice. Then, 10 μl of ProtoScript II Reaction mix (2x) and 2 μl of ProtoScript II Enzyme mix (10x) (New England Biolabs #E6560S) were added. Finally, the samples were incubated for 1 hour at 42°C and the enzyme was then inactivated at 80°C for 5 minutes. The cDNA samples were stored at -20°C.
2.3.4. RT-qPCR

This step was performed by Celia Lujan, she prepared a master mix for the RT-qPCR with 10 μl Power SYBR Green PCR Master Mix (ThermoFisher Scientific #4367659), 1 μl of the forward primer (100 μM) and 1 μl of the reverse primer (100 μM) (Table 2.5). 2 μl of cDNA were added to calculate a standard curve with concentrations from 1:1 to 1:32; the rest of the cDNA was diluted to 1:2 with nuclease-free water and also 2 μl were loaded in the plate. The plate was then read in an Eppendorf Realplex Mastercycler® (Eppendorf EPPE6300000.507) and a programme of 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds plus a melting curve were run.

The same primers were used for the S2R+ cell and the flies RT-qPCRs

Table 2.5. List of primers and their sequence.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>654</td>
<td>Act5c qPCR F</td>
<td>GAGCGCGGTTACTCTTCAC</td>
</tr>
<tr>
<td>655</td>
<td>Act5c qPCR R</td>
<td>GCCATCTCTGCTCAAAGTC</td>
</tr>
<tr>
<td>696</td>
<td>eIF4E qPCR1 F</td>
<td>TGTTGAGACGGGAAGACC</td>
</tr>
<tr>
<td>697</td>
<td>eIF4E qPCR1 R</td>
<td>CCTGGGCTTGACATCTTA</td>
</tr>
<tr>
<td>484</td>
<td>eIF4G qPCR F</td>
<td>GGTATGATAGGTTATGCTTCG</td>
</tr>
<tr>
<td>485</td>
<td>eIF4G qPCR R</td>
<td>ACTGATGACTGGGCTTTAACAG</td>
</tr>
<tr>
<td>419</td>
<td>eEF2 qPCR F</td>
<td>AGCTAATGAACCGATTGG</td>
</tr>
<tr>
<td>420</td>
<td>eEF2 qPCR R</td>
<td>AGCGTTTGTGTCCAGCTCT</td>
</tr>
<tr>
<td>429</td>
<td>Rps23 qPCR F</td>
<td>CGCTTCAAGGTGTCAAGGT</td>
</tr>
<tr>
<td>430</td>
<td>Rps23 qPCR R</td>
<td>AGATCTTGGCCGTTCCTTCT</td>
</tr>
</tbody>
</table>

2.4. Protein purification and analysis

2.4.1. Protein extraction

The flies used for western blots were snap frozen in liquid nitrogen and stored at -80°C. To prepare the samples 3 whole flies or 6 abdomens without ovaries were homogenized in 60 μl Laemmli buffer 2x (BioRad #1610737) with 50 mM dithiothreitol (DTT) (Sigma D0632). The samples were then boiled at 95°C for 5 minutes and centrifuged at 11000 rpm for 3 minutes.

When whole flies were used for the western blot, protein quantification was omitted as equal loading was achieved by using the same number of flies per samples. However, for dissected tissue, sample quantity was more variable so protein amount was determined using
the Pierce™ BCA (bicinchoninic acid) Protein Assay (ThermoFisher Scientific #23227). For this assay, samples were prepared as above in Laemmli sample buffer, but without DTT, as at concentration of 50mM DTT interferes with the Pierce™ BCA Protein Assay. Different amounts of bovine serum albumin was used for the standard curve. Once the protein content was calculated, DTT was added and samples were boiled for additional 2 minutes, and adequate number of samples loaded to the polyacrylamide gel.

2.4.2. SDS-PAGE electrophoresis

The loaded precast gels (BioRad #5678124) were run for 33 minutes at 200 V in running buffer (28.8 g glycine (Sigma #G8898), 6.06 g TRIS 1M HCl pH 7.4 base (Sigma #93362) and 2 g sodium dodecyl sulphate (SDS) (Sigma L3771)).

2.4.3. Immunoblotting

Separated samples transferred to a nitrocellulose membrane using a “wet” transfer at 100 V for 1 hour in cold transfer buffer (28.82 g glycine, 6.06 g TRIS 1M HCl pH 7.4 base and 2% EtOH). The membranes were stained with Ponceau S solution (Sigma #P7170) to verify efficiency of the transfer, and then blocked for 1 hour with 5% milk (Sigma #70166) in TBS-Tween (50 ml TRIS 1 M HCl pH 7.4 base, 30 ml NaCl 5 M (Sigma #S3014)) with 0.05% Tween (Sigma #P1379). Once blocked, a solution of the primary antibody in 5% Bovine Serum Albumin (BSA) (Sigma #A7906) (Bovine Serum Albumin dissolved in TBST) was added and incubated overnight at 4°C. The primary antibodies were dissolved as follows: phospho-eIF2α (Ser51) 1:1000 (CellSignaling #3398S); phospho-4EBP1 (Thr37/46) 1:500 (CellSignaling #2855S); phospho-Drosophila p70 S6 Kinase (Thr398) 1:500 (CellSignaling #9206S); non-phospho-4EBP1 (Thr46) 1:500 (CellSignaling #4923); β-actin 1:2000 (CellSignaling #8457S); GAPDH 1:2000 (GeneTex #GTX100118); Firefly 1:500 (GeneTex #GTX125849); Renilla 1:500 (GeneTex #GTX125851). Subsequently, the membranes were washed 3 times for 5 minutes in TBST and incubated for 1 to 2 hours with the secondary antibody anti-rabbit (CellSignaling #7074S) or anti-mouse (CellSignaling #7076S) antibodies at 1:2000 dilution in 5% milk in TBST. After washes, images were taken after adding Chemiluminescence ECL Substrate (ThermoFisher Scientific #34077) or Clarity™ Western ECL (enhanced chemiluminescence) substrate (BioRad #170-5060) in an ImageQuant LAS 4000 (GE Healthcare Life sciences # 28955810)

2.5 Overall translation measurements

To measure the level of global protein synthesis, a puromycin-based assay was used. Puromycin dihydrochloride is a tRNA analogue that gets incorporated into newly-synthesised
peptides causing premature chain termination and hence inhibiting protein synthesis, allowing its detection in immunoblotting with a puromycin monoclonal antibody (Deliu et al., 2017).

2.5.1. Sample preparation

The level of global protein synthesis was measured in S106>RNAi flies and therefore the fat body and intestines are the tissues of interest: however, we only used the fat bodies to avoid any cross-contamination from the intestinal microbiota. For this, 3 flies’ abdomens were dissected in a drop of Schneider’s Drosophila medium (ThermoFisher Scientific #21720024) and transferred to a microcentrifuge tube with 15 μl of puromycin dissolved in Schneider medium (10 μg/ml) (Sigma #P8833). The concentration of puromycin was based on previous literature (Deliu et al., 2017, Filer et al., 2017). The samples were incubated shaking at 750 rpm for 30 minutes at 25°C in an Eppendorf ThermoMixer. After this incubation, the samples were snap frozen in dry ice and stored at -80°C.

When this was performed in S2R+ cells, the plates were centrifuged for 5 minutes at 600 g media of the cells was aspirated and replaced with Schneider containing 10 μg/ml of puromycin. The cells were incubated for 30 minutes at 25°C. After the incubation, 6 wells of the same condition were pulled together into a 1.5 ml microcentrifuge tube. The samples were centrifuged at 900 rpm for 5 minutes, and then the media was aspirated. The pellets were washed with 100 μl PBS and centrifuged again at 900 rpm for 5 minutes. The PBS was aspirated, and the pellets frozen in dry ice. The samples were then stored at -80°C.

2.6. Translation fidelity assay in vivo

To assess the translation fidelity of the flies, a dual luciferase reporter was incorporated in Drosophila. This reporter is based on a Renilla luciferase followed by a firefly luciferase that are separated by an in-frame linker sequence that results in the simultaneous expression of both proteins (Fig. 2.3) (Salas-Marco and Bedwell, 2005). This linker sequence codes for a sense codon in the controls and the misinformation reporter or a stop-codon (UGA) to allow assessment of stop codon readthrough. There is also a reporter with a mutation in the active site of firefly (H245K) that allows measuring misincorporation levels. In all cases, Renilla luciferase is used to normalize the level of mRNA abundance and efficiency of translation initiation (Salas-Marco and Bedwell, 2005).
The role of decreased protein synthesis in delaying ageing | Martínez Miguel, V.E.

2.6.1. Cloning of the translation fidelity reporters for *Drosophila*

The translation fidelity reporters were cloned in the pattB pUAST vector under the hsp70 promoter by Ivana Bjedov.

---

**A)** Stop codon readthrough reporter

![Stop codon readthrough reporter diagram]

**B)** Misincorporation reporter

![Misincorporation reporter diagram]

Figure 2.3. Dual luciferase reporter constructs to measure translation fidelity. There are four dual luciferase reporters in total. One has the two luciferases that are linked by a readthrough with a control amino acid (Arg) A). Another, the stop codon readthrough reporter has a UGA Stop codon in the readthrough A). There is another control with a Gln in the readthrough B). The fourth is the misincorporation reporter and it has a mutation in the active site of firefly so it renders it inactive B). Figure based on (Salas-Marco and Bedwell, 2005).
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 2.4. Dual luciferase constructs cloned into the final vector. The Dual-Luciferase constructs were cloned into the pattB – pUAST vector under the hsp70 promoter.

2.6.2. Fly generation and backcrossing of the translation fidelity reporters

The fidelity reporters were generated by sending the dual luciferase reporters cloned in the pUAST-attB vector to be injected by BestGene. They were injected in the chromosome 2L in the attP33 site.

These flies were backcrossed to the white *Dahomey* background as follows:

F1: 100 ♀ Dual luciferase reporter (DLR) x 60 ♂ wDah/wDah

F2-F9: 100 ♀ wDahomey x 60 ♂ DLR/+ or

F10: 100 ♀ DLR/+ x 60 ♂ DLR/+
In order to check the translation fidelity of the RNAi lines, a double mutant of the dual luciferase reporters and the S1106 driver had to be generated. These genes are both in the second chromosome, so the chromosome had to recombine to contain both. The crosses were as following:

**F1:**

\[
\frac{100 \varphi}{S1106^{+}} + \frac{60 \sigma}{\text{Dual luciferase reporter (DLR)}} \times \frac{+}{+} \text{ Dual luciferase reporter (DLR)}^{+} +
\]

**F2:**

\[
\frac{20 \varphi}{S1106^{+}} \frac{DLR}{+} \times 20 \sigma \frac{sp}{cyo} \frac{MKRS}{TM6B}
\]

**F3:**

\[
\frac{1 \varphi}{S1106.DLR^{+}} \frac{cyo}{+} \times 1 \sigma \frac{+}{cyo} \frac{MKRS}{+}
\]

**F4:**

\[
\frac{20 \varphi}{S1106.DLR^{+}} \frac{cyo}{+} \times 20 \sigma \frac{S1106.DLR^{+}}{cyo} \frac{+}{+}
\]

**PCR detection of S1106**

In addition, S1106 have a red eye marker and the dual luciferase reporters have an orange eye marker, so visual identification was not possible and a PCR had to be done to find which flies had both S1106 and DLR genes. Flies from the F3 cross were snap frozen at in dry ice and their genomic DNA was extracted by adding 50 µl of Squishing Buffer (10 mM Tris HCl pH8 (Affymetrix #22638)); 1 mM EDTA (Sigma #EDS-100G); 25 mM NaCl (Sigma #S3014) ) with 0.2 mg/ml proteinase K (Biotechnology #E195). The flies were mashed and incubated for one hour at 37°C and then the proteinase K was inactivated by heating up the samples at 95°C for 15 minutes. Then, 0.5 µl of this DNA was then mixed with 2.5 µl of Taq Buffer, 0.5 µl dNTPs (deoxyribonucleotide triphosphate) (10 mM), 0.5 µl of primer 131 (100 µM) and 435 (100 µM), 0.25 µl Standard Taq Polymerase and water up to 25 µl (Table 2.6). The samples were then run in a PCR for 3 minutes at 95°C, 37 cycles of 95°C for 30 seconds, 60 seconds at 54°C, 2 minutes at 68°C and a final annealing for 5 minutes at 68°C. The PCR products were then run for 30 minutes in a 1.5% agarose gel (Fig. 2.5.).
Table 2.6. Primers and their sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>131</td>
<td>Firefly_F</td>
<td>GGAAGATCTATGACTCGAAAGTTTATGATCCAG</td>
</tr>
<tr>
<td>435</td>
<td>Firefly_R</td>
<td>GCCTATGCAGTTGCTCTCC</td>
</tr>
</tbody>
</table>

Figure 2.5. Representative image of the gel for the PCR products of the screen for positive S1106, dual luciferase reporters double mutants. The PCR with primers 131 and 435 amplifies a 1048bp fragment if the DNA has the dual luciferase reporter.

2.6.3. Fly crossing scheme

Virgin female flies of the four different fidelity reporters were collected and crossed to males of the flies of interest as described in ‘2.1. Drosophila stocks and maintenance’ section. The adult and mated progeny were split to their corresponding food (15 female flies/vial).

F0: ♀ DLR x ♂ RPS32 K60R or control  
F1: DLR, RPS23 K60R or DLR, control

Or

F0: ♀ S1106, DLR x ♂ RNAi lines  
F1: S1106, DLR/+; RNAi/+  

2.6.4. Sample preparation for the dual luciferase assay

Four flies per sample were mashed with 35 μl of 1x passive lysis buffer (PLB) (Promega #E1910) and left shaking for 4 hours at room temperature.
2.6.5. Dual luciferase assay *in vivo*

30 μl of sample was transferred to a 96 well white microplate (Greiner Bio-one #655074) leaving an empty well between samples to avoid signal cross-talk.

The plate was read in a Varioskan™ LUX microplate reader (ThermoFisher Scientific #VLOLO0TD0). The program used was: Well loop, 10,000 ms luminescence read, 50 μl Luciferase Assay Buffer II (LAR II) (Promega #E1910) dispensed to each well, 10,000 ms luminescence read, 50 μl Stop&Glo® buffer dispensed to each well, 10,000 ms luminescence read.

2.7. *Drosophila* translation fidelity mutant generation

2.7.1. CRISPR design

2.7.1.1. S2R+ cells and Cas9 flies sequencing

In order to design the guiding RNA (gRNA) and single-stranded oligodeoxynucleotides (ssODN), we had to sequence the flies that were going to be injected and the S2R+ cells to check if there were any mutations differing from the database sequence in RPS2 and RPS23. The genomic DNA of snap frozen nos-Cas9 Flies (BDSC #54591) was extracted by using QIAgen DNeasy Blood & Tissue Kit (Qiagen #69506). 5 flies per sample were crushed 180 μl buffer ATL, then, 20 μl Proteinase K was added and the mixture was vortexed and incubated at 56°C for 3 hours. After this, the samples were vortexed and 200 μl Buffer Al was added to the sample. 200 μl Buffer AL were added to the samples and immediately mixed. This mixture was then added to the DNeasy Mini spin column and the manufacturer’s instructions were followed.

S2R+ cells were grown in 6 well plates (Corning Costar #3516) and a maximum of 5 x10⁶ cells was transferred to a microcentrifuge tube and spun for 5 minutes at 1200 rpm. The media was removed, and the pellets were resuspended in 200 μl PBS. 220 μl Buffer AL with 10% Proteinase K was added to the samples and they were incubated at 56°C for 1 hour. 200 μl ethanol was added to the samples and the mixture was pipetted into a DNeasy Mini Spin column. Then, the manufacturer’s instructions were followed. The obtained DNA was then sent to Sanger sequence to Source BioScience.

2.7.1.2. gRNA design and screen

An efficient guiding RNA should be around 20bp and close to the mutation and it needs to start with the PAM sequence NGG. In order to clone the gRNA oligoes in the gRNA-
pAC-Cas-puro vector (Addgene #49330) the oligos were annealed in a PCR (95°C for 5 minutes, 85°C and then ramped -0.1°C/second for 1.4 minutes, then the temperature decreased in steps of 10°C until reaching 25°C) with the primers described in Table 2.7 and then phosphorylated with T4 Polynucleotide Kinase (NEB #M0201S) for 30 minutes at 37°C. In parallel, the vector was restricted with BspQI (NEB #R0712S) enzyme for 1 hour at 37°C and treated with calf intestinal phosphatase (CIAP) (NEB #M0290S) for 10 minutes at 37°C. After purification using QIAquick® PCR Purification Kit and following manufacturer’s instructions, (Qiagen #28104), the gRNA and the vector were ligated using T4 DNA ligase (NEB #M0202S) for 30 minutes at room temperature and DH5-α competent cells were transformed and plated on lysogeny broth (LB)-ampicillin (100 μg/ml) plates.

Table 2.7. List of primers and their sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>258</td>
<td>RPS2 sgRNA1 F</td>
<td>TTCGCCCCAGTAGCCACGGCGCA</td>
</tr>
<tr>
<td>259</td>
<td>RPS2 sgRNA1 R</td>
<td>AACTGCGCCGTGGCTACTGGGGC</td>
</tr>
<tr>
<td>260</td>
<td>RPS2 sgRNA2 F</td>
<td>TTCGCCCCACACCGTGCCCTGCA</td>
</tr>
<tr>
<td>261</td>
<td>RPS2 sgRNA2 R</td>
<td>AACTGCGGACGGCAGGGTGTTGGGGC</td>
</tr>
<tr>
<td>262</td>
<td>RPS2 sgRNA3 F</td>
<td>TTCGATGGGCACCAGGATGCGG</td>
</tr>
<tr>
<td>263</td>
<td>RPS2 sgRNA3 R</td>
<td>AACCCGCCATCCGTGTTGCCATC</td>
</tr>
<tr>
<td>264</td>
<td>RPS23 sgRNA1 F</td>
<td>TTCGATGGGCAGGTTAGGCTGCT</td>
</tr>
<tr>
<td>265</td>
<td>RPS23 sgRNA1 R</td>
<td>AACAGCAGCTAACTCTGCCATC</td>
</tr>
<tr>
<td>266</td>
<td>RPS23 sgRNA2 F</td>
<td>TTCGCTACCCTGACGGGGCAGCA</td>
</tr>
<tr>
<td>267</td>
<td>RPS23 sgRNA2 R</td>
<td>AACTCGTGCCCCGTCGACTGCGTG</td>
</tr>
<tr>
<td>291</td>
<td>pAC-sg-C9 F</td>
<td>AAAAAAGCACCGACTCGGTG</td>
</tr>
<tr>
<td>292</td>
<td>pAC-sg-C9 R</td>
<td>GTTCGACTTGCGCTGAAATACG</td>
</tr>
</tbody>
</table>

The isolated colonies were then subjected to PCR (95°C 30 seconds, and then 20 cycles of 95°C for 30 seconds, 61°C 30 seconds, 68°C 1 minute and final extension of 68°C) using Standard Taq polymerase (NEB # M0273L) and the number 259, 261, 263 and 265 primers from Table 2.7 (100 μM) were used as the forward primer of the corresponding reaction with number 292 (100 μM) as the reverse primer for all reactions. The PCR products were then run in a 1.5% agarose gel for 25 minutes at 100 V. The positive colonies were then cultured in 5 ml
of LB with ampicillin (100 μg/ml) at 37°C overnight. For plasmid extraction, GeneElute™ Plasmid Miniprep Kit (Sigma-Aldrich #PLN350) was used.

In order to select for the best gRNA we tested their DNA cutting efficiency using T7 endonuclease. To this end, 1x10⁶ S2 cells/well were seeded in a 6 well plate and left to grow for 24 hours. After this, they were transfected with the purified gRNA-pAC-Cas-puro plasmids as explained above. 48 hours after transfections, the medium was changed to fresh Schneider medium and puromycin was added to a final concentration of 9.2 μM. The cells were 6 days being selected in this antibiotic, and then their genomic DNA was extracted using DNeasy Blood Tissue Kit (Qiagen #69506) incubating the samples with Proteinase K and AL buffer for 2 hours at 56°C. This DNA was then subjected to PCR (98°C 30 seconds, and then 30 cycles of 98°C for 10 seconds, 63°C 20 seconds, 72°C 20 seconds and a final extension of 72°C 2 minutes) with the primers described in Table 2.8 and using Q5® High fidelity Polymerase (NEB #M0491L).

Table 2.8. List of primers and their sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>310</td>
<td>RPS2 gRNA1 F</td>
<td>TCGTCGCCAGCAGTCAGATGTTGATAGAGACAGATTGGGTCTCGGCTGGTTAAGTG</td>
</tr>
<tr>
<td>311</td>
<td>RPS2 gRNA1 R</td>
<td>GTCTCGTGAGGCTGAGATGATGATGATAAGAGACAGCGAGACAATGC CAGTACCAC</td>
</tr>
<tr>
<td>312</td>
<td>RPS2 gRNA3 F</td>
<td>TCGTCGCCAGCAGTCAGATGTTGATAGAGACAGCTTGGGTCTACGTA CCCCAGTT</td>
</tr>
<tr>
<td>313</td>
<td>RPS2 gRNA3 R</td>
<td>GTCTCGTGAGGCTGAGATGATGATGATAAGAGACAGCGACACTTGGCGGTGACCT</td>
</tr>
<tr>
<td>314</td>
<td>RPS23 gRNA1 F</td>
<td>TCGTCGCCAGCAGTCAGATGTTGATAGAGACAGCGAGGAAGAGAGAGCGACTGAT</td>
</tr>
<tr>
<td>315</td>
<td>RPS23 gRNA1 R</td>
<td>GTCTCGTGAGGCTGAGATGATGATGATAAGAGACAGCGAGGAAGAGAGGTGATCTTCT</td>
</tr>
<tr>
<td>316</td>
<td>RPS23 gRNA2 F</td>
<td>TCGTCGCCAGCAGTCAGATGTTGATAGAGACAGCGAGGAAGAGAGAGTAACCTG</td>
</tr>
<tr>
<td>317</td>
<td>RPS23 gRNA2 R</td>
<td>GTCTCGTGAGGCTGAGATGATGATGATAAGAGACAGCGAGGAAGAGAGAGTGACAAACCT</td>
</tr>
</tbody>
</table>

The PCR products were then run in a 1.5% agarose gel for 35 minutes at 100 V. The DNA was then extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen #28704). 200 ng of the purified DNA was then subjected to a PCR (95°C for 5 minutes, -2°C/s ramp to 85°C, -0.1°C/s ramp to 25°C and hold at 4°C) with 2 μl NEBuffer 2 and dH₂O up to 19 μl. After
this, 10 U T7 endonuclease I (NEB #M0302S) were added to the PCR product and incubated at 37°C for 15 minutes. The reaction was stopped by adding 2 μl 0.25 M EDTA and immediately loaded into a 2% agarose gel, which was run for 45 minutes at 120 V and visualized with Syngene UV transilluminator (Fig. 2.6.).

![Image of the T7 assay to test the efficiency of different gRNAs. The upper part shows the samples with T7 whereas the bottom part is the control showing the samples without T7.]

**2.7.1.3. ssODN design**

We designed and made constructs for the CRISPR-Cas9 system to make three different mutants affecting translation accuracy. The single amino acid mutation in RPS2 is reported to decrease accuracy in yeast (von der Haar et al., 2017). In RPS23 we aimed to generate one single amino acid mutant that also decreases translation accuracy and one that improved translation fidelity. In order for this mutations to be introduced in the flies' genome, *Drosophila* embryos having Cas9 under germline nosGAL4 driver were injected close to germline nuclei with a gRNA and ssODN.

The ssODN were designed to have around 80 nucleotides surrounding the mutation of interest, but also several additional mutations: one disrupting the protospacer adjacent motif (PAM) sequence and hence stop the Cas9 from cutting it once it is recombined into the genome, a silent mutation introducing a novel restriction site (PvuII) for easy PCR screening and stop the heterology block (Fig. 2.7.).
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

A) RPS2 FBgn00041867

5’ AA6TGATTGCCCAAGCCTACAAAAGGAGATTGCTGGGCAATTCTCTCTGTTTTCTCCACCCCAATAAAGGCTATAGACCTTACAAATGACATTTCAGGTGGAATATGCAAGAAATATTTGGTTTTAATAATTATTCACAGCATGCGCCCATACGA

B) RPS2 Tyrosine mutated to Cysteine (V152C)

5’ AA6TGATTGCCCAAGCCTACAAAAGGAGATTGCTGGGCAATTCTCTCTCTCCTTCCACCCCAATAAAGGCTATAGACCTTACAAATGACATTTCAGGTGGAATATGCAAGAAATATTTGGTTTTAATAATTATTCACAGCATGCGCCCATACGA

*indicates *AaeI* restriction sites
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 2.7. RPS2 and RPS23 sequences and mutations introduced by CRISPR. **A)** Sequence from FlyBase (FBgn0033912). Nucleotides changed in the CRISPR mutant highlighted in yellow. **B)** B) The sequence of the desired CRISPR mutation. Mutated nucleotides highlighted in green. ssODN sent to inject highlighted in grey and italics. The novel restriction site for screening written in blue.
2.7.2. gRNA cloning into pCFD3 vector

The gRNAs with the best performance in the T7 assay were cloned into the pCFD3U3 vector for fly embryo injections (Fig 2.8.). The gRNAs oligoes were designed to have 5’ GTCG-N19/20 in the sense oligo and 5’AAAC-N19/20 reverse complement in the antisense oligo. This allows cutting with BbsI enzyme. First, 1 µg of the pCFD3U6 empty vector was digested with 1 µl of BbsI enzyme (NEB #R0539), 5 µl of NEBuffer 2.1 (NEB #B7202S) and water up to 50 µl. After 25 minutes of incubation at 37°C, the products were purified with the QIAquick® PCR Purification Kit (Qiagen #28104). Then the inserts were annealed and phosphorylated by mixing 2.5 µl of the forward primer at 100 µM (primer 344 for RPS2 and primer 346 for RPS23), 2.5 µl of the reverse primer (100 µM) (primer 350 for RPS2 and primer 347 for RPS23) (Table 2.9), 2.5 µl of 10x PNK ligation buffer (NEB #M0201S), 1.25 µl T4 PNK enzyme (NEB #M0201S) and up to 15 µl of water. These mixtures were then subjected to a PCR (37°C for 30 minutes, 95°C for 5 minutes, ramp down to 25°C 5°C/minute). Then, the samples were diluted 1:200. These diluted annealed samples were ligated to the restricted and purified samples by mixing 50 ng of digested pcFD3U6 vector with 1 µl of the diluted inserts, 1.5 µl 10x T4 ligation buffer, 1 µl T4 DNA ligase and up to 15 µl of water. These samples were left 30 minutes at room temperature. Finally, 50 µl of competent DH5-α cells were transformed with 15 µl of the annealed mixture. These were incubated 20 minutes on ice, heat shocked for 30 seconds at 42°C, cooled down on ice for 2 minutes, 250 µl SOC media (NEB #B9020S) added and then they were incubated for once an hour at 37°C in a shaker. The transformed bacteria were then plated in LB-ampicillin (100 µg/ml) plates and left for 16 hours at 37°C.

The day after, single colonies were plated into LB-agar and also left to grow for 16 hours at 37°C. The isolated colonies were then subjected to a PCR to test if they were positive for both the insert and the vector. For this, 2.5 µl 10x Standard Taq Buffer were mixed with 0.5 µl dNTPs (10 mM), 0.5 µl forward primer (100 µM) (primer 344 for RPS2 and primer 346 for RPS23), 0.5 µl reverse primer (100 µM) (primer 352 for both RPS2 and RPS23), 0.125 µl Standard Taq Polymerase, DNA from a single colony and up to 25 µl of water. These mixtures were subjected to 95°C for 30 seconds, 30 cycles of (95°C for 25 seconds, 49°C for 40 seconds, 68°C for 20 seconds) and 68°C for 5 minutes. Once completed, 15 µl of the PCR product was loaded into a 1.5 % agarose gel and run for 40 minutes to find the positive colonies. Positive colonies were also left to grow overnight at 37°C in 6 ml of LB-ampicillin (100 µg/ml).
Table 2.9. List of primers and sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>344</td>
<td>RPS2 gRNA pCFD3 F</td>
<td>GTCGCCACACCGTGCCCTGCA</td>
</tr>
<tr>
<td>346</td>
<td>RPS23 gRNA pCFD3 F</td>
<td>GTCGCTACCGTCACGGGGCAGCA</td>
</tr>
<tr>
<td>347</td>
<td>RPS23 gRNA pCFD3 R</td>
<td>AAACCTCGCCCGGACAAGGTAG</td>
</tr>
<tr>
<td>350</td>
<td>RPS2 gRNA pCFD3 R</td>
<td>AAACCTCAGGGGCAAGGTGAGG</td>
</tr>
<tr>
<td>351</td>
<td>pCFD3 F</td>
<td>ACCTACTCAGCCAAGGCG</td>
</tr>
<tr>
<td>352</td>
<td>pCFD3 R</td>
<td>GTCGCTAATGCGTATGCA</td>
</tr>
</tbody>
</table>

1 ml of these gRNA-pCFD3U6 cultures were prepared as glycerol stocks by adding 500 μl of 60% glycerol and stored at -80°C. In order to isolate the plasmid DNA from the *E. coli* cultures, the remaining 5 ml were subjected GenElute™ Plasmid Miniprep Kit (Sigma #PL350) and following the manufacturer’s instruction. Concentration and purity was determined using NanoDrop One™ spectrophotometer (ThermoFisher Scientific). These samples were then sent to sequence with primer 351 (100 μM).
2.7.3. Embryo injection

We have used a CRISPR method in which flies expressing Cas9 are injected in the germline with the gRNA and ssODN of interest. The Cas9 flies selected were BDSC #54591 stock and they express the Cas9 protein during oogenesis under control of the nanos GAL4 driver and have a red eye marker.
2.7.4. Positive candidates screen

In order to screen for positive CRISPR mutants, a restriction site has been introduced in the ssODN so when the DNA is digested with the specific restriction enzyme and then run on a gel, if it has this restriction site, two bands can be seen. In addition, to screen for positive candidates that might have the RPS23 K60R/T mutation but not the Pvull restriction site, a PCR with a primer that 5’ begins with the mutation we introduced was also designed, as mismatches at the front of the primer impede DNA replication, so this primer should only amplify the mutant sequence and not the control.

2.7.4.1. Fly crosses scheme

Once these flies are adults, they are separated and crossed to double balancers. Then, their progeny is also single crossed to

\[
F_0: \quad 1^\text{♂} \text{ or } 1^\text{♀} \quad \text{Injected Cas9} \quad x \quad 4^\text{♂} \text{ or } 4^\text{♀} \quad \text{sp cyo}^\text{cyo} \quad \text{MKRS TM6B}
\]

\[
F_1: \quad 1^\text{♂} \text{ or } 1^\text{♀} \quad \frac{\text{Translation fidelity mutant/+}}{\text{cyo}} \quad \frac{\text{MKRS}}{\text{TM6B}} \quad x \quad 1^\text{♂} \text{ or } 1^\text{♀} \quad \frac{\text{sp cyo}}{\text{MKRS TM6B}}
\]

\[
F_2: \quad \frac{\text{Translation fidelity mutant cyo}}{\text{cyo}} \quad \frac{\text{MKRS}}{\text{TM6B}}
\]

2.7.4.2. Screen for positive mutants with the mutations of interest

The genomic DNA of snap frozen F1 Flies was extracted by crushing them individually in 10 µl Squishing Buffer with 0.1% Proteinase K (10 mM Tris HCl pH 8.0; 1 mM EDTA; 25 mM NaCl). Further 40 µl Squishing Buffer with 0.1% Proteinase K was added to each fly and they were incubated for 1 hour at 37°C. Proteinase K was inactivated at 95°C for 15 minutes.

The DNA was amplified by PCR using specific primers near the mutations of interest. For RPS2, 1 µl of primer 397 (100 µM) and 1 µl of 398 (100 µM) (Table 2.10) were mixed with 2 µl DNA, 5 µl 10x Taq Buffer, 0.5 µl Standard Taq Polymerase and water up to 50 µl. These mixtures were subjected to a PCR: 98°C 30 seconds, and then 30 cycles of 95°C for 30 seconds, 50°C 60 seconds, 68°C 30 seconds and a final extension of 68°C for 5 minutes. The products were then digested using 20 units of BamHI-HF (NEB # R3136L) per sample for 1 hour and 30 minutes at 37°C. The digested products were then run in a 1.5% agarose gel and imaged (Fig. 2.9. A).
For RPS23 (Table 2.10) 1 µl of primer 555 (100 µM) and 1 µl of 556 (100 µM) were mixed with 2 µl DNA, 5 µl 10x Taq Buffer, 0.5 µl dNTPs (10 mM) 0.5 µl Standard Taq Polymerase and water up to 50 µl. These mixtures were subjected to a PCR: 95°C 3 minutes and then 38 cycles of 95°C for 30 seconds, 47°C 60 seconds, 68°C 45 seconds and a final extension of 68°C for 5 minutes. 25 µl of these PCR products were restricted with 8 units of PvuII (NEB #R0151) and left overnight at 37°C. Then, the digested products were run in parallel to the non-digested controls in a 1.5% agarose gel for 30 minutes at 95V; if the candidate had the PvuII restriction site it gave the 646 bp band corresponding to rp23 plus two extra bands of 400 bp and 246 bp corresponding to the restricted fragments, whereas the candidates without the restriction site gave only the rps23 band of 646 bp. (Fig. 2.9. B)

Table 2.10. List of primers and their sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>397</td>
<td>RPS2 F</td>
<td>TGAGGTGCTGAAGATCATGC</td>
</tr>
<tr>
<td>398</td>
<td>RPS2 R</td>
<td>CCGAGTATGCTGGTGAAAGGA</td>
</tr>
<tr>
<td>555</td>
<td>RPS23 F</td>
<td>CGACAAGGACTACAAGAAGG</td>
</tr>
<tr>
<td>556</td>
<td>RPS23 R</td>
<td>TGCTTGCTGGAAAAAGATT</td>
</tr>
<tr>
<td>582</td>
<td>RPS23 restriction F</td>
<td>GTCCGAAAATCGACAAAATCCAG</td>
</tr>
<tr>
<td>583</td>
<td>RPS23 restriction R</td>
<td>GGCTGAGTTGGGCTGGCG</td>
</tr>
</tbody>
</table>

With the aim of finding mutants of RPS23 that had the mutation of interest, an oligo primer was designed to start with the mutation sequence, so it only aligns and amplifies mutated DNA (Table X). To check this, 0.5 µl of the fly genomic DNA was mixed with 2.5 µl 10x Taq Buffer, 0.5 µl dNTPs (10 mM), 0.5 µl of primers 582 (100 µM) and 583 (100 µM) (Table 2.10), 0.25 µl Standard Taq Polymerase, and up to 25 µl of water. These mixtures were subjected to a PCR: 95°C 3 minutes and then 33 cycles of 95°C for 30 seconds, 60°C 15 seconds, 68°C for one minute and a final extension of 68°C for 5 minutes. The samples were then run in an agarose gel, if the candidate had the K60R mutation, it gave a 692 bp band, but not if it was negative (Fig. 2.8 A).
2.7.3. DNA purification of CRISPR mutants and sequencing

The previously explained procedures allowed us to choose possible candidates with our desired CRISPR-introduced mutations. To verify if they had the mutations, 2 µl of the genomic DNA of these flies was amplified by PCR using the same primers described in Table 2.10 (2.5 µl of each primer (100 µM)) and 0.5 µl Q5® High-Fidelity DNA Polymerase (NEB #M0491L) and water up to 50 µl. Then, these samples were subjected to a PCR: 98°C 3 minutes and then 35 cycles of 98°C for 10 seconds, 66°C [for RPS2] or 60°C [for RPS23] 30 seconds, 72°C 25 seconds and a final extension of 72°C for 2 minutes). These PCR products were then purified using QIAGen QIAquick® PCR purification kit (Qiagen #28106) following the manufacturer’s instructions. These were then sent to Sanger sequence by Source BioScience.

2.7.5. Backcrossing scheme of the mutants

To properly analyse the effects of the RPS23 K60R mutation and avoid interpreting the effects of cofounding mutations, these flies had to be backcrossed to the wild type (Partridge and Gems, 2007). RPS23 K60R flies were backcrossed to w^Dahomey flies following the single-cross scheme described below.

\[
\text{F1: } 1^\Psi \frac{\text{RPS23 K60R}}{\text{cyo}} ; \frac{\text{MKRS}}{+} \times 2^\sigma \frac{\text{cyo}}{+} ; \frac{\text{MKRS}}{+}
\]

\[
\text{F2: } 3^\Psi \frac{\text{wDahomey}}{\text{wDahomey}} \times 1^\sigma \frac{\text{RPS23 K60R}}{\text{cyo}} +
\]

\[
\text{F3-F8: } 1^\Psi \frac{\text{RPS23 K60R}}{+} \times 2^\sigma \frac{\text{wDahomey}}{\text{wDahomey}}
\]
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

| \( \frac{\text{F9}}{\text{cyo}} + \text{MKRS} + \) |
| \( \frac{\text{F10}}{\text{RPS23} \text{K60R} \text{cyo}} \times \frac{\text{RPS23} \text{K60R} \text{cyo}}{} \) |
| \( \frac{\text{F11}}{\text{RPS23} \text{K60R}} \) |

RPS23 K60R flies have no visible marker so they had to be identified by sacrificing the mutant fly and following the PCR screen described above at each step.

2.8. S2R+ cells culture and maintenance

S2R+ cells were grown in T75 cell culture flasks (Corning #430641U) with Schneider media with 10% heat-inactivated foetal bovine serum (FBS) (ThermoFisher Scientific #16140071) and 50,000 U/l penicillin-streptomycin (ThermoFisher Scientific #15140122). These cells were kept at 25°C and passed to fresh medium once a week.

2.9. RNA interference (RNAi) in vitro

In order to downregulate the expression of several proteins, we used double-stranded (dsRNA)-mediated interference in S2R+ cells. This technique has proved successful in cultured Drosophila cells and it is based on the cell’s targeting and destruction of mRNA homologous to the dsRNA (Clemens et al., 2000, Kao and Megraw, 2004). The dsRNA was produced by Celia Lujan by making template DNA from the genomic DNA of our cultured S2R+ cells, then she extracted RNA from the S2R+ cells and made cDNA, which she finally converted to dsRNA. This was then used to treat the S2 cells.

2.9.1. dsRNA preparation

First, the RNA of S2R+ cells in culture was extracted by transferring \( 7.5 \times 10^6 \) cells to a 7.5 ml Falcon tube and following the same steps described in ‘2.3.2 RNA extraction’.

The RNA was then converted to cDNA. For this, first, the genomic DNA of S2R+ cells in culture was extracted following the same protocol described in ‘2.7.1.1. S2R+ cells and Cas9 flies sequencing’. This cDNA (at a concentration of 500 ng/\( \mu l \)) was then used as template (1 \( \mu l \)) for a PCR with 0.5 \( \mu l \) Q5® High-Fidelity polymerase, 10 \( \mu l \) 5x Q5® buffer, 1 \( \mu l \) dNTPs (10 mM), 10 \( \mu l \) forward primer (100 \( \mu M \)) and 10 \( \mu l \) reverse primer (100 \( \mu M \)) (Table 2.11), and up to 50 \( \mu l \). These mixtures were subjected to a PCR (30 seconds at 98°C, then 35 cycles of 10 seconds at 98°C, 20 seconds at 64-72°C (depending on the primer set, see Table 2.11) and 20 seconds at
72°C and a final annealing for 2 minutes at 72°C. After this, 5 µl of the PCR products were run on a 1.5% agarose gel to check the purity of these products.

Table 3.11. List of primers, their sequence and annealing temperature.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>709</td>
<td>eIF4E dsRNA F</td>
<td>TAATACGACTCATAA1GGGATAAACAAAGGGGGTTCCGT</td>
<td>67°C</td>
</tr>
<tr>
<td>710</td>
<td>eIF4E dsRNA R</td>
<td>TAATACGACTCATA1GGGAGATTGGACAAACATCGCGGCGGA</td>
<td></td>
</tr>
<tr>
<td>715</td>
<td>eIFG4 dsRNA F</td>
<td>TAATACGACTCATAAGGAGCAATGAATCGCAGCGGGAA</td>
<td>66°C</td>
</tr>
<tr>
<td>716</td>
<td>eIFG4 dsRNA R</td>
<td>TAATACGACTCATAAGGCGTCCACTTTGTCGGGTGATTC1</td>
<td></td>
</tr>
<tr>
<td>711</td>
<td>eEF2 dsRNA F</td>
<td>TAATACGACTCATAAGGGAGTTGGGAATGATCTGGG</td>
<td>69°C</td>
</tr>
<tr>
<td>712</td>
<td>eEF2 dsRNA R</td>
<td>TAATACGACTCATAAGGACCACGCTTGTATCCC1</td>
<td></td>
</tr>
<tr>
<td>727</td>
<td>RPS23 dsRNA F</td>
<td>TAATACGACTCATAAGGGCAGCAAGGACTGAA</td>
<td>72°C</td>
</tr>
<tr>
<td>728</td>
<td>RPS23 dsRNA R</td>
<td>TAATACGACTCATAAGGGCAGCCTTACGCGAAGAACC</td>
<td></td>
</tr>
<tr>
<td>717</td>
<td>Renilla dsRNA F</td>
<td>TAATACGACTCATAA1GGGATAACTGGGCGAGTGTTA</td>
<td>64°C</td>
</tr>
<tr>
<td>718</td>
<td>Renilla dsRNA R</td>
<td>TAATACGACTCATAAGGGATATCGCGATTTGACCA</td>
<td></td>
</tr>
<tr>
<td>719</td>
<td>Firefly dsRNA F</td>
<td>TAATACGACTCATAAGGGCTGGGCGTTAATCAGAG</td>
<td>64°C</td>
</tr>
<tr>
<td>720</td>
<td>Firefly dsRNA R</td>
<td>TAATACGACTCATAAGGGTGTTACCGTTTTGCGGCT</td>
<td></td>
</tr>
</tbody>
</table>

Afterwards, 95 µl of the PCR product were precipitated by adding 10 µl volume of sodium acetate (3 M), then 180 µl of cold 100% ethanol. These mixtures were then incubated for 15 minutes at room temperature to be later centrifuged at 12000 rpm for 5 minutes. The supernatants were discarded, and 1 ml of 70% ethanol was added to each sample. These were centrifuged at 12000 rpm for 5 minutes, the supernatants discarded, and the pellets let to air dry. Once dried, the pellets were dissolved in 20 µl of nuclease-free water to then measure the concentrations using NanoDrop One® spectrophotometer.

This DNA was then used as a template for the transcription reaction using MEGAscript® kit (ThermoFisher #AM1330). 1 µg of DNA was mixed with 4 µl 10x Reaction Buffer, 4 µl ATP solution, 4 µl CTP solution, 4 µl GTP solution, 4 µl UTP solution, 4 µl Enzyme Mix and water up to 40 µl and these reactions were left overnight at 37°C. After incubation, 1
µl of RNAse free-DNAse I (1 U/µl) was added and left to incubate for 15 minutes at 37°C. After this, the samples were annealed by leaving them for 10 minutes at 70°C, then cooled down to 25°C at a 0.1°C/sec rate. These dsRNA products were then purified by adding 2 volumes of nuclease-free water, 1/10 volume of sodium acetate (3 M) and 3 volumes of 100% ethanol and leaving these mixtures for 10 minutes at room temperature. After this incubation, the samples were centrifuged for 10 minutes at 12000 rpm, the supernatants were discarded and 500 µl of 80% ethanol were added. These samples were centrifuged for 5 minutes at 12000 rpm, the ethanol removed, and the pellets left to air dry. These dry pellets were then dissolved in 100 µl of nuclease free water and their concentrations measured using NanoDrop OneC spectrophotometer and then stored at -80°C.

2.9.2. dsRNA treatment in S2R+ cells

For the treatment, 100,000 cells/well were plated in a 48 well plate with 200 µl Schneider + 10% heat-inactivated FBS and penicillin G. These cells were left to sink to the bottom of the plates for 2 hours at 25°C. They were then centrifuged for 30 seconds at 600 g, their media was removed and 200 µl of Serum-free Schneider medium with the indicated amount of dsRNA were added. Then, these were incubated for 30 minutes at 25°C and 300 µl of Schneider + 10% heat-inactivated FBS and Penicillin G were added to each well. These plates were then left for 72 hours at 25°C before they were analysed.

2.10. Translation fidelity assay in vitro

2.10.1. Cloning of translation fidelity reporters

The translation fidelity reporters had to be adapted to work in Drosophila S2R+ cells. For this purpose, Celia Lujan had to insert the dual luciferase reporters into the pENTR™ 3C vector using pENTR™ Directional TOPO Cloning kit (Invitrogen #K2400-20) (Fig. 2.10. A). These constructs were then transferred to expression vectors with either the Heat Shock promoter (pHW) or Actin promoter (pAW) using the Gateway® Technology kit (Invitrogen #12535-019) (Fig. 2.9.). For transformation, stable competent E. coli cells (NEB #C3040H) were mixed with 2 µl of the clone mixture and left on ice for 30 seconds, followed by one minute at 42°C and 5 minutes on the ice again. Then, the samples were spread into LB agar plates and left at 37°C overnight. The day after, the colonies were isolated and left in LB media to culture overnight. The DNA was then purified using the GenElute™ Plasmid Miniprep kit (Sigma-Aldrich #PLN350).
The role of decreased protein synthesis in delaying ageing

The promoter was then changed to the metallothionein (pMT) promoter, as this is a copper-inducible promoter that allows expression only when desired. A vector containing the pMT promoter was kindly provided by Prof. Buzz Baum. This promoter was amplified using two primers (394 and 395) that had tails containing NotI and Stul restriction sites (Table 2.12) (Fig. 2.10. B). For this, 2 µl of template DNA was mixed with 10 µl of Q5® Buffer (NEB #M0491S), 2.5 µl of each primer (100 µM), 1 µl dNTPs (10 mM), 0.5 µl of Q5® High-Fidelity polymerase and water up to 50 µl. This mixture was subjected to a PCR (30 seconds at 98°C, then 35 cycles of 10 seconds at 98°C, 20 seconds at 72°C and 30 seconds at 72°C and a final annealing for 2 minutes at 72°C). These products were then purified using QIAquick® PCR Purification kit and 100 µg were digested with 1 µl of NotI-HF® (NEB #R3189) and 1 µl of Stul (NEB #R0187S), 5 µl of CutSmart® Buffer (NEB #B7204S) and water up to 20 µl.

The dual luciferase reporters were in the pHW vector (688pHW= misincorporation control reporter, 868pHW= misincorporation reporter, 690pHW= stop codon readthrough control reporter and 691pHW= stop codon reporter) (Fig 2.3) (Salas-Marco and Bedwell, 2005), so 1 µg were also digested with 1 µl of NotI-HF® and 1 µl of Stul, 5 µl of CutSmart® Buffer and water up to 20 µl. These digested vectors with the dual luciferase reporters were then run in a 1.5% agarose gel for 50 minutes at 100V and the 6kb band was extracted and purified using QIAquick Gel Extraction Kit.

These purified vectors containing the dual luciferase reporters were then ligated to the pMT promoter mixing them in a 1 DNA vector (ng):3 DNA insert (ng) ratio with 3 µl of T4 Ligation Buffer (NEB #M0202), 1.5 µl T4 DNA Ligase enzyme and up to 30 µl water. These were incubated at room temperature for 30 minutes; after this, the enzymes were inactivated by heating the samples for 10 minutes at 65°C. Then, 20 µl of the ligated mixtures were added to 50 µl of competent DH5-α cells. These were transformed as described in section ‘2.8.1.3. gRNA cloning into pCFD3 vector’.

Table 2.12. Table of the primers and sequences.

<table>
<thead>
<tr>
<th>#</th>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>394</td>
<td>pMT NotI</td>
<td>AAGGAAAAAAGCGGCCGCAATTCGTGCAAGGACAGG</td>
</tr>
<tr>
<td>395</td>
<td>pMT Stul</td>
<td>GAAGGCTCCTTTAGTTGCACCTGAGATG</td>
</tr>
</tbody>
</table>
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
Figure 2.10. Dual luciferase constructs cloned into their final vectors. **A)** The Dual-Luciferase constructs were cloned into the pENTR™ vector and then into the expression vectors using Gateway LR Technology. The expression vectors for S2R+ cells contained either a pAW promoter or pHW promoter. **B)** However, after optimization, the pHW promoter was replaced by the inducible pMT promoter.
2.10.2. Stable cell generation

In order to get better luminescence signal, stable cell lines expressing the translation fidelity reporters were made. For this purpose, 1 x 10^6 cells were seeded in a 6 well plate with 3 ml Schneider with 10% heat-inactivated FBS medium. After 24 hours, they were transfected with 0.4 μg of the translation fidelity reporter plasmid (690pMT, 691pMT, 688pMT or 868pMT) and 0.4 μg of the pAC5-pCO-Blast plasmid (kindly provided by Dr. Yuu Kimata) using the Effectene® Transfection Reagent kit (Qiagen #301425) and following manufacturer’s instruction. After 48 hours, the cells were centrifuged at 1200 rpm for 10 minutes at 25°C; the media was removed and replaced by 3 ml of Schneider with 10% heat-inactivated FBS and 30 μg/ml Blasticidin S HCl (ThermoFisher Scientific #A1113903). After one week, the cells were seeded in a T-25 cell culture flask (Corning Costar #430639) with 5 ml of fresh Schneider with 10% heat-inactivated FBS and 25 μg/ml Blasticidin. The cells were then passed to new plates with fresh media once a week.

2.10.3. Sample preparation

Cell viability was measured using Countess™ II Automated Cell Counter (ThermoFisher Scientific #AMQAX1000) and 100,000 cells with 500 μl Schneider medium with 10% heat-inactivated FBS and penicillin G (ThermoFisher Scientific #BP2955-5) were seeded in 48 well plates (Corning Costar #3548). After 2 days, 0.5 μl of different drugs at the indicated concentration in the Chapter 4. Results section were added to the cells together with 5 μl CuSO₄ (Sigma #I2852) 110 mM (to give a final concentration of 500 μM).

To prepare the samples for the dual luciferase assay, the plates were centrifuged for 5 minutes at 1200 rpm at 25°C. The medium was removed and the pellets washed with 1x PBS; then, the plates were centrifuged again at 1200 rpm for 5 minutes. The PBS was then removed and the pellets were frozen at -80°C for 30 minutes to enhance lysis. After 30 minutes 20 μl of PLB (Passive Lysis Buffer) from the Dual-Luciferase Reporter Assay System (Promega #E1910) kit were added to the cells and incubated at room temperature for approximately 15 minutes.

2.10.4. Dual luciferase assay in vitro

Once lysed, 10 μl of sample was transferred to a white 96 well plate and read using Varioskan™ LUX Microplate Reader. The reader was set to read the luminescence for 2000 ms,
then dispense 50 μl of LARII substrate, read luminescence again for 2000 ms and 2 ms lag; dispense 50 μl of Stop&Glo® and read luminescence for 2000 ms and 2 ms lag.

### 2.10.5. Drugs used in the dual luciferase assay in vitro.

The effect on translation fidelity of different drugs was tested. A stock solution and then a dilution series were done in order to add the same volume (0.5 μl) of each concentration (Table 2.13).

#### Table 2.13. Drugs used in the in vitro dual luciferase and their concentrations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stock concentration</th>
<th>Drug amount</th>
<th>Solvent volume</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>10 mM</td>
<td>4.3 mg</td>
<td>1.5 ml EtOH</td>
<td>Sigma (C7698)</td>
</tr>
<tr>
<td>Diazaborine</td>
<td>50 mM</td>
<td>2 mg</td>
<td>133.27 μl DMSO</td>
<td>Calbio</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>4 mM</td>
<td>2 mg</td>
<td>547 μl EtOH</td>
<td>LC Laboratories</td>
</tr>
<tr>
<td>Salubrinal</td>
<td>10 mM</td>
<td>2 mg</td>
<td>46.84 μl DMSO</td>
<td>CruzChem</td>
</tr>
<tr>
<td>Torin 1</td>
<td>2 mM</td>
<td>1 mg</td>
<td>822.88 μl DMSO</td>
<td>Adooq BioScience</td>
</tr>
<tr>
<td>Torin 2</td>
<td>2.5 mM</td>
<td>1 mg</td>
<td>926 μl DMSO</td>
<td>LC Laboratories</td>
</tr>
</tbody>
</table>

As a positive control, an error-inducing antibiotic, paromomycin sulphate salt (Sigma #P5057) was added to the cells. For this, a 50 mM stock solution was done dissolving 5 mg in 140 μl of water; then, 5 μl was added to the cells to obtain a 500 μM final concentration.
Chapter 3: Genetic screen for translation-associated factors that can extend lifespan in *Drosophila*
3.1. Introduction

Since the discovery that ageing is a malleable process, (Kenyon et al., 1993, Klass, 1983, Friedman and Johnson, 1988, Morris et al., 1996), there have been many studies trying to find what processes govern longevity, and in particular if it is possible to improve it. This has led to the significant discovery that there are highly evolutionarily conserved mechanisms regulating ageing, namely dietary restriction, downregulation of TOR and the IIS pathway (Bodkin et al., 2003, Colman et al., 2009, Miller et al., 2005, Grandison et al., 2009, Clancy et al., 2001, Tatar et al., 2001, Bluher et al., 2003), which importantly, all lead to reduced translation. Therefore, several labs around the same time directly downregulated translation, mostly genetically but also pharmacologically, in yeast and C. elegans and they saw that these interventions can extend lifespan in these organisms (Hansen et al., 2007, Pan et al., 2007, Henderson et al., 2006, Hamilton et al., 2005, Curran and Ruvkun, 2007, Steffen et al., 2008, Smith et al., 2008, Osterwalder et al., 2001, Syntichaki et al., 2007a, Syntichaki et al., 2007b). Therefore, to study if decreasing individual translation factors can increase lifespan as it does in yeast and C. elegans, we performed a focused longevity screen using the inducible GAL4-UAS system. Resembling the studies in C. elegans, because of the importance of protein synthesis during development and since any anti-ageing interventions in humans would be done in adults, we downregulated the expression of the translation-associated factors only during adulthood.
3.2. Results

3.2.1 The effect of downregulation of translation-associated proteins by RNAi on lifespan and healthspan in *Drosophila*

3.2.1.1. Downregulation of translation-associated proteins by RNAi in metabolic tissues can extend lifespan

In order to gain an insight into the role of protein translation in ageing, a focused longevity RNAi screen was performed using female flies. The downregulated transgenes were selected due to their reported effects on longevity in other model organisms and because of their implication in translation (Lasko, 2000, Kaeberlein and Kennedy, 2008). The targeted proteins can be grouped into three categories: translational initiation and elongation factors, ribosomal proteins and components of cellular pathways related to translation (see Table 2.3 Materials and Methods).

The Gene-Switch inducible driver flies were crossed with flies carrying an UAS-RNAi construct targeting the silencing of a specific mRNA. I am aware that there are some RNAi lines from the genetic stocks centres are not very effective in silencing gene expression and that some might have off target effects. In addition, a few of the VDRC KK library stocks may have additional plasmid integration sites, which can cause phenotypes not specific to the gene of interest (Green et al., 2014). Because of that, wherever possible we tested two independent RNAi lines; however, due to the numerous genes tested, we did not performed RT-qPCR testing for all the RNAi lines used in the screen, but only in the positive candidates.

The Gene-Switch system was chosen because of its ability to activate the transgene in an inducible manner, and hence downregulate the expression of the protein of interest, only during adulthood. In addition, this system is ideal for lifespan experiments because flies of the same genotype are put on food with RU486 (experimental flies) and without RU486 (control flies) (Materials and Methods 2.1). This avoids extensive backcrossing prior to lifespan analysis. To study the effect of downregulating these translation-associated proteins on different tissues two drivers were used, the Actin Gene-Switch driver, that is ubiquitously expressed, and the S106 driver, which is expressed only in the fat body and the intestine and it was successfully used in the past for longevity studies (Roman et al., 2001, Giannakou et al., 2004).

In the longevity screen, it was found that under the Actin Gene-Switch driver, none of the factors had a statistically significant lifespan extension; in fact, they lived shorter than the
controls (Fig. 3.1). In addition, we realised that the factor referred as eEF2 (CG4849) is actually eEFTuD2, a pre-mRNA splicer (Frazer et al., 2008). This shortening of lifespan observed in all lines tested could be due to the ubiquitous driver ActGS being too strong, therefore we next used a milder driver, the metabolic tissue-specific S106 driver (see Materials and Methods 2.1.3). When the same proteins were downregulated using the S106 driver several candidates were found to have statistically significant extensions of their lifespans compared to their controls, such as S106>RPS23-mit (CG1842) RNAi, S106>eIF1 (CG17737) RNAi, S106>eIF3-S8 (CG4954) RNAi, S106>eIF3-S10 (CG9805) RNAi, S106>eIF4E (CG4035) RNAi, S106>eEF2 (CG2238) RNAi and S106>RPS23 (CG8415) RNAi (Fig. 3.2) (Table 3.1). In parallel, myc (CG10798) RNAi was also used as a positive control due to involvement of myc in translation regulation and because myc downregulation extend lifespan in flies and mice (Averous and Proud, 2006, Greer et al., 2013, Hofmann et al., 2015), which we confirmed in our experiments and which validated our experimental set up (Fig 3.2. D) (Table 3.2).
Table 3.1. Median and maximum lifespan data from RU486-induced ActGS>RNAi flies. The factors shown here were ubiquitously downregulated via RNAi when RU486 was present in the food. Around 150 flies were used per condition. Median and maximum lifespans were calculated, and the p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control were calculated doing χ²-log-rank tests (statistically significant values highlighted in yellow).

<table>
<thead>
<tr>
<th>Factor</th>
<th>RU486</th>
<th>CG Stock</th>
<th>Medium LS (days)</th>
<th>Maximum LS (days)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>eEF2</td>
<td>-</td>
<td>CG2238</td>
<td>78.0</td>
<td>94.0</td>
<td>2.8E-24</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>47</td>
<td>77.5</td>
<td></td>
</tr>
<tr>
<td>eEF2</td>
<td>-</td>
<td>CG4849</td>
<td>87.0</td>
<td>94.0</td>
<td>1.6E-14</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>73</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>eEF2</td>
<td>-</td>
<td>CG4849</td>
<td>82.0</td>
<td>101.0</td>
<td>2E-07</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>71</td>
<td>84.5</td>
<td></td>
</tr>
<tr>
<td>eEF2</td>
<td>-</td>
<td>CG4849</td>
<td>67.0</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>69.0</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>elf1</td>
<td>-</td>
<td>CG17737</td>
<td>84.5</td>
<td>96.0</td>
<td>8.21E-09</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>71.0</td>
<td>84.5</td>
<td></td>
</tr>
<tr>
<td>elf2alpha</td>
<td>-</td>
<td>CG9964</td>
<td>82</td>
<td>96</td>
<td>2.9E-05</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>63.5</td>
<td>84.5</td>
<td></td>
</tr>
<tr>
<td>elf3-s9</td>
<td>-</td>
<td>CG4878</td>
<td>82</td>
<td>91.5</td>
<td>2.67E-17</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>61</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>elf3-s10</td>
<td>-</td>
<td>CG9805</td>
<td>69.0</td>
<td>78.5</td>
<td>2.58E-49</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>43.5</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>elf4A</td>
<td>-</td>
<td>CG7483</td>
<td>74.0</td>
<td>78.5</td>
<td>1.17E-07</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>64.5</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>elf4G</td>
<td>-</td>
<td>CG10811</td>
<td>82</td>
<td>91.5</td>
<td>1.68E-38</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>24</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Gcn2</td>
<td>-</td>
<td>CG16096</td>
<td>84.5</td>
<td>98.5</td>
<td>0.086087</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>77.5</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>RPL9</td>
<td>-</td>
<td>CG6141</td>
<td>80</td>
<td>93</td>
<td>2.08E-07</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>56.5</td>
<td>84.5</td>
<td></td>
</tr>
<tr>
<td>RPL19</td>
<td>-</td>
<td>CG2746</td>
<td>69.0</td>
<td>76.0</td>
<td>0.196528</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>69.0</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>RPS2</td>
<td>-</td>
<td>CG5920</td>
<td>67.0</td>
<td>78.5</td>
<td>2.28E-40</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>25.5</td>
<td>63.0</td>
<td></td>
</tr>
<tr>
<td>RPS2</td>
<td>-</td>
<td>CG5920</td>
<td>76.0</td>
<td>85.5</td>
<td>7.21E-09</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>64.5</td>
<td>78.5</td>
<td></td>
</tr>
<tr>
<td>RPS3</td>
<td>-</td>
<td>CG6779</td>
<td>91.5</td>
<td>98.5</td>
<td>2.46E-37</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>66.5</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>RPS11</td>
<td>-</td>
<td>CG8857</td>
<td>69.0</td>
<td>78.5</td>
<td>0.171747</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>69.0</td>
<td>81.0</td>
<td></td>
</tr>
<tr>
<td>RPS26</td>
<td>-</td>
<td>CG10305</td>
<td>74.0</td>
<td>78.5</td>
<td>2.58E-12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>60.0</td>
<td>64.5</td>
<td></td>
</tr>
<tr>
<td>RPS26</td>
<td>-</td>
<td>CG10305</td>
<td>71.5</td>
<td>78.5</td>
<td>0.463889</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>69.0</td>
<td>78.5</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 (PART 1) Median and maximum lifespan data from RU486-induced S106>UAS-RNAi flies. The proteins shown here were downregulated in the gut and fat body via RNAi when RU486 was present in the food. Around 150 flies were used per condition. Median and maximum lifespans were calculated. Some of the lifespans were repeated several times. The factors that were subsequently further characterised are written in red font. The p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control was calculated doing a χ2-log-rank test (statistically significant values highlighted in yellow).
Table 3.2 (PART 2) Median and maximum lifespan data from RU486-induced S106-UAS-RNAi flies.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF1</td>
<td>-</td>
<td>45627</td>
<td>68.0</td>
<td>77.0</td>
<td>0.249081</td>
<td>70.5</td>
<td>81</td>
<td>0.102662</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raptor</td>
<td>+</td>
<td>34814</td>
<td>66.5</td>
<td>81.0</td>
<td>0.120877</td>
<td>73.0</td>
<td>84.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raptor</td>
<td>-</td>
<td>41912</td>
<td>66.0</td>
<td>74.0</td>
<td>0.70704</td>
<td>68.5</td>
<td>74.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raptor</td>
<td>+</td>
<td>34814</td>
<td>66.5</td>
<td>81.0</td>
<td>0.120877</td>
<td>73.0</td>
<td>84.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL9</td>
<td>+</td>
<td>36639</td>
<td>65.0</td>
<td>76.5</td>
<td>0.001602</td>
<td>73.5</td>
<td>83.0</td>
<td>0.091986</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL19</td>
<td>+</td>
<td>41915</td>
<td>63.5</td>
<td>69.0</td>
<td>0.017469</td>
<td>66.5</td>
<td>74.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS2</td>
<td>+</td>
<td>106108</td>
<td>58.0</td>
<td>67.0</td>
<td>0.00031</td>
<td>73.0</td>
<td>84.5</td>
<td>0.803215</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS2</td>
<td>-</td>
<td>20963</td>
<td>63.0</td>
<td>76.0</td>
<td>0.214347</td>
<td>70.0</td>
<td>86.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS3</td>
<td>+</td>
<td>37742</td>
<td>65.0</td>
<td>75.0</td>
<td>0.180622</td>
<td>63.5</td>
<td>76.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS5</td>
<td>+</td>
<td>35395</td>
<td>37742</td>
<td>65.0</td>
<td>75.0</td>
<td>0.180622</td>
<td>63.5</td>
<td>76.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS11</td>
<td>+</td>
<td>23475</td>
<td>79.5</td>
<td>84.5</td>
<td>0.058526</td>
<td>62.5</td>
<td>74.0</td>
<td>0.094125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS23</td>
<td>+</td>
<td>35421</td>
<td>63.0</td>
<td>70.0</td>
<td>5.17E-11</td>
<td>60.0</td>
<td>71.5</td>
<td>8.17E-07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS23-mt</td>
<td>+</td>
<td>21429</td>
<td>72.5</td>
<td>84.0</td>
<td>0.005653</td>
<td>60.0</td>
<td>70.0</td>
<td>8.17E-07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPS26</td>
<td>+</td>
<td>16012</td>
<td>65.0</td>
<td>76.5</td>
<td>0.320002</td>
<td>63.5</td>
<td>83.5</td>
<td>0.015371</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S106 n W118</td>
<td>+</td>
<td>33933</td>
<td>62.5</td>
<td>74.0</td>
<td>0.001571</td>
<td>60.0</td>
<td>69.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S106 n W118</td>
<td>+</td>
<td>23475</td>
<td>79.5</td>
<td>84.5</td>
<td>0.058526</td>
<td>62.5</td>
<td>74.0</td>
<td>0.094125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
The role of decreased protein synthesis in delaying ageing  
Martínez Miguel, V.E.

Figure 3.1. Representative survival curves of ActGS>eIF4G RNAi (CG10811), ActGS>eEF2 RNAi (CG2238), ActGS>eIF3-S9 RNAi (CG4878), ActGS>EFTuD2 RNAi (CG4849), ActGS>RPS2 RNAi (CG5920), ActGS>eIF3-S10 RNAi (CG9805), ActGS>RPS26 RNAi (CG10305), ActGS>eIF4A RNAi (CG7483) and ActGS>RPS11 RNAi (CG8857). Dashed lines represent the survival of flies fed control food, and the solid lines represent the survival of the flies fed experimental 200 µM RU486-supplemented food. Around 150 mated female flies per condition were tested in each experiment. The p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control food were calculated doing χ2-log-rank tests: A) ActGS>eIF4G RNAi p=1.68 x10^{-38}. B) ActGS>eEF2 RNAi p=2.8 x10^{-24}. C) ActGS>eIF3-S9 RNAi p=2.67 x10^{-5}. D) ActGS>EFTuD2 RNAi (stock line #21963) p=1.6 x10^{-14}. E) ActGS>EFTuD2 RNAi (stock line #108596) p=2 x10^{-7}. F) ActGS>RPS2 RNAi (stock line #100308) p=2.28 x10^{-60}. G) ActGS>eIF3-S10 RNAi p=2.58 x10^{-49}. H) ActGS>RPS26 RNAi p=2.58 x10^{-12}. I) ActGS>eIF4A RNAi p=1.17 x10^{-7}. J) ActGS>RPS2 RNAi (stock line #20963) p=7.2 x10^{-9} and K) ActGS>RPS11 RNAi p=0.17.

We chose to focus on the initiation factor eIF4E (CG4035), the elongation factor eEF2 (CG2238) and the ribosomal protein RPS23 (CG8415) that showed to increase lifespan in several independent experiments and independent lines (Table 3.2, marked in red). In Figure 3.3., survival curves representative of each line are shown: S_{106}>eIF4E RNAi had 70.5 days medium and 82 days maximum lifespan compared to 63.5 days median and 72 days maximum lifespan of the control (p=4.51 x10^{-11}, χ2 log-rank test), S_{106}>eEF2 RNAi had 63 days median and 74 days maximum lifespan compared to the control's 60.5 days median 67 days maximum lifespan (p=2.44 x10^{-16}, χ2 log-rank test), S_{106}>RPS23 RNAi had 67.5 days median and 72 days maximum lifespan compared to the control’s 63 days median 70 days maximum lifespan (p=5.17 x10^{-11}, χ2 log-rank test) in the represented experiments.
Unexpectedly, downregulating eIF4G (CG10811), even with the milder S[106 driver, was detrimental to the flies’ lifespan. At the normal 200 µM RU486 concentration, S[106>eIF4G RNAi flies had 40 days median and 61 days maximum lifespan compared to the control’s 70.5 days median 80 days maximum lifespan (p=4.09 x10^{-49}, χ2 log-rank test) (Fig. 3.3.C). To achieve longevity, sometimes gene expression needs to be altered slightly, hence, we used a ten-fold lower RU486 concentration for S[106>eIF4G RNAi. Nevertheless, we still saw a decrease in the flies’ survival, from 70.5 days median and 80 days maximum lifespan in the control, to 59 days median and 70 days maximum lifespan in S[106>eIF4G flies (p=1.44 x10^{-16}, χ2 log-rank test) suggesting that eIF4G most likely cannot be targeted to delay Drosophila’s ageing, at least not in the fat body and intestine.

Figure 3.2. Representative survival curves of S[106>eIF1 RNAi (CG17737), S[106>eIF3-S8 RNAi (CG4954), S[106>RPS23-mit RNAi (CG1842) and S[106>myc RNAi (CG10798). Dashed lines represent the survival of flies fed control food, and the solid lines represent the survival of the flies fed experimental 200 µM RU486-supplemented food. Around 150 mated female flies per condition were tested in each experiment. The p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control food were calculated using χ2-log-rank tests: A) S[106>eIF1 RNAi p= 1.13 x10^{-8}. B) S[106>eIF3-S8 RNAi p= 2.7 x10^{-3}. C) S[106>RPS23-mit RNAi p= 0.005 and D) S[106>myc RNAi p= 6.93 x10^{-5}.

138
Figure 3.3. Representative survival curves of the control, $S_{106}>eIF4E$ RNAi (CG4035), $S_{106}>eEF2$ RNAi (CG2238), $S_{106}>RPS23$ RNAi (CG8415) and $S_{106}>eIF4G$ RNAi (CG10811). Dashed lines represent the survival of flies fed control food and the solid lines represent the survival of the flies fed experimental 200 µM RU486-supplemented food. In the case of $S_{106}>eIF4G$ RNAi, flies fed with 20 µM RU486-supplemented food were also tested (dotted line). Around 150 mated female flies per condition were tested in each experiment. The p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control food were calculated using χ2-log-rank tests: A) Control n.s. B) $S_{106}>eIF4G$ RNAi 200 µM p= 4.09 x10^{-49} and $S_{106}>eIF4G$ RNAi 20 µM p= 1.44 x10^{-16}. C) $S_{106}>eIF4E$ RNAi p= 4.51 x10^{-11}. D) $S_{106}>eEF2$ RNAi p= 2.44 x10^{-16} and E) $S_{106}>RPS23$ RNAi p= 5.17 x10^{-11}.

To corroborate that the RNAi used was silencing the expression of our genes of interest, the levels of specific mRNAs were analysed with the help of Celia Lujan. The abdomens without ovaries of the $S_{106}>UAS$-RNAi flies were dissected, their RNA extracted and converted to cDNA (Materials and Methods 2.3.4). Specific primers for each RNAi line were designed to be in exon-exon junctions and to avoid amplification of the RNAi hairpins.
constructs. In addition, a set of primers for Actin 5 (Act5C) was designed to normalise the levels of RNA. Using these primers, the cDNA was subjected to RT-qPCRs and after quantification, we saw that all our lines have statistically significant downregulated expression of their target genes in flies fed RU486 compared to their sibling non-induced controls: S_{106}>eIF4E RNAi 37% (p=0.0013, Welch’s test), S_{106}>eIF4G RNAi 41.5% (p=0.0024 Welch’s test), S_{106}>eEF2 RNAi 33.8% (p=0.0224 Welch’s test), S_{106}>RPS23 RNAi 57.4% (p=0.003 Welch’s test) (Fig. 3.4.).

Figure 3.4. Relative abundance of mRNA in the S_{106}>UAS-RNAi lines. The abundance of mRNA for the targeted genes from our longevity screen was quantified using RT-qPCR and then normalised by the abundance of Actin 5C-RNA for each sample. For each line, 6 samples were used with 4 flies in each, the calculated average is plotted ±SEM. The p values between the samples from the flies fed ethanol-supplemented control food (blue bars) and RU486+ (red bars) were calculated using Welch’s t-tests * p<0.05, ** p<0.005: S_{106}>eIF4E RNAi p=0.0013, S_{106}>eIF4G p=0.0024, S_{106}>eEF2 test p=0.0224, S_{106}>RPS23 test p=0.003.

3.2.1.2. Downregulation of translation-associated proteins by RNAi in metabolic tissues can improve health in Drosophila but does not confer resistance to heat shock and tunicamycin stresses

Very often long-lived mutants are also resistant to certain stresses, so in order to study this matter, the transgenic flies were subjected to heat-shock and tunicamycin stresses; these specific stresses were chosen because of their link to protein folding and stability (Chow et al., 2013, Tower, 2011).
Tunicamycin is an antibiotic that can inhibit N-linked glycosylation, a process that happens in the endoplasmic reticulum (ER) membrane and that is important for protein folding (Koizumi et al., 1999). Therefore, when cells are exposed to tunicamycin the cell’s defence mechanism is to induce the unfolded protein response (UPR); this response consists in transiently reducing overall protein translation and inducing the expression of several specific genes such as ER chaperones (Iwata and Koizumi, 2005). We tested how our flies responded to the presence of tunicamycin dissolved in a holidic medium that was also supplemented with 200 µM RU486 or ethanol as control (see Materials and Methods 2.1.5.). Usually, tunicamycin is dissolved in sugar agar medium (Girardot et al., 2004, Appocher et al., 2014), but we used the holidic medium as the lack of amino acids in sugar-agar medium could downregulate TOR and induce a dietary restriction-like response masking the effects of downregulating our translation-associated proteins. None of the lines was resistant when tested, and in fact, S106>eEF2 RNAi was more sensitive to this stress, having the experimental flies a medium lifespan of 11.7 days and maximum of 16.1 days compared to the control that had 14.7 days median and 18 days maximum lifespan (p=5.09 x10\textsuperscript{-10}, χ\textsuperscript{2} log-rank test); similarly, the shorter-lived S106>eIF4G RNAi experimental flies had 17.2 days median and 22.6 days maximum lifespan compared to the control 18.7 days median and 21.6 days maximum lifespan (p=0.008, χ\textsuperscript{2} log-rank test) (Fig. 3.5.). Thus, both one of the longer-lived lines and the shorter-lived line were sensitive to induced-ER stress.

Exposure to high temperature happens in the wild to many organisms, which try to cope with this stress by inducing the highly evolutionary conserved heat shock response (Akerfelt et al., 2010). Thus, it is often examined if longer-lived organisms have also improved survival under heat shock. Our S106>UAS-RNAi flies were exposed to 38°C as described in Materials and Methods 2.2.2. Only eIF4G showed an increased resilience to heat shock, having S106>eIF4G RNAi experimental flies a median survival of 27.5 minutes compared to the control’s 22.5 minutes (p=0.006, χ\textsuperscript{2} log-rank test) (Fig. 3.6.). The rest of the flies tested (S106>eIF4E RNAi, S106>eEF2 RNAi and S106>RPS23 RNAi) had no statistically significant changes compared to the control flies (Fig. 3.6.). This means that only the shorter-lived flies were resistant to heat shock stress and hence they probably have a better heat shock response.
Figure 3.5. Survival of the S106>UAS-RNAi flies exposed to the ER stressor tunicamycin. Survival curves showing the survival over a 30 day period of the S106 flies treated with tunicamycin dissolved in holicular media and supplemented either with RU486 (solid lines) or ethanol (dashed lines). 150 mated female flies were tested. The p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control were calculated doing a $\chi^2$-log-rank test: A) Control p = 0.5, B) S106>eIF4E RNAi p = 0.19, C) S106>eIF4G p = 0.0088, D) S106>eEF2 test p = 5.09 x 10^{-10} and E) S106>RPS23 test p = 0.266.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 3.6. The resistance of the S106>UAS-RNAi flies to heat shock. The flies were subjected to 39°C and the number of irresponsive flies was scored every 5 minutes. There were 15 flies in each vial, and 3 vials per condition were tested. Solid lines correspond to flies that had been fed food supplemented with RU486 whereas the dashed lines correspond to flies fed the control ethanol food. The p values between the survivorship data of the flies fed RU486 and the ones fed ethanol control were calculated doing a χ²-log-rank test: A) Control p= 0.06, B) S106>eIF4E RNAi p=0.53, C) S106>eIF4G p=0.0026, D) S106>eEF2 test p= 0.037 and S106>RPS23 test p=0.88.

We then studied the healthspan of the S106>UAS-RNAi flies by assessing their fecundity. Fecundity is commonly linked to ageing, with several lifespan-extending interventions such as dietary restriction or downregulation of IIS leading to decreased fecundity (Partridge et al., 2005). Nevertheless, reduced fecundity and increased lifespan can be also uncoupled in Drosophila, with long-lived Indy mutants, flies treated with sirtuin activating factors or by excluding methionine from the diet having increased and non-affected...
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

A common way of studying fecundity in female flies is by counting the number of eggs they lay over a period of time, so we counted the number of eggs laid over 24 hours once a week during 6 weeks laid by mated flies that were also being assessed for lifespan. The fecundity measurement showed that, although the experimental flies had a slight tendency to have fewer eggs, this was not significant except in the case of the shorter-lived S106>eIF4G RNAi, which laid only an average of 12 eggs/female compared to an average 30 eggs/female (p=0.0049, Welch’s test) (Fig. 3.7.). Therefore, we obtained a lifespan extension independent of fecundity reduction.

Figure 3.7. Fertility assessment of the S106>UAS-RNAi flies. The number of eggs laid by 15 flies for 24 hours was counted once a week for 6 weeks, a total of five vials per condition were studied. The average of the sum number of eggs per average female was calculated and plotted here ±SEM. Experimental flies fed RU486 (red) can be compared to their control siblings fed ethanol food (blue). The p-value of the data for the RU486- compared to the data for the RU486+ was calculated doing a Welch’s t-test, **p≥0.005. Control p= 0.44, S106>eIF4E RNAi n.s, S106>eIF4G RNAi p=0.0049, S106>eEF2 RNAi n.s and S106>RPS23 RNAi n.s.

Another health parameter that was studied in our S106>UAS-RNAi flies was the senescence of their negative geotaxis behaviour. Negative geotaxis, or climbing assay, is an innate behaviour that flies show when they are contained in a vial and the vial is tapped, they climb up the surface to the top of the vial (Jones and Grotewiel, 2011). This behaviour has been widely studied and it is known to decline with age; thus, when a longer-lived mutant
climbs better it is considered to be healthier. When the climbing assay was performed in our flies (see Materials and Methods 2.2.1), the longer-lived S;106>eIF4E RNAi and S;106>eEF2 RNAi had improved performance index at older ages, especially at week 5 (p= 0.008 and p=0.014 respectively, Student t-test) (Fig. 3.8. B). In addition, S;106>eEF2 RNAi had also improved performance index at week 6 (p=0.0065, Student t-test) compared to its control. Nevertheless, the also longer-lived S;106>RPS23 RNAi showed a tendency to have better performance index at week 5 but it was not statistically significant (Fig. 3.8. D). On the other hand, the shorter-lived S;106>eIF4G RNAi had impaired performance index both at week 4 (p=0.0003, Student t-test) and 6 (p= 0.019, Student t-test) compared to the control (Fig. 3.8. C). This demonstrates that our lifespan results consisting of downregulation of translation-related factors is correlated with healthspan.

Figure 3.8. Assessment of the senescence of negative geotaxis in the S;106>UAS-RNAi flies. The average performance index (n=3) for each week was calculated and plotted ±SEM; solid lines correspond to experimental flies that had been fed food supplemented with RU486 whereas the dashed lines correspond to flies fed the control ethanol food. For the control, eIF4E RNAi and eEF2 RNAi 10 vials with 15 flies in each were tested, for eIF4G and RPS23 RNAi 5 vials were tested.
with 15 flies in each. The p-value of the data for the RU486- compared to the data for the RU486+ was calculated doing a student t-test, *p≥0.05: A) Control n.s. B) S106>eIF4E RNAi week1, 3, 4 and 6 n.s.; week 2 p=0.0216 and week 5 p=0.008. C) S106>eIF4G RNAi week 1 and 2 n.s.; week 4 p=0.02 and week 6 p=0.038. D) S106>eEF2 RNAi week 1, 2 and 4 n.s.; week 3 p=0.042; week 5 p=0.144 and week 6 p=0.0065. E) S106>RPS23 RNAi n.s.

3.2.1.3. Downregulation of translation-associated proteins by RNAi in metabolic tissues does not alter signalling pathways related to protein synthesis

To assess if our S106>UAS-RNAi flies had altered protein synthesis through other signalling pathways, we measured the levels of phosphorylation of eIF2α at serine 51, p70 S6K at threonine 389 and 4EBP at threonine 37/46. For this purpose, we dissected the fat body and intestines of the flies, as these were the tissue where translation factors were downregulated, and we subjected these tissues to immunoblotting as described in Materials and methods 2.4. eIF2α is phosphorylated, and hence inactivated, under stress conditions to inhibit protein synthesis (Kimball, 1999). In our flies, there was no statistical difference in the level of phosphorylation of eIF2α between the experimental flies and their respective controls, not even in the S106>eIF4G RNAi flies which had the most pronounced phenotypic changes in reduced lifespan, climbing and egg laying (Fig. 3.9.). pS6K is a Ser/Thr protein kinase that phosphorylates RPS6 and it is involved in the control of 5’ oligopyrimidine tract mRNAs (Pullen and Thomas, 1997). Its phosphorylation at Thr389 by mTOR and Akt leads to activation of pS6K (Miron et al., 2003, Fenton and Gout, 2011). S106>eIF4G RNAi showed a statistically significant increase in the level of phosphorylated pS6K (p=0.009, Student t-test) (Fig. 3.9.). Finally, 4EBP is an inhibitor of cap-dependent translation through binding and sequestration of eIF4E; when 4EBP is hyperphosphorylated, it becomes inactive and hence cap-dependent translation can occur (Pause et al., 1994). None of our flies showed a significant difference in the level of phospho-4EBP normalised to total 4EBP; however, eIF4E and eIF4G consistently showed a reduction of both phospho and non-phospho 4EBP compared to their respective controls (Fig 3.9. C). This could be due to degradation of any hypophosphorylated free 4EBP when the level of eIF4F is low (Salaun et al., 2003), as the stoichiometry of eIF4F and 4EBP is tightly linked (Josse et al., 2016, Alain et al., 2012).
Figure 3.9. Levels of phosphorylation of eIF2α (Ser51), pS6K (Thr389) and 4EBP (Thr37/42) in the gut and fat body of the S106>UAS-RNAi flies. A), B) and C) S106>UAS-RNAi flies fed RU486 are represented in red and flies fed the control food are represented in blue. The levels of phosphorylated protein and total protein were normalised to their corresponding GAPDH levels before calculating the ratio between phosphorylated and total protein. Three independent experiments with total six samples (with 6 dissected flies in each, approximately 30 µg of protein loaded per lane) for eIF2α and 4EBP and 3 samples for pS6K. The average were calculated and are represented here ±SEM. The p-value between the RU486- and the RU486+ data was calculated with a Student’s t-test, *p≤0.05: B) S106>eIF4G RNAi pS6K (Thr389) p=0.0091. D) Representative images of the anti-phospho and total proteins plus their corresponding anti-GAPDH.
3.2.1.4. Measurements of *de novo* protein synthesis in flies with downregulated translation-associated proteins by RNAi in metabolic tissues

Some of the longevity studies in *C. elegans* in which different translation-associated factors and ribosomal proteins were downregulated or knocked out showed that the overall newly synthesized protein was also reduced by comparing the relative levels of $^{35}$S-methionine incorporation (Hansen et al., 2007, Pan et al., 2007). Therefore, we tried to measure the level of *de novo* protein synthesis in the fat body, in which translation-associated proteins were downregulated by RNAi. Here we should note that we did not use the intestine, although S$_{1106}$ also drives expression of UAS-RNAi in this organ, in order to avoid potential labelling of the microbiota's peptides and hence cross-contaminate our samples. For this purpose we dissected the fat body and incubated them in a solution containing puromycin dihydrochloride, a tRNA analogue that can get incorporated into newly-synthesized polypeptide chains allowing its detection by immunoblotting with an anti-puromycin monoclonal antibody (Deliu et al., 2017, Filer et al., 2017) (see Materials and Methods 2.4).

We optimised the method by trying different ways of incubating the dissected samples in the puromycin solution; a) we tried pinning the tissue down and covering it with Schneider’s media containing puromycin, b) we left the tissues in microcentrifuge tubes with puromycin median without mixing or c) we incubated the samples in a Thermomixer with continuous gentle mixing (Fig. 3.10.). We saw that the highest measured levels of puromycin with the least variability were obtained when we vortexed the samples, so the rest of experiments were performed this way. When we tested the S$_{1106}$>UAS-RNAi flies, we saw that despite the tendency in reduced translation found in the experimental flies, especially S$_{1106}$>elF4E RNAi, S$_{1106}$>elF4G and S$_{1106}$>RPS23, we still saw variability within the replicate samples and hence no significant differences (Fig. 3.11.).
Figure 3.10. Sample preparation optimization for the puromycin-based assay to measure de novo protein synthesis. The samples had to be incubated in puromycin dihydrochloride dissolved in Schneider (10 µg/ml) for 30 minutes at room temperature; thus, to optimise tissue absorption we tried diverse ways of incubating the sample. We dissected the fat bodies of outbred w^{Dah} female flies and we either pinned the samples down and covered them with the puromycin-Schneider solution (left part of the membrane), we left the samples in microcentrifuge tubes with the solution (middle part of the membrane), or we had these samples in a thermomixer (right part of the membrane). We saw that the highest level of puromycin detection and less variability between samples was seen in the immunoblot of the samples that had been mixed throughout the incubation period.
Figure 3.11. Relative levels of de novo protein synthesis in the fat bodies of flies with downregulated translation-associated proteins and their relative controls. We treated the fat body samples with puromycin dihydrochloride and detected the level of de novo protein synthesis by using a monoclonal antibody against puromycin in A) control flies fed control food or RU486, B) S;106>eIF4E RNAi fed control or RU486 food; C) S;106>eIF4G RNAi fed control or RU486 food; D) S;106>eEF2 RNAi fed control or RU486 food; E) S;106>RPS23 RNAi fed control or RU486 food. F) We normalised the data by their corresponding levels of GAPDH and to the control fed control EtOH food. Eight samples (with 3 fat bodies in each) for each condition from two independent experiments were analysed and the average calculated and plotted here ±SEM. We calculated the p values between the flies fed RU486 and the flies fed the control food using 1 way ANOVA, and no statistical difference was found.
If variability of this assay comes from unequal puromycin administration in the fat body tissue, then puromycin translation assay in the S2R+ cells should give more consistent results. Therefore, we decided to downregulate these same proteins in Drosophila S2R+ cells using dsRNA and measure the consequent effect on de novo protein synthesis also using the puromycin-based assay. For this, we downregulated elf4E, elf4G, eEF2 and RPS23 using double-stranded RNA (dsRNA)-mediated interference (Clemens et al., 2000, Kao and Megraw, 2004) (see Materials and Methods 2.9.). Celia Lujan used the same primers that the RNAi lines we used in the flies to create the dsRNA and she corroborated the efficacy of the RNA interference by subjecting measuring the abundance of RNA using RT-qPCR (see Materials and Methods 2.3.). The RT-qPCR allowed us to see a clear dose response to the increasing concentration of dsRNA (Fig. 3.12.). The cells treated with 50 ng/ml dsRNA against elf4E had 95 % downregulation of this factor’s mRNA (p=5.7 x10^{-6}, Student t-test), with 12.5 ng/ml they had 90% reduction (p=1 x10^{-5}, Student t-test), with 3.125 ng/ml the cells had 83.5% reduction (p=1.9 x10^{-5}, Student t-test) and with 0.78 ng/ml they had 63.5% reduction (p=2.2 x10^{-5}, Student t-test). When treated with 50 ng/ml dsRNA against elf4G the cells had 90.5% downregulation of this factor’s RNA (p=1.4 x10^{-4}, Student t-test), with 12.5 ng/ml they had 89% reduction (p=1 x10^{-4}, Student t-test), with 3.125 ng/ml they had 82.5% reduction (p=1.2 x10^{-4}, Student t-test) and with 0.78 ng/ml the cells had 65.5% reduction (p=7.9 x10^{-5}, Student t-test). The cells treated with 50 ng/ml dsRNA against eEF2 had 92.5 % downregulation of this factor’s RNA (p=1.6 x10^{-5}, Student t-test), with 12.5 ng/ml they had 90.5% reduction (p=1.7 x10^{-5}, Student t-test), with 6.25 ng 85.5% reduction (p=2 x10^{-5}, Student t-test) and with 0.78 ng/ml they had 76% reduction (p=2 x10^{-4}, Student t-test). Finally, using 50 ng/ml dsRNA against RPS23 reduced the level of the RNA encoding this ribosomal protein by 66% (p=4 x10^{-6}, Student t-test), using 12.5 ng/ml by 56.5% (p=1.1 x10^{-5}, Student t-test), using 3.125 ng/ml by 47% (p=3 x10^{-4}, Student t-test) and with 0.78 ng/ml there was a 15.5% reduction (p=5.5 x10^{-4}, Student t-test). These results gave us an idea of how efficient the dsRNA treatment in S2R+ cells were, and surprisingly showed how relatively low concentrations of dsRNA suffice for potent downregulation of gene expression. To replicate the milder downregulation of the translation-associated proteins that we have in the S106>UAS-RNAi flies, we decided to lower the dose of dsRNA for the puromycin-based measurement of de novo protein synthesis.
Figure 3.12. Relative abundance of RNA encoding for each translation-associated protein in S2R+ cells treated with decreasing amounts of dsRNA downregulating eIF4E, eIF4G, eEF2 or RPS23. A RT-qPCR was performed to measure the RNA abundance of each factor of interest in cells treated with dsRNA. Two samples were studied (12 wells from a 48 well plate mixed in each sample) for each condition. The values were normalised by the level of Actin 5C RNA of each individual sample and then they were relativized to the normalised average of the control. The averages ±SEM are plotted here and the p values between the cells treated with dsRNA and the untreated control were calculated using a Student t-test, **≤0.005:  

A) eIF4E dsRNA 50 ng/ml p=5.7 x10^{-6}; 25 ng p=1 x10^{-5}; 3.125 ng/ml p=1.9 x10^{-5} and 0.78 ng/ml p=2.2 x10^{-5}.  

B) eIF4G dsRNA 50 ng/ml p=1.4 x10^{-4}; 25 ng p=1 x10^{-4}; 3.125 ng/ml p=1.2 x10^{-4} and 0.78 ng/ml p=7.9 x10^{-4}.  

C) eEF2 dsRNA 50 ng/ml p=1.6 x10^{-5}; 25 ng p=1.7 x10^{-5}; 3.125 ng/ml p=2 x10^{-5} and 0.78 ng/ml p=2 x10^{-4}.  

D) RPS23 dsRNA 50 ng/ml p=4 x10^{-6}; 25 ng p=1.1 x10^{-5}; 3.125 ng/ml p=3 x10^{-4} and 0.78 ng/ml p=5.5 x10^{-4}.  

The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 3.13. Relative levels of de novo protein synthesis in S2 cells treated with several concentrations of dsRNA against the translation-associated proteins, eIF4E, eIF4G, eEF2 and RPS23. We measured de novo protein synthesis by incubating with puromycin cells that had been previously treated with dsRNA for 72 hours: A) control (no dsRNA) treatment B) dsRNA interfering with eIF4E, C) dsRNA interfering with eIF4G, D) dsRNA interfering with eEF2 and E) dsRNA interfering with RPS23. These cells were then subjected to immunoblotting and we detected the level of puromycin in each sample. Eight samples were analysed for the control (for each sample 6 wells from a 48 well plate were mixed) and three samples were used for the rest of the conditions. F) The level of relative puromycin was normalised against the corresponding total protein in the membrane detected by Ponceau staining and the average calculated. The averages ±SEM are plotted here and the p values between the treated cells and the control were calculated using 1 way ANOVA and no statistical significance was found.

To mimic the RNAi efficiency obtained in the S106>UAS-RNAi flies, we aimed to downregulate the expression of each translation-associated protein to the same level that was found in the RT-qPCR previously shown. Therefore, we aimed to downregulate eIF4E by approximately 37%, so we used 0.4 ng/ml, 0.2 ng/ml and 0.1 ng/ml dsRNA in the S2R+ cells. For eIF4G, we aimed to downregulate this factor by 42%, so we used 0.4 ng/ml, 0.2 ng/ml and 0.1 ng/ml dsRNA too. For eEF2, the desired downregulation was of 34%, so we used 0.1 ng/ml,
0.05 ng/ml and 0.025 ng/ml dsRNA. For RPS23, we wanted to downregulate this factor by 58%, so we used 25 ng/ml, 12.5 ng/ml and 6.25 ng/ml dsRNA. After 72 hours treatment with the specific dsRNA, we supplemented the cells with puromycin and detected the level of this chemical through immunoblotting (see Materials and Methods 2.4.). Again, we saw a tendency in the cells treated with these factors to have reduced levels of puromycin incorporation; however, these values were not statistically significant nor dose responsive (Fig. 3.13.). In order to reliably assess translation in these long-lived mutants, translation measurements using puromycin in vivo and in vitro will be repeated and the assay optimized to achieve less variability.

3.2.1.5. Translation fidelity examination in flies with downregulated translation factors by RNAi in metabolic tissues

One of the main questions that we wanted to answer was if the downregulation of translation through a decrease in translation factors leads to improved translation fidelity. Unfortunately, it was not possible to measure the levels of translation accuracy in our S1106>UAS-RNAi flies. We adapted a dual luciferase reporter based on yeast studies to be injected in flies using the pUAST-attB vector, to which we cloned the Heat-Shock promoter (see Materials and Methods 2.6.1). Heat shock promoter is a mild promoter under optimal 25°C and we chose it over the stronger Actin and Tubulin promoters to avoid potential artefacts linked to very high levels of overexpression of the reporter protein. A double mutant of translation fidelity luciferase-reporter flies and the S1106 driver was generated to measure translation accuracy in S1106>UAS-RNAi flies. However, the level of luminescence was too low in the dissected intestine and fat body tissues where S1106 driver was active, as the reporters were mainly expressed in the head and thorax (data not shown). Nevertheless, in Chapter 5. Results below, the reporters were used for measurement of translation fidelity in young and old flies, based on the signal coming from the head and thorax. A novel reporter was constructed using the ubiquitin promoter and it will be used in the future to measure how downregulation of translation factors impacts translation fidelity.

3.2.1.6. Epistasis measurements using paromomycin

We then wondered how the S1106>UAS-RNAi flies respond to an increase in translation errors. For this, we fed the flies with paromomycin, an antibiotic known to increase the level of mistranslation by triggering conformational changes in the ribosome (Ogle et al., 2001). We decided to use 800 µM paromomycin dissolved in 1 SYA with or without RU486 based on our data with S2R+ cells and after testing three different concentrations in the
control S106 flies (see Chapter 4. Results). In this pilot, we saw no effect of 600, 800 and 1000 µM on the lifespan of our control flies (Fig. 3.14.). Nonetheless, when we later tested these concentrations in our S106-UAS-RNAi flies, we saw a small beneficial effect of the flies fed with paromomycin compared to the flies without it (data not shown). We then decided to see if this was due to the antibacterial action of paromomycin, so we supplemented the food of the flies with 60 mg/L ampicillin, as some antibiotics have already been reported to extend lifespan in Drosophila (Loch et al., 2017, Brummel et al., 2004). In fact, when we tested ampicillin-supplemented food, we also saw a beneficial effect of this antibiotic on our flies. The S106 control flies had 69.5 days median lifespans when fed either RU486 or ethanol-supplemented media, whereas when fed ampicillin either with or without RU486, they had 74 days median lifespans \( p=5.2 \times 10^{-10}, p=2.1 \times 10^{-6} \), \( \chi^2 \) log-rank test) (Fig. 3.14. A). When ampicillin was tested in S106>eIF4E RNAi flies they also lived longer when given the antibiotic. The control S106>eIF4E RNAi flies fed control food had 69.5 median lifespans, whereas when fed ethanol-ampicillin media they had 72 days median lifespan \( p=0.002, \chi^2 \) log-rank test) (Fig. 3.14. B). The long-lived S106>eIF4E RNAi had an even longer lifespan when fed ampicillin: the flies fed RU486 had 74 days median lifespan and the flies fed RU486 and ampicillin had 76.5 days median lifespan \( p=1.13 \times 10^{-5}, \chi^2 \) log-rank test) (Fig. 3.14. C).

Figure 3.14. Optimisation of the conditions required for the paromomycin epistasis experiment. A) The control flies were fed different concentrations of paromomycin (600 µM orange, 800 µM brown and 1000 µM grey lines), the \( p \) values between the survivorship data of
the experimental flies supplemented with paromomycin compared to the flies supplemented ethanol were calculated doing a χ²-log-rank test: Control 600 µM paromomycin; control 800 µM paromomycin and control 1000 µM paromomycin n.s. B) These control flies were also later fed ampicillin (green lines), with (solid line) or without RU486 (dashed line), the p values between the survivorship data of the experimental flies supplemented with ampicillin and/RU486 compared to the flies supplemented ethanol control food were calculated doing a χ²-log-rank test: Control 600 µM paromomycin; control 800 µM paromomycin and control 1000 µM paromomycin n.s.

B) These control flies were also later fed ampicillin (green lines), with (solid line) or without RU486 (dashed line), the p values between the survivorship data of the experimental flies supplemented with ampicillin and/RU486 compared to the flies supplemented ethanol control food were calculated doing a χ²-log-rank test: Control 600 µM paromomycin; control 800 µM paromomycin and control 1000 µM paromomycin n.s.

C) S₁₀⁶>eIF4E RNAi was also fed ampicillin in the presence of RU486 (green solid line) or not (green dashed line). The p values between the survivorship data of the experimental S₁₀⁶>eIF4E RNAi flies fed RU486+ (either also supplemented with ampicillin or control) and the sibling control flies fed ethanol-supplemented food (either also supplemented with ampicillin or control) were calculated doing a χ²-log-rank test: S₁₀⁶>eIF4E RNAi RU486+ vs. control on ethanol food p= 0.033; S₁₀⁶>eIF4E RNAi RU486+ with ampicillin vs. control on ampicillin food p= 0.0036; control on ampicillin vs. control eIF4E on control food p= 0.002. Around 150 mated female flies per condition were used.

After these results, we bleached both the S₁₀⁶ and the RNAi stocks to remove any harmful bacteria. Otherwise, paromomycin might have a dual effect on lifespan: lifespan prolongation mediated by removal of harmful bacteria in the gut and lifespan shortening through increase of erroneous proteins. The fly eggs were bleached, and a few generations post-bleaching the progeny were then used to test the effects of paromomycin. In this experiment, 800 µM paromomycin slightly shorted the lifespan of the control flies, when fed RU486 or the ethanol control (p=5.8 x10⁻⁷ and p=0.0007 respectively, χ² log-rank test). S₁₀⁶>eIF4E RNAi flies when they were fed paromomycin and RU486 or the ethanol supplemented food had 56 days median lifespan, whereas the S₁₀⁶>eIF4E RNAi flies given just RU486 had a longer 63 days median lifespan and the flies with ethanol and paromomycin had shorter 53.5 median lifespans (Fig. 3.15. B). Therefore, feeding paromomycin shortened the lifespan of both the experimental and control S₁₀⁶>eIF4E RNAi flies (p=8.39 x10⁻¹² and 1.4 x10⁻¹⁰ respectively, χ² log-rank test). Paromomycin also decreased the lifespan of both the experimental and control S₁₀⁶>eEF2: S₁₀⁶>eEF2 RNAi fed RU486 supplemented food had 63 days median lifespan, compared to S₁₀⁶>eEF2 RNAi fed both RU486 and paromomycin that had 60 median lifespans (p= 0.006, χ² log-rank test) (Fig. 3.15. D). The control eEF2 RNAi flies fed ethanol and paromomycin supplemented food had a shorter lifespan than the flies fed only ethanol, 56 and 60.5 days median lifespans respectively (p=8.3 x10⁻¹¹, χ² log-rank test) (Fig. 3.15. D). S₁₀⁶>RPS23 RNAi flies, however, had shorter lifespan on paromomycin only when they were also fed RU486 and hence RPS23 was downregulated; the flies fed paromomycin and RU486 had a median lifespan of 63 days compared to 67.5 days median lifespan of the experimental flies (p=3.3 x10⁻⁷, χ² log-rank test) (Fig. 3.15. E). The flies fed control ethanol-supplemented food with or without paromomycin both had 63 median lifespans; therefore they were not affected by this antibiotic. In addition, it should be noted that S₁₀⁶>eIF4E RNAi
lived longer than its control (p=6 x10^{-23}, \chi^2 \text{ log-rank test}), S_{1,106}>eEF2 also lived longer (p=2.4 x10^{-16}, \chi^2 \text{ log-rank test}) and S_{1,106}>RPS23 RNAi did too (p=5.2 x10^{-11}, \chi^2 \text{ log-rank test}). Therefore, paromomycin stopped the lifespan extension of downregulation in metabolic tissues of eIF4E, eEF2 or RPS23, making the lifespans be as the ones of the control flies. In addition, the shorter-lived S_{1,106}>eIF4G RNAi flies were even shorter-lived when they were supplemented with paromomycin (p=7 x10^{-4}, \chi^2 \text{ log-rank test compared to } S_{1,106}>eIF4G flies fed only RU486) (Fig 3.15. C). Interestingly, the control S_{1,106}>eIF4G RNAi flies were also shorter lived in the presence of paromomycin (p=5.44 x10^{-11}, \text{ log-rank test compared to control } S_{1,106}>eIF4G flies fed control food). Thus, paromomycin had a detrimental effect in both experimental S_{1,106}>eIF4G flies and controls.

Therefore, in the presence of paromomycin, the lifespans of eIF4E, eEF2 and eIF4G RNAi lines were shifted to the left and longevity proportionally shortened on both the control and the RU486-GS-inducing food; thus, increased protein errors by paromomycin equally affected controls and long-lived lines (Fig. 3.15.). This shift to the left is less pronounced in RPS23 RNAi line, where it appears that paromomycin treatment abolished longevity and where longevity of S_{1,106}>RPS23 RNAi on RU486 and paromomycin food is nearly undistinguishable from the non-induced condition and the control. It could be that increased accuracy has a role in longevity by RPS23 downregulation and not in the other RNAi lines. We aimed to use a concentration of paromomycin low enough that would not affect longevity of the control flies and that would abolish the lifespan extension of the mutants, in case their longevity was mediated by an increase in translation accuracy. However, it is difficult to achieve such careful paromomycin dosage, and in the future, it would be good to repeat these experiments with several paromomycin concentrations. An additional confounding factor in these experiments is that the feeding behaviour of these flies could affect paromomycin intake. In sum, future measurement of accuracy using the dual-luciferase reporters will clarify the role of accuracy in these mutants.
Figure 3.15. Effect of paromomycin on the survival of $S_{1}106$-UAS-RNAi flies. To activate the UAS-RNAi system flies were fed RU486 (red solid lines). As a control, the sibling flies were fed ethanol-supplemented food (blue solid lines). 800 µM paromomycin was given to flies fed RU486 (red dashed lines) or control food (blue dashed lines). Around 150 mated female flies per condition were used. The p values between the survivorship data of the flies were calculated doing a χ²-log-rank test: A) Control flies fed RU486+ vs. control flies on control food p= $3.9 \times 10^{-7}$; control flies on RU486 with paromomycin vs. control flies on RU486 p= $7 \times 10^{-4}$ and control flies on paromomycin vs. control flies on control food p= $5.84 \times 10^{-7}$. B) $S_{1}106$-eIF4E RNAi on RU486 vs. eIF4E RNAi control flies on control food p= $6 \times 10^{-23}$; $S_{1}106$-eIF4E RNAi on RU486+ and paromomycin vs. $S_{1}106$-eIF4E RNAi flies on RU486 p= $8.39 \times 10^{-12}$ and eIF4E RNAi control flies on paromomycin vs. eIF4E RNAi control flies without p= $1.48 \times 10^{-10}$. C) $S_{1}106$-eIF4G RNAi on RU486 vs. eIF4G RNAi control flies on control food p= 0.001; $S_{1}106$-eIF4G RNAi on RU486 with paromomycin vs. $S_{1}106$-eIF4G RNAi flies on RU486 p= $7 \times 10^{-4}$ and eIF4G RNAi control flies on paromomycin vs. eIF4G RNAi control flies without p= $5.44 \times 10^{-11}$. D) $S_{1}106$-eEF2 RNAi on RU486 vs. eEF2 RNAi control flies on control p= $2.44 \times 10^{-10}$; $S_{1}106$-eEF2 RNAi on RU486 with paromomycin vs. $S_{1}106$-eEF2 RNAi flies on RU486 p= 0.006 and eEF2 RNAi control flies on paromomycin vs. eEF2 RNAi control flies without p= $8.2 \times 10^{-11}$. E) $S_{1}106$-RPS23 RNAi RU486 vs. RPS23 RNAi control flies on control food p= $5.2 \times 10^{-11}$; $S_{1}106$-RPS23 RNAi on RU486 with paromomycin vs. $S_{1}106$-RPS23 RNAi flies on RU486 p= $3.3 \times 10^{-7}$ and RPS23 RNAi control flies on paromomycin vs. RPS23 RNAi control flies without p= 0.023.
In addition, we counted the eggs of the flies fed paromomycin to see how this error inducing antibiotic affected their fecundity. The only RNAi line that was significantly affected by paromomycin was eEF2, which when fed the control food with paromomycin, had fewer eggs than the flies fed just the control food (p=0.0096, Welch’s test) (Fig. 3.16. D). Interestingly, when the S106>eEF2 RNAi flies were fed RU486 and paromomycin there was a rescue of this phenotype and the flies did not lay significantly fewer eggs than the flies just fed RU486 (Fig. 3.16 D). The shorter-lived S106>eIF4G RNAi laid fewer eggs than their controls, independently of the presence or absence of paromomycin on the food (p=0.0028 and p=0.0079 respectively, Welch’s test) (Fig. 3.16. C).

![Figure 3.16. Effect of paromomycin on the fertility of S106-UAS-RNAi flies. The flies were fed 800 µM paromomycin supplemented food with (red dotted columns) or without (blue dotted columns) RU486 and the number of eggs laid over a period of 24 hours was counted once a week for 6 weeks. Five vials with 15 flies in each were studied for each condition, run in parallel to the lifespan analysis. Data shown here are the average cumulative number of eggs per average female ± SEM. The p values between were calculated with a Welch’s t-test * p≤0.05 **p≤0.005: A) Control, n.s. B) S106>eIF4E RNAi n.s. C) S106>eIF4G RNAi vs control eIF4G RNAi p=0.0079 and S106>eIF4G RNAi on paromomycin vs control eIF4G RNAi on paromomycin p=0.0028. D) Control eEF2 RNAi vs control eEF2 RNAi on paromomycin p=0.0096. E) S106>RPS23 RNAi n.s.](image-url)
3.2.1.7. Conclusions about the effect on *Drosophila*'s longevity of downregulating translation-associated proteins by RNAi

The results explained in this section show that downregulating eIF4E, eEF2 and RPS23 in the flies’ metabolic tissues can extend their lifespan and improve their health span, the latter shown by the negative geotaxis. However, this does not give the flies resistance against tunicamycin or heat shock stresses. Downregulating eIF4G in *Drosophila*'s metabolic tissue decreases the lifespan and health span of the flies (both negative geotaxis and fecundity), but it confers resistance against heat shock. This suggests that stress resistance does not always correlate with longevity. Also, only this short-lived genotype had higher levels of phosphorylated pS6K (Thr389). In addition, several attempts were carried out to measure the level of *de novo* protein synthesis, but unfortunately, no conclusive results were obtained. The error-inducing paromomycin decreases the lifespan (but not the fertility) of flies with downregulated eIF4E, eIF4G, eEF2 and RPS23 but the lifespan of their respective controls is also shortened by paromomycin.
3.3. Discussion

3.3.1. Ubiquitously downregulating certain translation-associated factors in adult flies can be detrimental, but their downregulation in the fat body and intestine can extend lifespan

We aimed to study the effect of downregulating translation in Drosophila’s lifespan, and resembling the studies carried out in C. elegans (Hansen et al., 2007, Hamilton et al., 2005, Curran and Ruvkun, 2007, Syntichaki et al., 2007a, Pan et al., 2007), we first used the ubiquitous inducible driver Actin GS. Unlike in the worm studies, we saw that all the RNAi lines tested had a negative or non-apparent effect on lifespan, with the exception of CG4849, which although it was annotated as eEF2, it actually corresponds to the splicing factor EFTUD2 (Lasko, 2000, Marygold et al., 2017). This means that the decrease of expression of these factors in the whole body of adult flies with the standard 200 µM concentration of RU486 we used, was detrimental; for this reason, we decided to use the S106 driver, which drives the expression of the GAL4-UAS system only in the flies’ metabolic tissues (i.e. the fat body and intestines) and that has been linked to longevity and it has already been used in lifespan experiments (Hwangbo et al., 2004, Giannakou et al., 2004, Alic et al., 2011).

In our screen using this driver, we found that downregulation in the fat body and intestine by RNAi of the expression of elf1 (CG17737), elf3-S8 (CG4954), elf3-S10 (CG9805), elf4E (CG4035), eEF2 (CG2238), RPS23 (CG8415), mitochondrial RPS23 (CG1842) and myc (CG10798) increased lifespan. It is important to note that the initiation factors elf1, elf3 and elf4E have already been shown to be related to ageing in adult C. elegans, but RPS23, mitochondrial RPS23 and eEF2 had not (Pan et al., 2007, Syntichaki et al., 2007a). Interestingly, flies with downregulated elf4G (CG10811) had shorter lifespan than the controls, even when 10 times less RU486 was supplemented, although worms with downregulated ifg-1 (worm’s homolog of elf4G) had improved longevity (Hansen et al., 2007, Curran and Ruvkun, 2007, Pan et al., 2007). Furthermore, some factors that had a robust lifespan extension when downregulated in adult worms did not increase lifespan in our screen, namely RPL9 (CG6141), RPL19 (CG2746) or RPS3 (CG6779). These inconsistencies between worms and flies could be explained by several reasons. One of the causes could be that these factors do not affect longevity through metabolism, so their downregulation in this tissue was not enough for lifespan extension. Another possibility is that the functions of these proteins are slightly different between yeast, worms and flies and hence their effect on longevity differs. Another
reason for these variances in the effects seen between model organisms could be that the level of downregulation mediated with the S106 RNAi driver was not appropriate for lifespan extension for these genes. It is common in ageing studies that too low levels of expression alteration do not lead to a longevity phenotype while too strong downregulation is detrimental, and it is challenging to determine the appropriate alteration of expression.

Finally, another possibility to take into account is that these RNAi lines might not be silencing the gene of expression of the protein of interest, as either, they do not work or have an off-target. Unfortunately, it was not feasible to corroborate this in all the lines of our focused screen, so we had to carry the RT-qPCRs only for the lines in which we decided to focus.

We decided to concentrate on eF4E, elf4G, eEF2 and RPS23 because these lines gave the most robust lifespan extension we observed and where possible, they had replicate results with various independent RNAi lines. In addition, the selection of these lines could give further insights into the longevity effects of downregulation of translation initiation, elongation and ribosomal biogenesis. We performed a quantitative PCR to measure the level of expression of these factors in our flies with downregulation by the S106-UAS-GAL4 system, and we saw that all of them were at least 30% downregulated. Nonetheless, we have still not corroborated if the levels of global protein synthesis are altered in these flies. Thus, we worked on optimizing for our tissues of interest a method based on the use of puromycin dihydrochloride, a tRNA analogue that gets incorporated into newly-synthesized peptides causing premature chain termination and allowing its detection in immunoblotting with a puromycin antibody (Deliu et al., 2017, Filer et al., 2017). This, together with the results obtained in S2R+ cells that have these translation-associated factors downregulated by dsRNA, have shown that despite the trend of reduced de novo protein synthesis seen when these factors are downregulated by RNAi, there is still too much variability in our results. Therefore, we are still working on the optimization of this technique and we are also considering using others (e.g. polysome profiling) to quantify translation.

We also studied the resilience of S106>elF4E RNAi, S106>elF4G RNAi, S106>eEF2 RNAi and S106>RPS23 RNAi flies to heat shock, as lessening translation, and particularly improving accuracy, are expected to render flies heat shock resistant. Notably, the longer-lived flies did not show resistance against this stress but the shorter-lived S106>eLF4G RNAi did. The longer-lived worms with downregulated translation were thermotolerant in the Hansen et al. experiments (i.e. treated with dsRNA against eIF4E, eIF4G, S6K, RPS6, RPS15, RPL19 and TOR), whereas Pan et al. and Syntichaki et al. did not see this heat shock resistance in worms treated with dsRNA against S6K, eIF4E and eIF4G (Hansen et al., 2007, Pan et al., 2007, Syntichaki et
al., 2007a). These discrepancies could be caused by the age of the worms in which the heat shock experiment was performed, Hansen et al. used 3 days old worms but Pan et al. used 5 days old worms (Syntichaki et al. do not specify the age) (Syntichaki et al., 2007a, Pan et al., 2007). In our experiment, it could also be that the age of the flies was not appropriate (we used 10-12 days old flies) or that the experimental set up that was used was not sensitive enough to see any differences, so we are considering repeating it with flies from different ages and using different protocols; for example, giving the flies a heat shock in which the temperature ramps up or in which the temperature is lower than 38°C to mimic physiologically normal conditions and to give the cells time to cope with the temperature-induced damage as the heat shock dose administered is not lethal to the fly (Colinet et al., 2013). Another possibility, however, is that the mechanisms behind the longevity effect are not related to increases in the heat shock response. In fact, a more recent study in C. elegans with downregulated eIF4G showed that the gene required for thermotolerance, hsf-1, was not involved in lifespan extension (Howard et al., 2016). It is true that one of the hypotheses suggests that decreased translation increases the folding capacity, yet, this does not mean that the damage produced by acute heat shock can be improved under the better folding seen in normal physiological conditions. For example, a recent paper found that paromomycin concentrations that shorten the lifespan of S. cerevisiae by a decrease in translation fidelity did not make the yeast sensitive to heat-shock. Therefore, to better explore this hypothesis we could perform a re-folding assay (for example, based on luciferase re-folding) (Suhm et al., 2018).

We also tested the resistance of these flies to UPR stress, using tunicamycin as a UPR inducer, as it has been shown to be enhanced in worms with downregulated eIF4G and yeast with ribosomal subunit deletions (Steffen et al., 2012, Howard et al., 2016). None of our S1106>UAS-RNAi flies showed resistance against tunicamycin. One possible explanation of this result is that the concentration of RU486 (200 µM) that we put in the tunicamycin-supplemented holidic medium was too high, as it is the same we put in 1 SYA medium, which, as already mentioned, shows less bioavailability than the holidic medium (Piper et al., 2014). Thus, we are planning to test it again with a lower concentration of RU486. Furthermore, tunicamycin, like other stressors such as paraquat, is administered by feeding and hence any flies that feed less may seem more resistant and on the contrary, if our mutants were feeding more because of downregulated translation they would be ingesting more tunicamycin. Nevertheless, it could still be that these flies are not resistant to this stress because their lifespan extension is not by reason of an improvement in the response to UPR or protein
folding. Another possible explanation between the discrepancy between the resistance shown by worms with eIF4G RNAi and our flies with S106>eIF4G RNAi is that in worms the downregulation was ubiquitous whereas in our case it was tissue-specific, meaning that maybe downregulation in more tissues could have led to resistance in our flies too.

In addition, we studied the fecundity of flies with downregulated eIF4E, eIF4G, eEF2 and RPS23, since this is a parameter of health span that is tightly linked to ageing and most long-lived mutants have impaired fecundity (Khazaeli and Curtsinger, 2013, Stearns et al., 2000). Remarkably, our longer-lived lines did not have decreased fecundity, whereas the shorter-lived eIF4G did. However, this result is different from what was seen in longer-lived adult worms with downregulated translation by RNAi, which had significantly reduced fecundity (Hansen et al., 2007, Pan et al., 2007). This discrepancy is probably caused by the worm treatment being ubiquitous and stronger than ours, and that the germline of our flies should be unaffected. It is important to note too that, although fecundity has been shown to be downregulated in longer-lived organisms, including flies, leading to theories of ageing such as ‘The disposable soma’, these two effects can also be uncoupled (Giannakou et al., 2004, Khazaai and Curtsinger, 2013, Mason et al., 2018, Sgro and Partridge, 1999, Stearns et al., 2000). Thus, it is preferable to have anti-ageing interventions that are not detrimental to fecundity, like in our case.

3.3.2. Downregulation in the fat body and intestines of eIF4E extends lifespan but downregulation of eIF4G shortens it

The eukaryotic initiation factors 4E, 4G and 4A belong to the eIF4F complex and have been shown to be related to ageing in yeast and in C. elegans (Howard and Rogers, 2014a, Curran and Ruvkun, 2007, Hansen et al., 2007, Syntichaki et al., 2007a); however, in our hands eIF4E and eIF4G had opposite effects in both lifespan and health span, eIF4E improving them and eIF4G worsening them. eIF4E binds the scaffolding eIF4G, which in turn recruits the helicase eIF4A factor (Aitken and Lorsch, 2012). When the eIF4F complex is formed, eIF4G binds PABP (Poly (A)-binding protein) circularizing the mRNA and facilitating eIF4E to recognize the 5’ cap structure and eIF4A to anchor the 5’ UTR of mRNA, hence cap-dependent translation can start (Aitken and Lorsch, 2012). In addition, eIF4G also binds eIF3, at least in mammalian cells, and eIF5 facilitating recruitment of the pre-initiation complex (PIC), and its interaction with eIF4A lead to conformational changes that can modulate its function (Svitkin et al., 2005, Aitken and Lorsch, 2012). Importantly, eIF4E is sequestered by 4EBP, when mTOR is inhibited, to downregulate cap-dependent initiation; however, in the context of reduced eIF4E, the eIF4G factor can still bind eIF4A and trigger IRES cap-independent translation or
work with eIF1 to facilitate TISU-mediated translation (Lomakin et al., 2000, Sinvani et al., 2015).

In *C. elegans* there is one gene encoding eIF4G, whereas in *Drosophila* and mammals there are two copies, eIF4G-1 and eIF4G-2 (Marygold et al., 2017). The latter is expressed in the testis of *Drosophila*, whereas eIF4G-1 is expressed in somatic tissues and it is the one that was downregulated in our experiments. Nevertheless, the downregulation of this factor in the fat body and intestines was sufficient to decrease the fertility and lifespan of *Drosophila* females. Similarly, the *C. elegans* with downregulated eIF4G by RNAi, although long-lived, also had decreased fecundity (Pan et al., 2007, Hansen et al., 2007). This could indicate that the downregulation of translation, both cap-dependent and independent, in our flies were too severe to exert its benefits, or perhaps that cap-independent translation is the mechanism positively impacting longevity. In fact, the furthest reduction in translation in the studies by Hansen et al. was seen in the eIF4G RNAi worms (Hansen et al., 2007). Furthermore, this factor has been shown to be essential in the development and its reduction can also lead to intestinal atrophy in worms and further neurodegeneration in ischemia mice models (Contreras et al., 2008, Long et al., 2002, Vosler et al., 2011). It is important to remark that the eIF4G flies were the only flies that showed heat resistance in our assay; this could be due to eIF4G, as suggested by Howard et al. 2016, behaving like a ‘switch’ that regulates the expenditure of energy in response to perturbed protein homeostasis (Howard et al., 2016). This means that when there are cellular stresses that threaten the proteome, eIF4G halts protein synthesis and shifts translation to express factors that can protect the cell, at least in yeast and *C. elegans*, and possibly in flies. In fact, silencing of eIF4G leads to altered gene expression, including the upregulation of proteins involved in catabolism or heat shock factors (Ramirez-Valle et al., 2008). This is also interesting in the context of our results, which indicate that the most pronounced resistance does not correlate with an improvement in longevity.

On the other hand, downregulating eIF4E in the fly’s fat body and intestine was sufficient to extend lifespan in our experiments, as it was in adult *C. elegans* treated with RNAi (Syntichaki et al., 2007a, Hansen et al., 2007). Furthermore, overexpression of 4EBP, and hence inactivation of eIF4E, can also extend lifespan in flies (Zid et al., 2009). There are seven genes encoding different isoforms of eIF4E in *Drosophila*, five in *C. elegans* and three in mammals, which have different biochemical properties and expression patterns (Marygold et al., 2017, Rhoads et al., 2006). We studied the effects of eIF4E-1 (CG4035), the canonical form that is expressed ubiquitously and it is essential for cap-dependent translation, especially during embryogenesis (Hernandez et al., 2005). One of the lines (VDRC #17003) we used and that had
extended lifespan, targeted elf4E-1 but also off-targeted elf4E-7, a less expressed isoform that has a weaker affinity for elf4G and that might work to support translation initiation (Hernandez et al., 2005).

The lifespan extension seen in our S106>elf4E RNAi flies was accompanied by improved performance in the negative geotaxis assay. This assay is used as a parameter to measure locomotor senescence and it has been linked with improved ageing (Piper and Partridge, 2018, Gargano et al., 2005). Furthermore, the fecundity of our flies was not reduced, as it was not reduced in long-lived adult worms treated with elf4E RNAi (Syntichaki et al., 2007a). Therefore, both lifespan and healthspan were affected in the opposite direction when either elf4E or elf4G were downregulated. It will be important to measure in the future both the level of overall translation by puromycin and their effects on cap-independent translation, for this we can use a common method which is based on bicistronic luciferase reporters (usually Renilla and firefly) that that have the upstream cistron translated via canonical cap-dependent initiation followed by an internal translation initiation sequence that allows the translation of the downstream cistron (Jackson, 2013, Van Eden et al., 2004).

We also tested the level of phosphorylation of eIF2α (Ser51), S6K (Thr389) and 4EBP (Thr37/46) and we saw that neither the level of phospho eIF2α (Ser51) nor 4EBP (Thr37/46) was changed in S106>elf4E RNAi nor S106>elf4G RNAi flies. Nevertheless, the level of both phospho 4EBP and total 4EBP was strongly reduced in the flies with lower expression of elf4G and elf4E. Interestingly, it has been shown that these factors are stoichiometrically regulated with the amounts of 4E BP (Josse et al., 2016). Additionally, phospho S6K increased in the S106>elf4G RNAi flies. S6K is a regulator of cell growth and energy metabolism by signalling of IIS and mTOR (Um et al., 2006). This kinase requires phosphorylation to be active, and it promotes ribosome biogenesis and protein synthesis; therefore, the upregulation of its phosphorylation in the S106>elf4G RNAi flies could be an attempt by the cell to upregulate protein synthesis to safer levels. Although a direct interaction between S6K and elf4G has not been reported to the best of our knowledge, elf4F complex has been implicated in the upstream inactivation of mTORC1 through TSC2 (Tsokanos et al., 2016); consequently, it could be possible that elf4G leads to activation of S6K but not 4EBP.

Therefore, there are striking differences in the longevity effects of reducing either elf4E or elf4G, even if they form part of the same complex. Notably, we tested a line (CG7483) that was annotated as elf4A, but that recently has been shown to not have any initiation factor activity (Marygold et al., 2017). These contradictory effects could be due to ageing being
regulated by differentially translated genes, which are expressed in a cap-independent manner when elf4E is inhibited but that cannot be expressed when elf4G is inhibited, as this factor regulates both cap-dependent and cap-independent initiation of translation. In fact, Drosophila under dietary restriction have elf4E repressed by 4EBP binding, which leads to an upregulation of cap-independent translation of IRES-containing mRNAs, and interestingly one of these mRNAs belongs to the Drosophila insulin-like receptor (dInR), which is only translated through this cap-independent mechanism in some tissues (Marr et al., 2007). Furthermore, elf4G also leads to increased expression of certain genes, especially heat shock factors such as hsp70 and hsfl (Howard et al., 2016, Hernandez et al., 2004), that although they are probably the ones leading to thermoresistance in our flies, they are not sufficient to extend lifespan. This hypothesis could be tested by performing a translation state array analysis (TSAA), or ribosome profiling, a technique that uses ribosome foot-printing and RNA deep sequencing (Rogers et al., 2011, Zid et al., 2009, Xiao et al., 2016). Another possibility is that these interventions that downregulate translation lead to differential translation that, although still governed by cap-dependent initiation mechanisms, depend on the structure of the mRNA; for example, Drosophila with upregulated 4EBP had reduced translation of mRNA with highly structured 5’ UTRs and increased translation of mRNAs with simpler secondary structures, such as mitochondrial ribosomal proteins and genes from the mitochondrial Complexes I and IV (Zid et al., 2009). This would mean that the processes involved in improved ageing, such as mitochondrial metabolism or tissue-specific dInR are deregulated with reduced elf4E, but not with reduced elf4G.

Furthermore, although the overall speed of translation depends on the rate of initiation, elongation and termination, initiation has been long considered to be a rate-limiting step (Gingold and Pilpel, 2011). In addition, at higher initiation rates there is a higher likelihood of ribosome “jamming” or stalling (Tuller et al., 2010), and one of the cellular responses against stalling is halting translation initiation (Joazeiro, 2017). Therefore, downregulating elf4F could be leading to a reduction of ribosome stalling that can be beneficial for longevity. Although the role of ribosomal stalling is not known in ageing, it has already been linked to neurological diseases (Kapur and Ackerman, 2018); a loss-of-function mutation in GTP Binding Protein 2 (GTPBP2), which is a ribosome rescue protein that acts to prevent stalls, has been found in patients with mental retardation, and neuronal degeneration (Jaberi et al., 2016). Interestingly, mice with deficient GTPBP2 or tRNAArg^UCU (which also leads to ribosome stalling) activate GCN2 (Ishimura et al., 2016, Dong et al., 2000), which in turn has been found to be
linked to ageing in yeast, *C. elegans* and *Drosophila* (Kang et al., 2017, Vlanti et al., 2013, Hu et al., 2018).

Our main hypothesis is that improved translation fidelity leads to amelioration of ageing; however, a direct link between translation initiation and fidelity is not immediately apparent. Nevertheless, it is important to note that translation fidelity can also affect start codon recognition (i.e. being more or less prone to start translation in other than AUG codons) (Valente and Kinzy, 2003) and that this could be more efficient in our flies with downregulated eIF4E and eIF4G. In addition, initiation factors have been directly linked with stop codon readthrough: eIF3 can promote stop codon readthrough by interfering with eRF1 and eRF3 and stimulating the incorporation of sense codons (Beznoskova et al., 2015). PABP1, which is recruited to the poly (A)-cap of mRNA by eIF4F has also been found to negatively regulate stop codon readthrough by interacting with eRF3 (Roque et al., 2015). Interestingly, overexpression of Pab1 (yeast’s PABP1) leads to decreased stop codon readthrough and to slowed cell growth in *S. cerevisiae* (Cosson et al., 2002, Roque et al., 2015). It is also important to mention that PABP1 has been found to decrease with age and in patients with oculopharyngeal muscular dystrophy, both leading to accelerated muscle cell ageing (Anvar et al., 2013). Thus, our eIF4E flies could have more efficient functioning of PABP1 leading to improved accuracy and longevity. Besides, Ctk1, a kinase subunit of the carboxyl-terminal domain kinase I (CTDK-1) complex, was found to increase translation fidelity by phosphorylating RPS2 in yeast and to lead to severe growth defects when knocked out (Rother and Strasser, 2007). Moreover, later it was also shown to be necessary for efficient canonical translation initiation, and although the specific mechanism is still unknown, it has been suggested that Ctk1 regulates initiation by a process involving eIFs, since cricket paralysis virus (CrV) IRES (i.e. a type of IRES that is totally independent of eIFs) function was not affected by Ctk1 depletion (Coordes et al., 2015). These results suggest that translation fidelity and initiation can be biologically regulated by the same protein, although a link between fidelity and eIF4F has not been shown yet, it is a possibility that cannot be ruled out yet.

### 3.3.3 Downregulation of eEF2 in the fat body and intestines extends lifespan and healthspan

Translation elongation is a process highly conserved between prokaryotes and eukaryotes. In eukaryotes, there are only two major elongation factors, eEF1 (bacterial EFTu) and eEF2 (bacterial EFG) (Dever and Green, 2012). eEF2 is a GTP-binding translocase that facilitates the movement of the tRNA to the E and P sites, and the mRNA to move down the ribosome (Dever and Green, 2012). In our longevity screen, we saw that downregulation of
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

169

eEF2 by RNAi in the metabolic tissues extended lifespan, improved the senescence of negative geotaxis and it did not affect fecundity. In fact, elongation has been linked to the nutrient sensing and longevity regulator, mTOR; phosphorylation and inactivation of eEF2 by a highly selective eEF2 kinase, eEF2K, renders this factor inactive, slowing the rate of elongation (Browne et al., 2004, Leprivier et al., 2013, Rhoads et al., 2006). This kinase is in turn phosphorylated and inactivated by mTOR; nevertheless, eEF2K belongs to the α-family of kinases, which is not found in Drosophila, and to date, no other kinase has been identified to phosphorylate eEF2 in flies (Lasko, 2000, Marygold et al., 2017, Dever and Green, 2012). This, however, does not mean that eEF2 is regulated by an insect-specific protein totally different from eEF2K.

Another question to solve is how downregulating eEF2 can extend lifespan. One of the possibilities is linked to the involvement of translocation in fidelity and how this step of elongation can lead to errors. When mTORC1 is inhibited by rapamycin in mammalian cells, eEF2 is also inhibited, and protein accuracy is improved (Conn and Qian, 2013, Browne and Proud, 2002). In fact, a mutation in eEF2 (P596H) found in patients with autosomal dominant spinocerebellar ataxia leads to impaired translocation and increased frameshifting errors (Liu et al., 2014, Hekman et al., 2012). eEF2 has also been directly implicated in translation accuracy: this factor has a unique and highly conserved between archaea and eukaryotes histidine to diphthamide modification that, if blocked, leads to a halt in protein synthesis and cell death (Greganova et al., 2011, Mateyak and Kinzy, 2013, Van Ness et al., 1980). Interestingly, this diphthamide has been proposed to support fidelity of translation, since losing it leads to an increase in frameshifting errors, especially when eEF2 is depleted (Uthman et al., 2013, Hawer et al., 2018, Liu et al., 2012, Ortiz et al., 2006). It is also important for this discussion to note that the yeast without the diphthamide modification, are not only hypoaccurate but also very sensitive to high temperatures (Kimata and Kohno, 1994, Hawer et al., 2018). It should also be noted that the blocking of this modification is of great biomedical relevance, as it can cause embryonic lethality, neurological and developmental disorders and it has also been found in ovarian and prostates cancers (Liu et al., 2017, Nakajima et al., 2018, Sekiguchi et al., 2018, Webb et al., 2008, Chen and Behringer, 2004). In addition, the mechanisms exploited by some aminoglycosides such as sordarin or anisomycin is to inhibit translocation by directly binding eEF2, causing frameshifting (Dinman et al., 1997, Harger et al., 2001). Therefore, it will be interesting to see if the lifespan extension of our flies is due to increased translation fidelity; for this reason, we are planning to measure the level of stop
The role of decreased protein synthesis in delaying ageing                      | Martínez Miguel, V.E.

codon readthrough and misincorporation in our S106>eEF2 RNAi flies with our dual-luciferase based reporters.

Reducing eEF2 could also be beneficial because of a reduction in elongation speed. Decreasing elongation speed has been linked to increased folding capacity, for example, the presence of non-optimal codons facilitates co-translational folding by reducing the speed of the ribosome (Zhang et al., 2009, Shabalina et al., 2013). In another interesting study, it was shown that eukaryotic proteins can be properly folded by bacteria when elongation speed is slowed down (Siller et al., 2010). In this study, Siller et al. aimed to increase the folding efficiency of eukaryotic proteins (firefly, green fluorescent protein (GFP) and Cdc13) which are inadequately folded by prokaryotes; thus, they used streptomycin pseudo-dependent *E. coli* hyperaccurate mutants (RPS12 R86S and P91Q), which increase their elongation speed in a streptomycin dose-dependent response. They saw that slowing down elongation had a clear increase in the folding proficiency of these proteins and that the streptomycin-treated *E. coli* were not less proficient due to increased mistranslation nor enhanced degradation but merely because of speed. Furthermore, downregulating translation by a 15-20% reduces the accumulation of protein aggregates and misfolded species in mammalian cells (Meriin et al., 2012a, Meriin et al., 2012b). It is important to note, that this prevention of misfolding is achieved by using elongation inhibitors, such as cycloheximide but not initiation inhibitors, demonstrating the importance of this step on co-translation folding (Meriin et al., 2012a, Meriin et al., 2012b). Nevertheless, this does not rule out an increase in translation fidelity too, as it has been discussed in the introduction, increased protein fidelity can lead to improved folding capacity.

Finally, downregulating eEF2 could trigger differential translation of certain genes involved in ageing. It has been recently shown that, in *Drosophila*, drops in initiation rates are related to the lower translation of mRNAs containing weak Kozak sequences compared to strong Kozak sequences, and on the contrary, drops in elongation rates lead to the opposite translation preferences (Acevedo et al., 2018). This means that not only downregulating overall translation, but specific steps of this process can promote the differential translation of certain subpools of mRNAs that could be affecting ageing differently. In addition, phosphorylation of eEF2 in neurons has been related to altered control of gene expression, especially of genes encoding microtubule-related proteins (Kenney et al., 2016).
3.3.4 Downregulation in the fat body and intestines of RPS23 extends lifespan

Contrary to what was seen in *C. elegans* and yeast, where several ribosomal proteins extended lifespan (RPS6, S10, S11, S15, S18, S22, S26, RPL4, L6, L7, L9, L13, L19, L22, L23, L29, L30, L31, L34 and L43), (Curran and Ruvkun, 2007, Smith et al., 2008, Hansen et al., 2007, Kaeberlein et al., 2005, Steffen et al., 2008b), in our screen, we tested RPS2, S3, S9, S11, S23, S26, RPL9 and L19 and the only ribosomal protein that extended the lifespan of our flies was RPS23. RPS23 is a ribosomal protein located in the decoding centre of the small 40S ribosomal subunit, which in certain regions it has striking amino acid similarity with evolutionary distinct organisms and it has been involved in the maintenance of translation fidelity (Alksne et al., 1993, Carter et al., 2001, Selmer et al., 2006, Gorini and Davies, 1968, Zimmermann et al., 1971).

In our screen, although the flies with downregulated RPS23 had their lifespan extended, they were the only longer-lived line that had not a significant improvement of the senescence of negative geotaxis. Nonetheless, the climbing assay was only performed until week 5 in these flies, whereas in the rest it was followed until week 6, so it could be that the effect on health span could be seen in later weeks if this had been studied.

An interesting point to discuss is how decreasing the number of ribosomal proteins can extend lifespan. In yeast, when genes encoding 60S subunit proteins were deleted, or when the ribosome biogenesis disruptor diazaborine was given to the cells, there was a response similar to dietary restriction that was dependent on Gcn4 activation (Steffen et al., 2008b). Moreover, Hansen et al. saw that the lifespan extension in worms caused by the reduction of ribosomal proteins was DAF-16 (FOXO) independent, whereas the lifespan extension by downregulation of initiation factors was DAF-16-dependent (Hansen et al., 2007). This suggests that the longevity effects of these two interventions are distinct and that the subset of genes transcribed by DAF-16/FOXO is not activated in longer-lived organisms with downregulated expression of ribosomal proteins.

Interestingly, a group of mutants in *Drosophila* were discovered by T. Morgan and C. Bridges in 1919 and they were described to have thinner and shorter bristles, to be developmentally delayed and to have reduced fertility, these were the common phenotypes of the *minute* mutants (Bridges et al., 1923, Morgan and Bridges, 1919, Schultz, 1929). Later, it was discovered that the minute mutants had in common the haploinsufficiency of ribosomal proteins but, to the best of our knowledge, there has not been any minute discovered to have
RPS23 insufficiency (Kongsuwan et al., 1985, Marygold et al., 2007, Marygold et al., 2017). Although our S106>RPS23 RNAi flies cannot be considered a minute, since the RPS23 reduction has been restricted to adulthood and it is not ubiquitous, certain effects seen in minutes could apply to our flies too; for example, a recent study has found that the growth-related phenotypes seen in several minutes are due to the increased expression of a transcription factor, Xrp1, which is a master regulator of the expression of genes involved in glutathione metabolic processes, iron-sulfur cluster assembly, DNA recombination and telomere maintenance (Lee et al., 2018). Therefore, it would be interesting to check if the levels of this transcription factor are only required during development or if even in our flies with reduced RPS23 only in adulthood also have elevated levels of Xrp1.

Furthermore, ribosomes are not considered an unalterable entity, but distinct ribosomal subpopulations with different compositions, properties and functions have been found to exist between and within cells (Xue and Barna, 2012). This means that some ribosomal proteins, such as RPL10 are either enriched or depleted in certain ribosomal subsets which trigger differential translation of specific mRNAs, especially those involved in metabolism, cell cycle and development (Shi et al., 2017). For example, S. cerevisiae in response to stress produces ribosomes lacking RPS26, which interestingly is involved in the translation of strong Kozak sequences (Ferretti et al., 2017). Another example is how a deletion of RPL38 in mice does not change the level of global protein synthesis but it suppresses the translation of a specific Hox mRNA set, leading to defects in skeletal patterning (Kondrashov et al., 2011). Nevertheless, to date, RPS23 has not been reported to be involved in ribosomal heterogeneity, and since it is an essential protein of the decoding centre its downregulation might just lead to a global decrease in ribosomal biogenesis. Suggesting that there is a tendency towards overall translation downregulation when the expression of this factor is reduced, we have the data from the puromycin-based de novo protein synthesis experiment in S2R+ cells and flies. Therefore, to test this hypothesis is crucial to finalize optimizing the puromycin assay to unravel if S106>RPS23 flies have significantly lower translation than the controls.

Recently, RPS23 has been proposed to be an effector of the hypoxic response, at least in fungi, through modulation of Sre1 (sterol regulatory element 1) transcription factor, which regulates lipid metabolism (Clasen et al., 2017). Ofd1 (S. pombe nomenclature) is an oxygenase that controls the activity of Sre1, but it also hydroxylates newly synthesized RPS23 at P62 (S. pombe nomenclature), facilitating the formation of a complex formed by Ofd1-RPS23-Nro1 that moves to the nucleus leading to the incorporation of RPS23 to assembling
40S ribosomal subunits (Clasen et al., 2017). Nevertheless, in situations of hypoxia, Ofd1 cannot hydroxylate RPS23, which stabilizes the complex with Nro1 and hence it becomes sequestered and unable to hydroxylate Sre1, which in turn is free to act as a transcription factor of genes involved in the hypoxic response and sterol metabolism. Furthermore, RPS23 expression can modulate the function of Sre1, with higher levels of unassembled RPS23 leading to higher level of Sre1 activity, which Clasen et al. suggest is regulated by nutrient levels (i.e. in nutrient deprivation, lower RPS23 expression leads to higher sequestration of Sre1 and hence lower Sre1 activity, so lipid metabolism matches this nutritional state) (Clasen et al., 2017). Remarkably, Ofd1 has homologs in S. cerevisiae (Tpa1), humans (OGFOD1) and flies (Sudestada1) and they all can hydroxylate RPS23 (Katz et al., 2014, Loenarz et al., 2014, Singleton et al., 2014); however, metazoans do not have Nro1, so the sequestration and regulation of Sre1 might be different from fungi (Clasen et al., 2017). In addition, hydroxylation of RPS23 by Tpa1 in budding yeast is necessary for transition fidelity, as deletion of this enzyme leads to increased stop codon readthrough (Loenarz et al., 2014). Therefore, our flies with downregulated expression of RPS23 could be mimicking a state of nutrient deprivation in which SREBP (sterol regulatory element binding protein, Drosophila’s Sre1 homolog) is sequestered more readily by Sudestada1 and hence its transcription activity is dampened leading to downregulation of sterol synthesis genes. Remarkably, a few years ago SREBP was found to protect C. elegans against the accelerated-ageing phenotype produced by high glucose diets through a shift in lipid metabolism (Lee et al., 2015), and recently it has been reported that the synergistic effect of a combination of three drugs rifampicin psora-4 and allantoin, that strongly extend lifespan in worms, acts through overexpression of SREBP1 (Admasu et al., 2018). Thus, RPS23 has other cellular functions other than translation that could be involved in the ageing phenotype of the S1106>RPS23 RNAi flies.

3.3.6. Translation fidelity in flies with downregulated eIF4E, eIF4G, eEF2 and RPS23 in the metabolic tissues

One of the hypotheses of why decreasing protein synthesis leads to improved longevity is that this occurs through enhanced translation accuracy; for this reason, we downregulated several translation factors and ribosomal proteins and we saw that some of them can extend lifespan. We tried to measure the level of translation errors by expressing a mistranslation and a stop codon readthrough reporter based on the dual-luciferase assay as they are a gold standard in measuring these kinds of errors (Salas-Marco and Bedwell, 2005, Kramer et al., 2010). Nevertheless, although accuracy has been measured in flies by using 2D-
gels (Parker et al., 1981), to the best of our knowledge, the dual-luciferase technique for translation fidelity has never been used in flies, and hence we had to optimize the system for its use in *Drosophila*. To minimize possible artefacts from very high overexpression of a non-endogenous protein, we cloned these luciferase reporters under a mild promoter, the heat shock promoter (Qin et al., 2010, Brand and Perrimon, 1993). Unfortunately, we were unable to detect luminescence that is sufficiently higher than the background levels in the fat tissue, as most of its expression was detected in the thorax and heads. We have since then cloned these vectors under a stronger promoter, the ubiquitin promoter, and we already have the flies in the laboratory so they can be processed. In case the measurements are still not high enough to be reliable to measure luminescence in the flies’ metabolic tissues, we are planning to downregulate our specific factors in S2R+ cells by dsRNA and using the dual luciferase assay that has already been optimized in cells to measure fidelity.

An indication of whether long-lived flies having lower eIF4E, eEF2 and RPS23 have increased translation fidelity can be achieved by performing an epistatic experiment using the error-inducing antibiotic, paromomycin. Epistasis is defined as the phenomenon by which a mutation’s phenotype depends on a gene at a different locus (Cordell, 2002). In our case, we wanted to see if artificially increasing the level of errors returned the lifespan of our long-lived flies to the level of the control flies. We first optimized the concentration of paromomycin to be the highest possible without disturbing the lifespan of the control flies. The first time we tested paromomycin, counterintuitively, had a beneficial effect on our flies. This, as it was briefly mentioned in the results section, was suspected to be due to the antibiotic effect of paromomycin. In fact, it has been previously tested how antibiotics can extend the lifespan of *Drosophila* through clearing certain malignant bacteria (Loch et al., 2017, Brummel et al., 2004), and in our experiments, when the flies were supplemented with ampicillin, their lifespans were also extended. Moreover, when we bleached the eggs to remove any bacteria and repeated the lifespan with wild-types on paromomycin, we no longer saw any effect of this antibiotic neither on lifespan nor on egg-laying.

When we tested the same concentration of paromomycin in our experimental flies, we saw that the S106>RPS23 RNAi flies fed control non-RU486 food lived the same independently of the presence of paromomycin, as expected by the optimization assay. Interestingly, S106>RPS23 RNAi flies fed inducing-drug RU486 and paromomycin did not have increased lifespan anymore. This can be explained by two hypotheses; first one, the lifespan extension caused by downregulation of RPS23 is due to an improvement in fidelity and hence when an error-inducing drug is given, the longevity effect is no longer seen, or the second, the
downregulation of RPS23 in the metabolic tissues renders the flies sensitive to paromomycin. These hypotheses will be clarified by the results of our direct fidelity measurements.

For the rest of the lines tested, however, we saw that paromomycin also affected all the flies fed the non-RU468 control food supplemented with this antibiotic. The difference between this experiment and the experiment in which we optimized the concentration of paromomycin was that in the former we cooked the paromomycin food fresh each week; therefore, maybe the potency of the error-promoting antibiotic was enhanced this time. Nevertheless, although S106>eIF4E RNAi flies were negatively affected by the antibiotic regardless of the presence or absence of RU486, the lifespan of the flies fed RU486 and paromomycin was reduced to the same level of the control lifespan. Therefore, we can conclude that either lowered eIF4E exert its lifespan-extending effect through improved translation accuracy, or that these flies are neither more resistant nor more sensitive to the antibiotic. In addition, both the experimental and the control S106>eIF4G RNAi flies had also a decrease in lifespan because of supplementation of this antibiotic. However, it was interesting to see that the S106>eIF4G RNAi flies fed control food with paromomycin had a shorter lifespan that was almost identical to S106>eIF4G RNAi fed RU486, meaning that translation errors can provoke the same reduction in lifespan than downregulated eIF4G. Therefore, it would be interesting to repeat these experiments with a wider range of paromomycin concentrations and see if the effect follows a dose-response. We could also monitor the feeding status of these flies, because, as it was explained in the tunicamycin discussion, any fly that is feeding more will become more susceptible to the toxic agent. Nevertheless, we will have to wait until the direct measurement of translation fidelity to see if the lifespan reduction of lowered eIF4G is due to a deterioration of translation accuracy.

Remarkably, although both the S106>eEF2 RNAi flies fed either RU486 or non-RU486 control food were negatively affected by paromomycin, the flies with the activated suppression of eEF2’s expression showed certain resistance to the antibiotic compared to the control. In addition, the number of eggs laid by flies fed the control food with paromomycin was significantly lower than the flies fed just the control food, whereas the number of eggs laid by the flies fed RU486 and paromomycin was not significantly affected compared to flies fed RU486. Thus, there is a differential effect of paromomycin in the longevity and the fecundity of the flies with reduced eEF2 in the metabolic tissue.
Chapter 4. Pharmacological screen for drugs that improve translation fidelity and their potential to be anti-ageing treatments in *Drosophila*
4.1. Introduction

Due to the improvements in lifestyle and sanitation, the life expectancy of humans has been rapidly increasing since the 19th century (Oeppen and Vaupel, 2002), and this has led to a dramatic increase in the proportion of elderly in the world, both in less and more developed countries (United Nations, 2017). Although these are great news, there is a socioeconomic impact caused by the higher prevalence of ageing-related diseases, and hence it is crucial to look for anti-ageing interventions that prevent these (Partridge, 2014, Castillo-Quan et al., 2015). One way of tackling these plethora of diseases is by looking for drugs that extend lifespan and health span (Fontana et al., 2010, Barzilai et al., 2016), and this search usually starts by finding drugs that extend lifespan in an evolutionary conserved way in different model organisms. Therefore, we wanted to investigate if some of the drugs that can extend lifespan also alter translation fidelity in S2+ cells, which could also eventually lead to a new in vitro anti-ageing drug screen.
4.2. Results

4.2.1. Drugs that extend lifespan improve translation fidelity \textit{in vitro}

4.2.1.1. Validation of translation fidelity reporters for \textit{Drosophila} derived S2R+ cells

One of the main aims of this thesis was to study how drugs that affect lifespan or translation alter translation fidelity. For this purpose, we adapted for \textit{Drosophila} a set of dual luciferase reporters that are a gold standard to measure the level of stop codon readthrough and amino acid misincorporation (Salas-Marco and Bedwell, 2005). In order to optimise the dual luciferase assay, we tested several conditions that could affect our set-up. First, we wondered how the number of plated \textit{Drosophila}-derived S2R+ cells could affect the assay, so we seeded 80,000, 100,000 and 130,000 cells. These cells were then transiently transfected with the translation fidelity reporters or a GFP-containing vector control (Fig. 4.1. A). We saw that the best levels of luminescence intensity were measured when 100,000 cells were seeded in a 48 well plate, and so was the transfection efficiency.

S2R+ cells are normally cultured in Schneider medium containing heat-inactivated FBS and a mixture of streptomycin and penicillin; however, streptomycin, as discussed in Introduction 1.2.2.1.1., is known to increase the level of errors in bacteria, so we wondered if this could interfere with our translation fidelity assay as it could perhaps affect mitochondrial ribosomes. We first tested stop codon readthrough in cells seeded at different densities with or without this antibiotic (at the standard concentration of 85 µM), and we did not see much difference between the stop codon readthrough in the presence or absence of streptomycin (Fig. 4.1. B). Nevertheless, when streptomycin was given together with paromomycin, especially at the highest concentrations of paromomycin (Fig. 4.1. C), the level of stop codon readthrough increased, so in later assays, we used Schneider media with heat-inactivated FBS and just penicillin.

For several reasons we tested different concentrations of paromomycin: first, to validate our dual luciferase assay in measuring translation fidelity, since, to the best of our knowledge, this has never been used in \textit{Drosophila}, second, to see which concentration of paromomycin would work best as a positive control in our future assays and third, to see if the combination of streptomycin and paromomycin had an effect compared to streptomycin on its own (Fig. 4.1.). Doing this, we validated our novel system for measurements of translation accuracy in \textit{Drosophila} cells — the level of stop codon readthrough increased in a dose-
The role of decreased protein synthesis in delaying ageing  

Martínez Miguel, V.E.

dependent manner when the cells were given paromomycin. We also decided to use 500 μM in subsequent assays, as it gave one of the highest increases in stop codon readthrough.

Figure 4.1. Dual luciferase assay optimisation. A), B) and C) Panel showing the density and plated number of S2R+ cells and the transfection efficiency with GFP. D) We tested the effect on stop codon readthrough of streptomycin (85 μM) in addition to paromomycin (500 μM) at 70,000, 100,000 and 130,000 cells densities. E) We also tested the effect on stop codon readthrough of different concentrations of paromomycin on their own or with streptomycin (85 μM), when 100,000 cells were seeded.

4.2.1.2. Several drugs that extend lifespan decrease the level of translation errors

We aimed to explore the effect of several drugs on translation errors, both stop codon readthrough and misincorporation, so for this, we used the luciferase-based reporters that we adapted for Drosophila (Salas-Marco and Bedwell, 2005). After optimizing the cell density, the concentration of paromomycin required and the optimal media conditions, we improved the assay by stably transfecting the S2R+ cells with vectors containing the translation fidelity reporters (see Materials and Methods 2.10.2.). This stable transfection allowed us to increase the level of luminescence detected, which had been shown to be especially low for the misincorporation reporter when transiently transfected (data not shown) and that we expected to be even lower if any of the drugs improved the level of errors. In fact, when we
studied the transfection efficiency in transiently transfected cells with a control vector containing GFP, we saw that this was around 20% (Fig. 4.1. A). Furthermore, we cloned the translation fidelity reporters to use in the creation of stable cell lines under the *Drosophila* metallothionein (MT) copper (II) sulphate-inducible promoter (Bunch et al., 1988) (see Materials and Methods 2.10.1.). Thus, we were able to induce the expression of the reporters by adding this chemical just after treating the cells with the indicated drugs (see Materials and methods 2.10.3.). Thereby all luciferase produced upon induction by copper sulphate was in the presence of anti-ageing drugs and our results were not affected by the pre-existing luciferase. These treatments were carried out overnight and consisted of different drugs known to either extend lifespan in model organisms (rapamycin, Torin 1, trametinib and diazaborine) or to directly affect protein translation (cycloheximide, salubrinal and Torin 2). It should also be noted that firstly, a screen was performed for each drug with a larger number of concentrations (data not shown), and then a concentration window was selected to further study with more samples and repeats (which are the results that will be shown below).

Rapamycin is an inhibitor of TORC1, and hence an inhibitor of translation too, that has been repeatedly shown to extend lifespan in an evolutionary conserved way from yeast to mammals (Bjedov and Partridge, 2011, Bjedov et al., 2010, Johnson et al., 2013, Kapahi et al., 2004, Miller et al., 2011, Pan et al., 2007), and that decreases the level of stop codon readthrough and misincorporation in mouse fibroblasts (Conn and Qian, 2013). When the S2R+ cells were given rapamycin, the level of stop codon readthrough was reduced in all the concentrations tested (2.5 nM p=0.0028, 10 nM p=0.0003; 25 nM p=0.003; 100 nM p<0.0001 and 250 nM p<0.0001, Welch’s test) and the level of misincorporation was also reduced at several rapamycin concentrations (10 nM p=0.0044; 25 nM p=0.0028 and 100 nM p=0.037, Welch’s test) (Fig. 4.2. A). Importantly, although misincorporation seemed to be higher when the cells were treated with 250 nM rapamycin, this was not statistically significant (p=0.134, Welch’s test) (Fig. 4.2. B).

Torin 1 and 2 are more potent inhibitors of mTOR than rapamycin since they are catalytic inhibitors that block the action of both TORC1 and TORC2 (Guertin and Sabatini, 2009, Liu et al., 2011). In addition, Torin 1 has been shown to extend lifespan in flies (Mason et al., 2018). This drug decreased the level of stop codon readthrough in all the concentrations tested (0.2 nM p=0.0101; 0.5 nM p=0.0236; 1 nM p=0.0021; 5 nM p=0.0016 and 20 nM p=0.0049, Welch’s test) (Fig. 4.2. C), and it also decreased the levels of misincorporation when the cells were given the highest concentrations tested (1 nM p=0.0021; 10 nM p=0.044; 50 nM p=0.0007 and 100 nM p=0.0011, Welch’s test) (Fig. 4.2. D). Nevertheless, at the lowest...
concentrations it seemed to be an increase of mistranslation, which nonetheless was not significant (0.2 nM p=0.286; 0.5 nM p=0.069 and 5 nM 0.078, Welch’s test) except for 1 nM (p=0.0236, Welch’s test) (Fig. 4.2. E). Surprisingly, Torin 2 did not alter significantly the level of translation errors, neither up nor down (Fig. 4.2. F).

We also tested two drugs that have been reported to extend lifespan, trametinib and diazaborine. Trametinib is an inhibitor of MAPK/ERK Kinase (MEK) 1 and 2 and it has been shown to extend lifespan in flies (Slack et al., 2015, Yamaguchi et al., 2011). This drug decreased the levels of stop codon readthrough at several concentrations (1 nM p=0.0064; 5 nM p=0.0304 and 10 nM p=0.0022, Welch’s test) (Fig. 4.2. G) and the levels of misincorporation at the highest concentrations tested, 5 nM and 10 nM (p=0.0012 and p=0.0039, respectively, Welch’s test) (Fig. 4.2. H). Diazaborine is a drug that inhibits ribosome biogenesis (Loibl et al., 2014) and that extends lifespan in yeast (Steffen et al., 2008b). However, when this drug was tested in our S2R+ cells, it did neither decrease nor increase significantly the level of errors (Fig. 4.2. I and J).

We also tested two drugs that directly alter the levels of translation, salubrinal and cycloheximide. Salubrinal is a drug that increases eIF2α phosphorylation and blocks its dephosphorylation, leading to an arrest of translation initiation (Boyce et al., 2005). When this drug was given to the S2R+ cells, the highest concentration tested, 10 µM, significantly decreased the level of amino acid misincorporation but not stop codon readthrough (p=0.0082, Welch’s test) (Fig. 4.2. K and L). Cycloheximide is a molecule that binds the large ribosomal subunit inhibiting translation elongation (Schneider-Poetsch et al., 2010a). When we tested its effect on translation fidelity, this drug showed to decrease the level of stop codon readthrough in the lowest concentrations (25 nM p=0.0002; 100 nM p<0.0001 and 250 nM p<0.0001, Welch’s test) (Fig. 4.2 M). However, despite being a drug that decreases translation, it did not affect amino acid misincorporation, and even the concentrations that seemed to increase the level of this error were not significant (25 nM p=0.4225 and 100 nM p=0.173, Welch’s test) (Fig. 4.2. N).
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
Figure 4.2. Drugs known to affect lifespan in model organisms, translation or both, can also alter the level of stop codon readthrough or amino acid misincorporation. S2R+ cells were treated with different concentrations of certain drugs overnight. In each experiment, there are two negative controls (black columns), one in which nothing was added (0) and other representing the solvent in which the drug was dissolved (Ethanol or DMSO) plus a positive control, 500 µM paromomycin (blue column). The signal of firefly luminescence was divided by the signal of Renilla for each individual sample to calculate the firefly/Renilla ratios. Then, the percentage stop codon readthrough was calculated by dividing each sample’s firefly/Renilla ratio of the stop-codon reporter by the average firefly/Renilla ratio of the control reporter of each condition. Similarly, the percentage misincorporation was calculated by dividing each sample’s firefly/Renilla ratio of the misincorporation reporter by the average firefly/Renilla ratio of the control reporter. The background luminescence was always subtracted to the firefly luminescence values. Total n=18 from at least three independent experiments were studied. The p values were calculated with a Welch’s t-test between the corresponding solvent control and each drug concentration * p≤0.05 **p≤0.005. A) Stop codon readthrough paromomycin p=0.003; rapamycin 2.5 nM p=0.0028, 10 nM p=0.0003; 25 nM p=0.0003; 100 nM p<0.0001 and 250 nM p<0.0001. B) Misincorporation paromomycin p=0.0321; rapamycin 10 nM p=0.0044; 25 nM p=0.0028 and 100 nM p=0.037. C) Stop codon readthrough paromomycin p=0.0001; Torin 1 0.2 nM p=0.0101; 0.5 nM p=0.0236 and 1 nM p=0.0021; 5 nM p=0.0016; 20 nM p=0.0049. D) Misincorporation paromomycin p<0.0001; Torin 1 1 nM p=0.0236; 10 nM p=0.044; 50 nM p=0.0007 and 100 nM p=0.0011. E) Stop codon readthrough paromomycin p=0.0004; Torin 2 n.s. F) Misincorporation paromomycin p<0.0001; Torin 2 n.s. G) Stop codon readthrough paromomycin p<0.0001; trametinib 1 nM p=0.0064; 5 nM p=0.0304 and 10 nM p=0.0022. H) Misincorporation paromomycin p=0.03; trametinib; 5 nM p=0.0012 and 10 nM p=0.0039. I) Stop codon readthrough paromomycin p=0.0042; diazaborine n.s. J) Misincorporation paromomycin p<0.0001; diazaborine n.s. K) Stop codon readthrough paromomycin p<0.0001, salubrinal n.s. L) Misincorporation paromomycin p=0.0069; salubrinal 10 µM p=0.0082. M) Stop codon readthrough paromomycin p<0.0001; cycloheximide 25 nM p=0.0002; 100 nM p<0.0001 and 250 nM p<0.0001. N) Misincorporation paromomycin p=0.0037; cycloheximide n.s.
Our results strongly suggest that there is a correlation between the drugs that increase fidelity in flies (rapamycin, Torin 1 and trametinib) and an improvement of both stop codon readthrough and misincorporation. On the contrary, other drugs that decrease translation but have not been shown to increase lifespan in flies (cycloheximide, diazaborine and salubrinal), do not reduce the level of errors, or only reduce stop codon readthrough (as cycloheximide), at least at the tested concentrations and treatment duration. This suggests that to achieve longevity, both types of translation errors need to be reduced, and to test this further we examined longevity in the presence of these drugs.

4.2.1.3. Effect on lifespan of the drugs tested on the translation fidelity assay

Some of the drugs we tested in our dual luciferase assay for translation fidelity measurements, such as rapamycin, trametinib, Torin 1 and diazaborine, have been reported to extend lifespan in model organisms, while Torin 2, cycloheximide and salubrinal have not been tested for ageing to the best of our knowledge. We decided to study the effect of these drugs on longevity in our conditions using the outbred strain w<sup>Dah</sup> flies. For this, once the flies became adults, they were split into media supplemented with a few different concentrations of each drug (see Materials and Methods 2.1.5.). It is critical to test several drug concentrations because it is usually mild downregulation of a signalling pathway that has anti-ageing effects, while high drug concentrations may have undesirable side effects that can be detrimental for lifespan (Castillo-Quan et al., 2015).

We used rapamycin as a positive control, as this is a drug that has been several times reported to extend lifespan in different model organisms, including flies. We dissolved this drug in holidic medium as it increases the bioavailability of drugs, compared to standard sugar-yeast food, and therefore it allows for lower drug concentrations to be used (Piper et al., 2014). For example, Bjedov et al. supplemented rapamycin to flies dissolved in 1 SYA, and 200 µM extended the lifespan significantly (Bjedov et al., 2010); however, Piper et al. showed that only 5 µM was required for lifespan extension when dissolved in holidic media (Piper et al., 2014). Similarly, in our experiment, flies fed 5 µM rapamycin-supplemented food lived longer, 68.5 days median and 75.5 days maximum lifespan, compared to the flies fed the control ethanol-supplemented medium, which had 64 days median lifespan and 78 days maximum (p=6.4 x10<sup>-9</sup>, χ<sup>2</sup> log-rank test) (Fig. 4.3. A). The flies fed 10 µM rapamycin-supplemented medium had 71 days median and 85 days maximum lifespan, so they were also longer-lived compared to the control flies fed ethanol-supplemented medium (p=2.28 x10<sup>-15</sup>, χ<sup>2</sup>log-rank test).
While our experiments were in progress, Torin 1 was reported to extend the lifespan of flies (Mason et al., 2018). In our experiment, we supplemented this drug dissolved in holidic media and we saw that the highest concentration tested, 10 µM, had a longer lifespan compared to the flies fed control DMSO-supplemented medium (Fig. 4.3. B). The flies were given 10 µM Torin 1 had 68.5 days median and 80.5 days maximum lifespan compared to the control flies that had 66 days median and 78 days maximum lifespan (p=0.037, χ² log-rank test). Torin 2 has not been reported to extend lifespan, but in our experiment, the highest concentration tested, 4 µM very slightly but statistically significantly extended the lifespan of the flies compared to the flies fed the control DMSO (Fig. 4.3. C): 66 days median and 80 days maximum lifespan compared to the control’s 64 days median and 80 days maximum lifespan (p=1.6 x10⁻⁵, χ² log-rank test).

Diazaborine and cycloheximide both shortened the flies’ lifespan compared to their respective controls at several concentrations. When the flies were given 3 µM diazaborine dissolved in the holidic medium, they had 64 days median and 75.5 days maximum lifespan compared to the DMSO controls 64 days median and 78 days maximum lifespan (p=0.014, χ² log-rank test). At the highest concentration, 30 µM, they lived even shorter, 61.5 days median and 75.5 days maximum lifespans (p=0.002, χ² log-rank test compared to the control) (Fig. 4.3. D). Flies treated with cycloheximide had significantly shorter lifespan at almost all the concentrations tested than the flies given the control ethanol-supplemented 1 SYA medium (Fig. 4.3. E). The control flies had 63 days median and 75.5 days maximum lifespan, whereas the flies treated with 0.1 µM lived 61 days median and 72.5 days maximum (p=0.318, χ² log-rank test), the 1 µM had 58.5 days median and 69.5 days maximum lifespan (p=0.00066, χ² log-rank test), with 10 µM they had 58.5 days median and 75.5 days maximum lifespan (p=0.039, χ² log-rank test), with 100 µM they had 58.5 days median and 69.5 days maximum lifespan (p=0.0005, χ² log-rank test) and with 1 mM they had 47 days median and 69.5 days maximum lifespan (p=6.6 x 10⁻¹⁸, χ² log-rank test). Finally, when we treated the flies with salubrinal dissolved in holidic medium, none of the concentrations tested had any significant effect on the flies’ lifespan (Fig. 4.3. F).
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 4.3. Effect on lifespan of rapamycin, Torin 1, Torin 2, diazaborine, cycloheximide and salubrinal. Flies were given several concentrations of each drug, and the average survival date of these have been plotted together with the average survival data of the flies fed the control media with the solvent used to dissolve each drug. Around 150 mated female flies per condition were tested, the p values between the survivorship data of the flies fed the drug of interest and the ones fed the solvent control medium were calculated doing a χ²-log-rank test: A) Rapamycin 5 µM p= 0.02 and rapamycin 10 µM p= 1.9 x 10⁻⁶. B) Torin 1 0.8 µM, 0.4 µM, and 2 µM were n.s. and 10 µM p= 0.0369. C) Torin 2 0.25 µM and 1 µM n.s. and 4 µM p=0.02. D) Diazaborine 0.3 µM n.s., 3 µM p= 0.014 and 30 µM p= 0.0023. E) Cycloheximide 0.1 µM n.s., 1 µM p=0.0006; 10 µM p=0.039; 100 µM p=0.0005 and 1 mM p=6.6 x 10⁻¹⁸. F) Salubrinal n.s.
4.2.1.4. Conclusions about the effect of longevity-related drugs on translation fidelity.

Our *in vitro* study of how different drugs affect translation fidelity has shown that rapamycin, Torin 1 and trametinib are potent downregulators of both stop codon readthrough and amino acid misincorporation. These three drugs also extend lifespan in model organisms including flies. Cycloheximide only improved the level of stop codon readthrough and it shortened the lifespan of flies. Diazaborine and Torin 2 had no effect on translation fidelity, despite a range of concentrations tested, but diazaborine shortened lifespan and Torin 2 slightly increased lifespan at the highest concentration tested. Salubrinal, however, affected neither translation errors nor lifespan. Therefore, it can be concluded that the improvements of stop codon readthrough and misincorporation can be uncoupled and that the drugs that lead to fewer errors of both misincorporation and stop-codon readthrough also have the most robust lifespan extensions in *Drosophila*. Importantly, this suggests that our in vitro luciferase assay for translation fidelity could be used to evaluate if a translation-related drug is a potential anti-ageing drug. In addition, given that these drugs affect translation differently, and that only some of them lead to potent improvements in fidelity and in longevity, future work is expected to reveal complex anti-ageing mechanisms.
4.3. Discussion

4.3.1. The lifespan extending effect of the drugs tested correlates with increased translation fidelity

Anti-ageing interventions in humans are a step closer to come true, as there are already several trials on the run and many scientific groups around the world searching for promising drugs (Fontana et al., 2010, Longo et al., 2015, Barzilai et al., 2016). For this reason, we decided to study the effect on translation fidelity of drugs already known to either extend lifespan or that reduce protein synthesis. For this purpose, we treated S2R+ cells with different concentrations of these drugs and we checked if the levels of stop codon readthrough and misincorporation changed. First, we tested rapamycin, which is known to extend lifespan in several organisms (Bjedov et al., 2010, Harrison et al., 2009, Johnson et al., 2013, Sharp and Strong, 2010, Robida-Stubbs et al., 2012). Rapamycin is an inhibitor of mTORC1, preferentially inhibiting the phosphorylation of S6K and in mammalian cells, it also inhibits the partial phosphorylation of 4EBP at Ser65 (Guertin and Sabatini, 2009, Liu et al., 2011, Kang et al., 2013); in addition, when given chronically, in some mammalian cells at least, it also inhibits mTORC2 (Sarbassov et al., 2006). mTORC1 regulates cap-dependent translation through eIF4E; when nutrients are abundant mTOR is active, so 4EBP is hyperphosphorylated and it cannot bind to eIF4E, which can become part of eIF4F and hence initiate translation (Ben-Sahra and Manning, 2017); however, 4EBP is largely insensitive to rapamycin (Saxton and Sabatini, 2017). In addition, mTORC1 phosphorylates S6K promoting translation too by the inactivation of eEF2K, in a rapamycin-sensitive manner (Saxton and Sabatini, 2017). Interestingly, rapamycin has already been shown to decrease the level of stop codon readthrough and misincorporation in mouse fibroblast in an S6K dependent manner (Conn and Qian, 2013). We also found that rapamycin decreased the level of both stop codon readthrough (UGA) and misincorporation in S2R+ cells, but in our case, it might not be by inhibition of eEF2K as it was suggested by Conn and Qian, since this kinase has not been found in *Drosophila* (Ryazanov, 2002, Conn and Qian, 2013). Furthermore, our assay was different from Conn and Qian’s in the fact that they used a single luciferase reporter plasmid, not a dual luciferase reporter, which is used in translation accuracy field and which can detect more subtle differences and at the same time reduces experimental variability. The plasmid they used contained a firefly that either had a stop codon (UAG) or an R218S substitution in the middle of firefly’s sequence. This means that rapamycin can also improve another kind of stop codon readthrough and that the improvement in misincorporation was also based in changes at the first, second or both codons (the
misincorporation that could give firefly its activity back was a UCU→Arg (CGC, CGU, CGA, CGG, AGA or AGG)) (Conn and Qian, 2013).

In addition, we tested another drug that was recently shown to extend lifespan in *Drosophila*, Torin 1 (Mason et al., 2018); this is a mTOR inhibitor more potent than rapamycin, as it a catalytically inhibits the phosphorylation of all mTORC1 substrates, including S6K, 4E-BP (at both Thr37/46 and Ser65), Grb10 (growth factor receptor-bound protein 10), PRAS40 (proline-rich Akt substrate 40) and ULK1 (unc-51 like autophagy activating kinase 1) and it also inhibits mTORC2 (Guertin and Sabatini, 2009). Torin1 also decreased the level of both translation errors in some of the highest concentrations tested. Interestingly, when we checked the effect of this drug on *Drosophila*’s lifespan in our laboratory, we also found that only the highest tested concentration extended lifespan. The lowest concentration that we tested, 1 nM, increased the level of misincorporation but it still lowered the level of stop codon readthrough. This could have been caused by this low concentration affecting only certain substrates of mTOR that lead to a stress response that increases the level of translation errors. However, the results obtained at the highest concentrations still indicate a correlation between translation fidelity and extended lifespan. It is interesting to note that this more potent mTOR inhibitor had less pronounced effects on both lifespan and accuracy than rapamycin. The stronger effect of rapamycin compared to Torin 1 could be due to rapamycin specifically activating the pathways that are the most beneficial for longevity, namely, autophagy, reprogramming of protein synthesis and upregulation of certain genes (e.g. mitochondrial metabolism genes) (Guertin and Sabatini, 2009, Wang and Proud, 2006, Kapahi et al., 2004, Zid et al., 2009, Bjedov and Partridge, 2011, Cope et al., 2014). Similarly, the weaker, although significant, improvement of accuracy seen in cells treated with Torin 1 compared to rapamycin treatment could arise from supplementary targets of this drug acting through mTORC2 that mask the fidelity effects.

Another drug that we tested was Torin 2, since, like Torin 1, inhibits both TORC1 and TORC2 (Liu et al., 2011). This molecule was developed to be more potent than rapalogs and than other ATP-competitive kinase inhibitors such as Torin 1, and at certain concentrations it can inhibit ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), DNA-protein kinase and PI3Kα and it shows prolonged kinetics, meaning that the effect on the substrates is kept for longer than for rapamycin but shorter than Torin 1 (Liu et al., 2013). This drug had not been previously reported to extend lifespan, but in our experiments, the highest concentration slightly extended the lifespan of our flies; however, the level of translation errors was unchanged in all the concentrations. Here, there is no correlation
between lifespan extension and accuracy, at least at the tested concentrations. One possibility is the in vitro set up did not replicate the in vivo one, and that we did not hit the right concentration window to see a change in translation accuracy. Another possibility is that Torin 2 affected longevity through a mechanism independent of translation accuracy, as at high concentrations it has been shown that it becomes a pan-inhibitor of not only mTOR but other kinases of the same family (Liu et al., 2013). Clearly, this drug did not behave as Torin 1 or rapamycin, and in fact, Torin 1 has been reported to be one of the second generation mTOR inhibitors that behaves the most like rapamycin in both the concentration required for the inhibition of the phosphorylation of mTORC1’s substrates and also in its anti-ageing effects, whereas Torin 2 is the most different to rapamycin, as it is more potent and less selective for the anti-ageing effects (Leontieva and Blagosklonny, 2016).

Trametinib has also been shown to extend the lifespan of Drosophila and to protect mice against some of the detrimental effects of obesity (Slack et al., 2015, Banks et al., 2015). Trametinib binds and blocks Mek1 and Mek2 (mitogen-activated protein kinase kinase 1 and 2) hence it is an inhibitor of the Ras-Erk-ETS (E-twenty-six) signalling pathway (Yamaguchi et al., 2011). Interestingly, Mek2 phosphorylates ERK1 and ERK2, which have been shown, in mammalian cells, to phosphorylate MetRS in two sites that render this tRNA synthetase more promiscuous, mischarging Met to other tRNAs and hence triggering misincorporation of this amino acid (Lee et al., 2014). Thus trametinib, by inhibiting Mek2 could be leading to less Met-misincorporation. In addition, Mek2 has also been shown to be directly inhibited by deacetylated tRNA levels, which increase in periods of lower nutrient availability, to slow down the cell cycle (Wang et al., 2016). Furthermore, this pathway has been shown to act downstream of the lifespan extension produced by downregulation of chico (Drosophila’s insulin receptor substrate), by regulating Aop (anterior open) and Foxo transcription factors (Slack et al., 2015). In our screen, this drug downregulated the level of both misincorporation and stop codon readthrough. This could have been by Aop regulating the expression of genes related to translation, as it has been recently shown that this gene takes part on the regulation of genes encoding proteins involved in translation and energy metabolism in Drosophila (Dobson et al., 2018). Importantly, these results correlate again translation accuracy with lifespan.

In addition, we tested diazaborine, which has not been shown to extend lifespan in flies, but it extended the lifespan of yeast (Steffen et al., 2008b). This drug is an inhibitor of ribosomal biogenesis that works through inhibiting Drg1 and hence blocking the release of Rlp24 from the 60S in fungi (Loibl et al., 2014). When we tested, if anything, it shortened the
lifespan of the flies and it did not change the level of translation errors. Its efficacy in insects has not been tested, but it has been recently reported that it also blocks the action of certain enzymes, the serine proteases, in human cells (Antonio et al., 2018). Therefore, it could be that it is not effective in insects or that it simply does not increase lifespan neither it decreases errors.

Similarly, we tested salubrinal, that although it has not been implicated in ageing, it arrests translation through an increase in eIF2α phosphorylation by inhibiting protein phosphatase 1 (PP1) (Boyce et al., 2005, Carrara et al., 2017). This drug did not increase the lifespan of our flies, but it decreased the level of misincorporation at the highest concentration tested. Although this drug works in zebrafish and C. elegans, it has been proposed to not be effective on Drosophila due to the lack of PP1 (protein phosphatase 1) in flies (Julien et al., 2016). Nevertheless, recently an eIF2α phosphatase specific for non-mammals has been found and it has certain common characteristics with mammalian PP1, in particular, the PP1R15a (PP1 regulatory subunit 15A region) that salubrinal inhibits (Malzer et al., 2013, Boyce et al., 2005). Therefore, it could still be that lowering the level of misincorporation is not sufficient to extend lifespan if stop codon is not decreased too.

Finally, we tested cycloheximide, which has not been implicated in ageing either, but it is an inhibitor of translation elongation through binding to the E-site in the large subunit (Schneider-Poetsch et al., 2010a). This drug did not extend Drosophila’s lifespan, and in the highest concentrations it shorted the flies’ lifespan. It has been found that elevated concentrations of cycloheximide block both translation and transcription (Schneider-Poetsch et al., 2010a). Cycloheximide has also been proposed to promote the depletion of ubiquitin, leading to cell death (Keeven et al., 2002, Hanna et al., 2003); therefore, it would be interesting to check if in our flies ubiquitin levels are affected and this is one of the causes of mortality. Interestingly, cycloheximide decreased the level of misincorporation when translation itself was reduced (Abraham and Pihl, 1983). Therefore it was suggested that it was not the elongation rates that affected misincorporation but the accumulation of cognate substrate when translation itself was reduced (Abraham and Pihl, 1983). This could mean that cycloheximide inhibits elongation,
but it does not exploit the two steps that ensure the accuracy of elongation: the initial selection step and the proofreading step.

In fact, there are more factors that can affect stop codon readthrough than misincorporation and, therefore, the drugs that decreased the level of stop codon readthrough in our screen (rapamycin, Torin1, trametinib and cycloheximide) could have done it through a plethora of mechanisms. The main players in stop codon recognition are release factors; after binding the ribosome their ‘switch’ loop docks in the domain closure pocket formed by RPS23, 18S rRNA and 28S rRNA and structural rearrangements trigger the open domain (Prabhakar et al., 2017). Then, peptidyl and RFs release need to also occur accurately. This means that there are some antibiotics, such as the oxazolidines, that increase only stops codon readthrough but not misincorporation, as they bind to 23S and 16S rRNA (Thompson et al., 2002). These two rRNAs are part of the peptidyl transferase centre, which in the termination step of translation is required to be activated by release factors, but when the drugs are bound, it is hyperactivated and hence aminoacylated tRNAs can be loaded and the termination missed (Thompson et al., 2002). In addition, it has been recently found that stop codon recognition in eukaryotes is influenced by nucleotides in the entry channel that can recognize certain nucleotides downstream of the stop codon itself, and hence they can interact with 18S rRNA, 25S rRNA, and other proteins of the large subunit (Panopoulos et al., 2004, Cridge et al., 2018).

In conclusion, we can infer from our data that lifespan-extending drugs also decrease the level of both stop codon readthrough and misincorporation. The effect of specific aspects of translation accuracy and longevity has already been reported. In a study using different rodent species, Ke et al. found that translation accuracy at the first and second codon correlate with the species lifespan, meaning that the more accurate species also had the longer lifespans (Ke et al., 2017). In our experiment, the misincorporation reporter had a CGC→His (CAC or CAU) substitution and consequently, the misincorporation necessary to give the activity back to firefly in our set up is also at the second codon. Importantly, our results differ from Ke. et al., as they did not see that improving in stop codon readthrough had coevolved with longevity. Nevertheless, our results together with what we have seen in RPS23 K60R flies, show that in Drosophila enhancing the fidelity of both stop codon and misincorporation play a role in longevity.
Chapter 5. Impact on development and longevity of a substitution point mutation that alters translation fidelity
5.1 Introduction

Translation is a process that has to happen fast and accurately, to provide the cell with functional proteins at efficient speed; however, due to the complex nature of this process and how two different alphabets need to be used, the error rates in vivo are still quite significant (Kramer and Farabaugh, 2007, Ogle and Ramakrishnan, 2005). Importantly, translation fidelity is mainly governed by the decoding centre, and this was first shown by a breakthrough study by Gorini and Kataja that found that aminoglycoside antibiotics bind the small subunit of the ribosome and induce errors (Gorini and Davies, 1968, Gorini and Kataja, 1964a, Gorini and Kataja, 1964b). This led to the discovery of streptomycin resistant, dependent and sensitive strains that had these phenotypes because of mutations in ribosomal proteins, which also caused them to be hyper or hypoaccurate (Gorini et al., 1966, Gorini et al., 1967, Funatsu and Wittmann, 1972, Hasenbank et al., 1973). So far, translation accuracy mutants have been isolated or generated in microorganisms, both bacterial and yeast (Liu and Liebman, 1996, Piepersberg et al., 1975, Smith et al., 2001, Alksne et al., 1993, Synetos et al., 1996, Anthony and Liebman, 1995, Bjorkman et al., 1999, Chiou and Jones, 1995, Gregory et al., 2001, Inaoka et al., 2001). However, to the best of our knowledge, there are no metazoan organisms with these mutations; thus, taking advantage of the CRISPR/Cas9 technique, we have aimed to develop ribosomal fly mutants that alter translation accuracy. These will be a powerful tool to directly study how translation fidelity can affect ageing.
5.2. Results

5.2.1. Generation and characterisation of the first metazoan translation fidelity mutant

5.2.1.1. Design of the translation fidelity mutations by CRISPR Cas-9

To further investigate the role of translation accuracy in ageing, three mutants with single point substitutions RPS2 (Y143C), RPS23 (K60R) and RPS23 (K60T) were designed (see Materials and Methods 2.7.1.). These mutations were chosen because they have been reported in *E. coli* and *S. cerevisiae* to alter translation accuracy and they are in very evolutionarily conserved regions of these ribosomal proteins (Alksne et al., 1993, Anthony and Liebman, 1995, Velichutina et al., 2000, von der Haar et al., 2017) (Fig. 5.1. A). With the purpose of introducing these mutations in the *Drosophila* genome, the CRISPR/Cas9 system was used (see Materials and Methods 2.7.). We decided not to introduce any visual marker to avoid any potential functional changes, but we introduced in our ssODN design a silent restriction site for each mutant that would allow for genetic screening (see Materials and Methods 2.7.4.). Unfortunately, after an extensive screening for the possible CRISPR-edited candidates, we found only flies with the RPS23 K60R mutation. We were also able to find mutants that either had just the RPS23 K60R mutation and those that had both RPS23 K60R and the restriction site PvuII mutation (introduced for easier screening) (Fig. 5.1. B). We decided to backcross all these mutants to *w*Dah flies (see Materials and Methods 2.7.5.). For backcrossing we used the PCR primers that contained the introduced amino acid substitution and silent mutation and that hence did not amplify wild-type *rps23* at a narrow temperature window.
Figure 5.1. A single point mutation in an evolutionarily conserved region of RPS23 can alter translation. A) The protein 23 of the small ribosomal subunit (40S) is located at the decoding centre of the ribosome. When a highly conserved lysine (RPS12 K42 in E. coli and RPS23 K62 in S. cerevisiae) is substituted by other amino acids, there are direct changes in the levels of translation errors. B) Sequences of some of the mutants we got by CRISPR-Cas9 are depicted. RPS23 K60R has CGC (arginine) instead of AAG (lysine) and an extra mutation to disrupt the guiding RNA sequence; the RPS23 K60R PvuII mutant has two extra mutations in the site of the guiding RNA also to disrupt it and a silent substitution T→G to become a PvuII restriction site.

RPS23 K60R was first discovered as a mutation conferring resistance to streptomycin (StrR) in *E. coli* (Gorini and Kataja, 1964a). This (K42R in bacteria) and other mutations in RPS12 (the bacterial homologue of RPS23), such as K42N, K42Q, K42T, R85R, K87R, P90L, P90Q, and P90R, were further characterised and they were found to decrease the level of translation errors and hence confer either resistant or even dependence to the error-inducing drug streptomycin (Bohman et al., 1984, Bjorkman et al., 1999, Tubulekas et al., 1991, Funatsu and
Wittmann, 1972, Timms et al., 1992). However, when these same mutations were introduced in *S. cerevisiae*’s RPS23 (in the old nomenclature it was known as RPS28), RPS23 K62R was found to decrease accuracy (Alksne et al., 1993). Importantly, the K60R mutation is in a highly conserved region of RPS23, the KQPNSA region (Alksne et al., 1993, Anthony and Liebman, 1995, Chumpolkulwong et al., 2004); thus, we decided to see the incidence of this lysine in nature. For this reason, we run the amino acid sequence through BLAST using Ensembl and we looked at the variations of the KQPNSA region across the tree of life (Ensembl, 2018). Interestingly, we saw that the lysine at that position is highly conserved across kingdoms, and that the only other amino acid that is found in that position in nature is arginine (Fig. 5.2 A and B). Arginine instead of lysine is only present in several extreme thermophilic archaea species (Fig. 5.2 C).
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 5.2. Highly conserved region across all living organisms in the homologues of Drosophila’s RPS23. A) and B) show that there is a highly conserved amino acid region in the homologues of RPS23 comprising KQPNSA residues. C) When several model organisms such as Neurospora, S. cerevisiae, S. pombe, C. elegans, chimpanzees, Xenopus or Arabidopsis are compared, this conservation is even more clear. There is only another possibility that occurs naturally, RQPNSA, which is seen in some thermophilic archaea (marked in red). BLAST comparison using Ensemble Metazoan (GeneTree code EGGT0005000005347) (Ensembl, 2018).
5.3.1.2. Translation fidelity is improved in RPS23 K60R flies

As already mentioned, there are some discrepancies in the reported alterations of translation fidelity due to the RPS23 K60R mutation, which is mostly considered to have a mild restrictive (i.e. hyperaccurate) effect in bacteria and a ram (i.e. hypoaccurate) phenotype in S. cerevisiae. Therefore, we studied the level of translation errors of our mutant flies. For this, we used the dual-luciferase stop codon readthrough reporters by crossing these flies to our RPS23 K60R mutants and control (see Materials and Methods 2.6.). We then used samples from whole flies, either young (10 days) or old (60 days), and the luminescence intensities were at least 5 times higher than the background, so we did not have the same problems reported in the samples from dissected tissues of S106>UAS-RNAi. Most likely this is because of the higher expression of the dual luciferase reporter in muscles and neuronal tissues compared to the fat body.

This assay allowed us to see that the level of stop codon readthrough in the RPS23 K60R PvuII compared to the RPS23 WT control was significantly decreased in young and old samples (p=0.0239 and p=0.0017, respectively, Welch’s test) (Fig. 5.3.). The RPS23 K60R young flies were not significantly different from the controls but there was a tendency towards improved accuracy. Nevertheless, the old RPS23 K60R flies had significantly less stop codon readthrough than the old controls (p=0.002, Welch’s test). Therefore, Drosophila’s RPS23 K60R substitution leads to improved stop codon readthrough, being this more similar to E. coli than to S. cerevisiae.

Importantly, measuring accuracy in the control flies enabled us to address a long-standing question in the field, whether translation errors increase with age in metazoan organisms (Ke et al., 2018). Interestingly, we saw that the level of stop codon readthrough drastically increased from a mean of 7.5% in young WT flies to 20.1% in the old WT flies (p=0.017, Welch’s test), while the level of this type of translational error did not significantly change between young and old RPS23 K60R nor K60R PvuII flies. It is also interesting to note that the measured percentage of stop codon readthrough in vivo was much higher (ranging from the average 2.1% in the old RPS23 K60R flies to the average 20.1% in the old WT flies) compared to the values obtained in vitro (ranging from the average 0.4% in cells treated with 250 nM rapamycin to the average 1.67% in cells treated with paromomycin).
Figure 5.3. The RPS23 K60R mutation leads to decreased stop codon readthrough in young and old flies. The WT control (blue columns) had increased levels in old flies (60 days) compared to young flies (10 days). The RPS23 K60R PvuII (grey columns) have less stop codon readthrough than the control at both, young and old ages. In addition, the RPS23 K60R flies (orange) had also decreased levels of errors compared to the control. The signal of firefly luminescence was divided by the signal of Renilla for each individual sample to calculate the firefly/Renilla ratios. Then, the percentage stop codon readthrough was calculated by dividing each sample’s firefly/Renilla ratio of the stop-codon reporter by the average firefly/Renilla ratio of the control reporter of each genotype and age. The background luminescence was always subtracted to the firefly luminescence values. For the young flies, 24 samples with 6 flies per sample for each genotype were analysed from two independent experiments. For the old flies, 12 samples with 6 flies per sample for each genotype were analysed from two independent experiments. The average ±SEM are represented in this graph. The p values were calculated with a Welch’s t-test*: p≤0.05 **p≤0.005: RPS23 K60R PvuII (young) vs WT (young) p= 0.0239; WT (old) vs WT (young) p= 0.017; K60R (old) vs WT (old) p= 0.0017; K60R PvuII (old) vs WT (old) p= 0.002.

5.3.1.3. Directly altering translation fidelity by a mutation in the ribosomal decoding centre delays development independently of the environment

*Drosophila* ribosomal protein mutants, called minutes are often developmentally delayed, as expected given the importance of translation in development (Dunn, 1937, Marygold et al., 2007, Schultz, 1929). Therefore, to further characterise RPS23 K60R mutation in *Drosophila*, we studied the flies’ developmental patterns. For this, we assayed the flies’ fecundity (defined by the number of eggs laid), the ratio of pupae: eggs, the ratio of flies: pupae and the developmental rates (the time of emergence of adult flies) in both
homozygotes and heterozygotes. Furthermore, we wanted to see if these fecundity and developmental parameters changed under different environmental conditions, since external stimuli have been shown to affect the growth rates of *Drosophila* minute mutants and also of bacterial hyperaccurate mutants (Isono et al., 1976, Sakka et al., 1987, Mikkola and Kurland, 1988, Sinclair and Kaufman, 1984). Therefore, we left the flies to lay eggs and then these eggs to develop either at different temperatures (18°C, 25°C and 30°C), on different diets (poor 0.5 SYA or standard 1 SYA media) or in the presence of the aminoglycoside paromomycin (see Materials and methods 2.1.2).

To assess fecundity of the female flies, we counted the number of eggs that mated flies laid over a 24 hours period time. The number of eggs was only decreased by the homozygous RPS23 K60R mutation compared to the control flies in normal 1 SYA medium at 25°C (p=0.0047, Welch's test) (Fig. 5.4. A). Interestingly, in the presence of the error inducing drug paromomycin, the number of eggs of the homozygous RPS23 K60R PvuII flies was higher than the control (p=0.0144, Welch’s test). The ratio pupae: eggs was reduced in the fidelity mutants under several conditions; it was reduced in the 0.5 SYA-fed homozygous K60R and K60R PvuII flies compared to the WT (p=0.03 and p=0.003 respectively, Welch’s test) and it was also reduced in the 0.5 SYA-fed heterozygous K60R PvuII flies compared to the control flies (p=0.0089, Welch’s test) (Fig. 5.4. C). The pupae: eggs ratio was also reduced in heterozygous K60R flies fed the standard 1 SYA medium at 25°C (p=0.0276, Welch’s test) (Fig. 5.4. A). There were also fewer pupae: eggs in both homozygous and heterozygous K60R and K60R PvuII compared to the control when they were given paromomycin (homozygous K60R p=0.003; homozygous K60R PvuII p=0.0123; heterozygous K60R p=0.0144 and heterozygous K60R PvuII p=0.0429, Welch’s test) (Fig. 5.4. E). The ratio flies: pupae was only lower in homozygous K60R flies supplemented with paromomycin compared to the respective control (p=0.0002, Welch’s test).

In conclusion, neither fecundity nor the ratio of flies: pupae were affected by the RPS23 K60R mutation in most of the conditions tested, but the ratio pupae: eggs was significantly reduced compared to controls in RPS23 K60R homozygous and heterozygous under the poor 0.5 SYA media and in the presence of paromomycin.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 5.4. The fertility and various stages of development of the RPS23 K60R flies are affected by several environmental conditions. The number of eggs, pupae and emerged flies were counted for RPS23 WT (blue columns), RPS23 K60R (orange columns) and RPS23 K60R Pvull (grey columns). For this, 5 female virgin flies and 5 males were crossed in a single vial (containing the indicated medium) and left to mate 24 hours in the studied condition. Around 10 vials per condition of the homozygous flies and 15 vials per condition of the heterozygous flies were tested, the averages are shown here ± SEM. To calculate the p values between the WT and the RPS23 K60R or K60R Pvull, Welch’s t-tests were used, *p=0.05, **p=0.005: A) Eggs laid by RPS23 K60R p=0.0047; B) n.s.; C) Pupae: eggs RPS23 K60R p= 0.0299 and RPS23 K60R Pvull p=0.003; D) Pupae: eggs RPS23 K60R p=3 x10^-4 and RPS23 K60R Pvull p=0.0123. E) n.s.; F) Pupae: eggs K60R Pvull p=0.0089; G) Pupae: eggs RPS23 K60R p=0.0114 and RPS23 K60R Pvull p=0.043.

We also studied the rate at which the adult translation accuracy mutant and control flies emerged from the pupae under different conditions. We saw a delay in the emergence of the RPS23 K60R and K60R Pvull flies compared to the controls in all the conditions tested, but it varied in the degree of delay. At 25°C and a standard 1 SYA diet, 50% of the control flies had emerged in day 10, while the homozygous K60R and K60R Pvull had approximately one day delay emergence, (Fig. 5.5. A and B). Whereas the heterozygous, under this same standard
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

condition, were only half day delay compared to the controls that emerged in day 10 (Fig. 5.5. C and D). Development of flies at 30°C is faster and control flies emerged in day 8.5, but still, at this elevated temperature, the homozygous K60R and K60R PvuII flies emerged almost 1 day later than the controls (Fig. 5.5. E and F), whereas the heterozygous emerged around half a day later (Fig. 5.5. G and H). At 18°C, where 50% of the flies have emerged by day 17.5, the delay of the ribosomal mutants was even more pronounced, the homozygous K60R and K60R PvuII emerged more than 2 days after the controls (Fig. 5.5. I and J). When a poorer 0.5 SYA food with half the yeast content was tested, 50% of the control flies had emerged by day 10, both homozygous and heterozygous K60R and K60R PvuII adult flies emerged only around half a day later (Fig. 5.5. K, L, M and N). However, the error-inducing antibiotic, paromomycin, slowed down emergence of the homozygous flies more than the heterozygous RPS23 mutants differently: homozygous K60R and K60R PvuII emerged more than 2 days later than the control flies, which had emerged by day 10 (Fig. 5.5. O and P), whereas the heterozygous K60R and K60R PvuII were only one day delayed (Fig. 5.5. Q and R). All the described effects were almost equal between the males and females of each condition. Thus, the fly accuracy mutant is developmentally delayed especially at low temperature and low yeast content.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 5.5. RPS23 K60R adult flies emerged later than the controls under all the conditions tested. The number of flies emerging was recorded and classified according to sex every few hours for the WT control (blue lines), RPS23 K60R (orange lines) and RPS23 K60R Pvull (grey lines). The cumulative frequency percentage of adult flies over time was then calculated for each condition. Around 10 vials per condition of the homozygous flies and 15 vials per condition of the heterozygous flies were examined.
5.3.1.4. Directly altering translation fidelity in *Drosophila* influences longevity in a condition-dependent manner

The main aim of this thesis is to explore how translation fidelity affects longevity, so for this purpose, we studied the lifespan of RPS23 K60R flies. In addition, we tested the effect of the accuracy mutation on lifespan under different environmental conditions, as these conditions had shown to alter the developmental phenotype of the K60R flies; this time we also tested the lifespan of these flies under a higher temperature, 30°C, as the K60R substitution naturally occurs in thermophiles.

We first tested the lifespan of both homozygous and heterozygous RPS23 control, RPS23 K60R and RPS23 K60R PvuII female and male flies at standard 25°C. We saw that at this standard temperature, female homozygous K60R flies had statistically the same lifespan than the control (65.5 days median, 77.5 maximum lifespan of the K60R flies compared to 65.5 days and 74.5 days maximum lifespan of the control), but the K60R PvuI had a shorter lifespan (56 median and 68.5 maximum lifespan) than the control (p=1.49 x 10^-6, χ² log-rank test) (Fig. 5.6. A). Interestingly, the homozygous K60R and K60R PvuII males had a shorter lifespan than the control (p=2.24 x10^-17 and p=5 x10^-33 respectively, χ² log-rank test). The control males lived 68.5 days median and 77.5 days maximum lifespan whereas the K60R male flies had 56 median and 71.5 maximum lifespan and the K60R PvuII flies had 53.5 median and 63 maximum lifespan. However, at 30°C, homozygous K60R females had 35 days median and 39.5 days maximum lifespan, which was significantly longer than the 32.5 median and 37 maximum lifespan of the controls (p=0.0028, χ² log-rank test). However, the homozygous K60R males lived the same than the controls, 32.5 days median lifespan for both and 37 days or 39.5 days maximum for K60R and the control respectively (Fig. 5.6. B).

The longevity effect of this mutation was different on heterozygous flies. The heterozygous female K60R and K60R PvuII flies at 25°C lived longer (70 days median, 82 days maximum and 72.5 days median, 86 days maximum lifespan, respectively) than the control (63.5 days median and 75 days maximum lifespan) (p=1.17 x10^-20 for K60R and p=6.93 x10^-28 for K60R PvuII compared to the controls, χ² log-rank test) (Fig. 5.6. C). Similarly, the males with the heterozygous K60R mutation had a 61 days median and 75 days maximum lifespan, which was longer (p=0.017, χ² log-rank test) than the control’s 59 days median and 70 maximum lifespan. This effect was very similar at 30°C for the heterozygous flies: female K60R and K60R PvuII flies lived longer than the controls (33 days median and 35.5 days maximum lifespan) (p=2.96 x10^-30 and p=3.54 x10^-9, respectively, χ² log-rank test) with a 38 days median and X maximum lifespan and a 35.5 median and 38 maximum lifespan respectively. The K60R males
had a 35.5 days median and 40 days maximum lifespan, which was longer than the control 28.5 days median and 35.5 days maximum lifespan ($5 \times 10^{-20}$, $\chi^2$ log-rank test) (Fig. 5.6. D).
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 5.6. Effect of RPS23 K60R mutation on lifespan under two different temperatures. The lifespans of both females (solid lines) and males (dashed lines) were studied in A) homozygous and B) and heterozygous C) and D) RPS23 WT control (blue lines), RPS23 K60R (orange lines) and RPS23 K60R PvuII (grey lines) reared at 25°C and 30°C. Around 150 mated flies per condition were tested, the p values between the survivorship data of the RPS23 K60R or K60R PvuII and the RPS23 WT controls were calculated doing a χ2-log-rank test: A) Homozygous RPS23 K60R females vs control females n.s.; RPS23 K60R PvuII females vs control females $p=1.49 \times 10^{-6}$; RPS23 K60R males vs control males $p=2.24 \times 10^{-17}$; RPS23 K60R PvuII males vs control males $p=6.93 \times 10^{-28}$. B) Homozygous K60R females vs control females $p=0.0028$; RPS23 K60R males vs control males n.s. C) Heterozygous RPS23 K60R females vs control females $p=1.17 \times 10^{-20}$; RPS23 K60R PvuII females vs control females $p=6.93 \times 10^{-30}$; RPS23 K60R males vs control males $p=0.017$; RPS23 K60R PvuII males vs control males n.s. D) Heterozygous RPS23 K60R females vs control females $p=2.96 \times 10^{-30}$; RPS23 K60R PvuII females vs control females $p=3.54 \times 10^{-9}$; RPS23 K60R males vs control males $p=5 \times 10^{-20}$; RPS23 K60R PvuII males vs control males n.s.
As we saw that food plays an important part in the development of RPS23 K60R mutant flies, we then tested how a diet of 0.5 SYA (poor yeast content), 1 SYA (standard yeast content) and 2 SYA (rich yeast content) could affect the lifespan of females. For this, homozygous and heterozygous flies at 25°C were studied. We saw that homozygous K60R flies lived shorter than the control when fed 0.5 SYA medium (19 days median, 62.1 days maximum lifespan compared to the control’s 49.2 median and 72.6 maximum lifespan, p=8.7 x10^{-8}, χ² log-rank test) (Fig. 5.7. A). Nevertheless, there was no statistical change in survivorship when they were given 1 SYA, 61 days median and 72.6 maximum lifespan for the K60R flies compared to 61 days median and 68.1 maximum lifespan of the control flies. On the contrary, they lived longer than the controls in a 2 SYA diet, the K60R flies had 58.6 median and 69.1 maximum lifespan whereas the controls had 51.6 median and 65.6 maximum lifespan in this diet (p=0.00018, χ² log-rank test) (Fig. 5.7. C). The results for the heterozygous K60R flies were slightly different; the K60R flies lived the same as the controls when fed 0.5 SYA medium (58.6 days median for both and 88 days maximum lifespan for the control and 75 days for the K60R flies), but the K60R PvuII flies lived significantly shorter than the control, with only 19 days median and 71 days maximum lifespan (p=2.2 x10^{-16}, χ² log-rank test) (Fig. 5.7. D). Nevertheless, when fed 1 SYA, the K60R flies and K60R PvuII flies lived the same, 68.1 median and 77 days maximum lifespans, but the K60R PvuII flies were statistically significantly longer lived than the control (p=0.03 compared to the control, χ² log-rank test) (Fig. 5.7.D). When the flies were given 2 SYA medium, both heterozygous K60R and K60R PvuII lived longer than the control (p=1.3 x10^{-10} and p=2.85 x10^{-5}, respectively, χ² log-rank test) (Fig. 5.7.F). The K60R flies had a 58.6 median, 72.5 maximum lifespan and the K60R PvuII flies had 56.2 median and 68.1 maximum lifespan compared to the control’s 51.6 median and 65.6 maximum lifespan. In sum, longevity of RPS23 K60R mutants was promoted on food with increased yeast content.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

Figure 5.7. Effect of RPS23 K60R mutation on lifespan fed 0.5 SYA, 1 SYA and 2 SYA. The lifespans of females were studied in RPS23 WT (blue lines), RPS23 K60R (orange lines) and RPS23 K60R Pvull (grey lines). We studied both homozygous A) fed 0.5 SYA medium, B) 1 SYA medium and C) 2 SYA, and heterozygous D) fed 0.5 SYA medium, E) 1 SYA medium and F) 2 SYA. Around 230 mated homozygous and 150 heterozygous female flies per condition were tested, the p values between the survivorship data of the RPS23 K60R or K60R Pvull and the RPS23 WT controls were calculated doing a χ²-log-rank test: A) Homozygous RPS23 K60R flies vs control at 0.5 SYA p=8.7 x10⁻⁸. B) Homozygous RPS23 K60R flies vs control at 1 SYA n.s. C) Homozygous
RPS23 K60R flies vs control at 2 SYA p=0.00018. D) Heterozygous RPS23 K60R flies vs control at 0.5 SYA n.s.; RPS23 K60R PvuII vs control at 0.5 SYA p=2.2 x10^-16. E) Heterozygous RPS23 K60R flies vs control at 1 SYA n.s.; RPS23 K60R PvuII vs control at 1 SYA p=0.03. F) Heterozygous RPS23 K60R flies vs control at 2 SYA 1.31 x10^-10; RPS23 K60R PvuII vs control at 2 SYA p=2.85 x10^-8.

5.3.1.5. RPS23 K60R female flies have improved healthspan when reared under standard conditions

In ageing, it is not only important to assess lifespan but also healthspan, and in flies, this is commonly measured by performing a negative geotaxis or climbing assay, which is known to decline with age (Gargano et al., 2005, Jones and Grotewiel, 2011). For this reason, we performed the negative geotaxis assay once a week for 5 weeks in female WT and RPS23 K60R flies reared at standard conditions (Fig. 5.8.). We saw that homozygous K60R flies performed significantly better than the control at week 2 and week 3 (p=7.7 x10^-4 and p=0.018 respectively, Student t-test); however, the mutant flies had a worse performance index than the control at week 5 (p=0.017, Student t-test). The heterozygous K60R flies had improved performance indices than the control flies at week 2 (p= 0.0065, Student t-test), week 3 (p=0.026, Student t-test) and week 4 (p=3.87 x10^-5, Student t-test). It should be noted that this experiment was performed in parallel to the lifespan shown in Fig. 5.6., in which the homozygous K60R flies lived the same than the control but the heterozygous K60R flies lived significantly longer than the control (p=0.0056). This means that the lifespan extension was accompanied by an improvement in healthspan in the accuracy mutants.
5.3.1.6. Homozygous RPS23 K60R female flies are sensitive to paromomycin but heterozygous RPS23 K60R flies were not

Accuracy mutants were first discovered because of their resistance to streptomycin, an antibiotic that increases the level of translation errors (Bilgin et al., 1992, Birge and Kurland, 1969, Gorini and Kataja, 1964b, Gorini et al., 1967); therefore, we decided to test the resistance of the accuracy RPS23 K60R flies to the error-inducing drug paromomycin, which is known to affect eukaryotic cells too (Carter et al., 2000, Chernoff et al., 1994, Fan-Minogue and Bedwell, 2008, Prokhorova et al., 2017, Tuite and McLaughlin, 1984). For this, we put adult female control flies and both homozygous and heterozygous RPS23 K60R flies on 1 SYA supplemented with 800 µM paromomycin, similarly to the epistasis experiments explained in Chapter 3. Results.

Figure 5.8. Assessment of the senescence of negative geotaxis in the RPS23 K60R flies. The average performance index (n=3) for each week was calculated and plotted ±SEM for RPS23 K60R (orange lines) and control flies (blue lines). 10 vials with 15 flies in each were tested. The p-values of the data for the K60R flies compared to the data for the controls were calculated doing a Student t-test,*p≥0.05: A) Homozygous K60R flies at week 1 n.s.; week 2 p=7.7 x10^{-4}; week 3 p=0.019; week 4 n.s. and week 5 p=0.017. B) Heterozygous K60R flies at week 1 n.s.; week 2 p=0.0065; week 3 p=0.025; week 4 p=3.87 x10^{-5} and week 5 n.s.
We saw that homozygous K60R flies were sensitive to paromomycin, with 46 days median and 57.5 days maximum lifespan compared to the control flies supplemented this drug, which had 60 days median and 71.5 days maximum lifespan (p=6.6 x10^{-31}, χ² log-rank test) (Fig. 5.9. A). The heterozygous K60R flies, however, did not show any significant difference in their resistance to paromomycin, they had 60 days median and 67 days maximum lifespan compared to the 57.5 days median and 73 days maximum lifespan of the controls supplemented with this drug (Fig. 5.9. B). Therefore, the RPS23 K60R are not resistant to this aminoglycoside as their bacterial homologues, and actually, they are sensitive when the mutation is homozygous.

Figure 5.9. Effect of paromomycin on the lifespan of RPS23 K60R flies. The lifespans of females were studied in RPS23 WT (blue lines) and RPS23 K60R (orange lines), fed control 1 SYA food (solid lines) or 800 µM paromomycin (dashed lines). The mutations were either homozygous A) or heterozygous B). Around 150 female flies per condition were tested, and the average survivorship calculated and plotted here. The p values between the survivorship data of the RPS23 K60R flies and the RPS23 WT controls (either fed control food or paromomycin accordingly) were calculated doing a χ²-log-rank test: A) Homozygous RPS23 K60R flies vs control n.s.; RPS23 K60R on paromomycin vs control on paromomycin p=6.6 x10^{-31}. B) Heterozygous RPS23 K60R flies vs control p=0.0056; RPS23 K60R on paromomycin vs control on paromomycin n.s.
5.3.1.7. Conclusions about the characterisation of the translation fidelity mutant RPS23 K60R

Our study of the effects on translation accuracy of the RPS23 K60R mutation in the highly evolutionarily conserved accuracy-region of this ribosomal protein showed that, at least, in heterozygous females, it leads to improved stop-codon readthrough that remains at very low levels even in old flies. Notably, we also saw that the level of this type of error increases drastically in aged wild-type flies, which had not been previously reported in a metazoan organism. In addition, we saw that the developmental patterns of the flies with this fidelity mutation are very sensitive to the environment (i.e. low temperature and poor yeast content diet), especially the pupa: egg ratio. Nevertheless, they are always delayed in the emergence of adult flies from pupae, being this more pronounced in homozygous flies and when they are reared at 18°C or when given paromomycin. Interestingly, the lifespan of the K60R mutant was longer than the control in heterozygous flies, especially at higher temperatures and under richer diets, and at the standard rearing conditions, it correlated with an improvement of healthspan. Nonetheless, unlike most hyperaccurate mutants, the homozygous K60R flies are sensitive to paromomycin, but the heterozygous are not. Therefore, we can conclude that introducing a mutation that alters translation fidelity has very significant phenotypical effects, both in delayed development and longer lifespan, and that these effects are exacerbated by the environment.
5.4. Discussion

5.4.1. K60R reduces stop codon readthrough in *Drosophila*

In order to study the direct link between translation fidelity and ageing, we designed three different mutants based on yeast and *E. coli* studies; one had a Y152C substitution in RPS2, which would lead to a *ram* hypoaccurate mutation, and then other two substitutions in RPS23, K60R and K60T, which would render the ribosome hypoaccurate and hyperaccurate respectively (Alksne et al., 1993, Synetos et al., 1996, Gorini and Kataja, 1964a, Ozaki et al., 1969, Funatsu and Wittmann, 1972, Birge and Kurland, 1969). For this purpose, we used the CRISPR/Cas9 system; we designed ssODNs with the specific substitutions and a silent mutation leading to a restriction site, and together with our guiding RNAs they were injected in embryos of germline-expressing Cas9 flies (Hsu et al., 2014). We had to screen for positive candidates within the progeny of these flies; unfortunately, we were able to find only flies with the RPS23 K60R mutation. The lack of positive candidates for RPS23 K60T or RPS2 Y152C could be due to either a lethal effect of these mutations in the flies, a detrimental effect in development that made them be outcompeted by the wild-type flies, or because the inefficiency of the CRISPR/Cas9 system requires more F1 progeny to be screened. Thus, we are planning to repeat the same process to obtain other accuracy mutants such as RPS23 K60N or K60Q (Kalapala et al., 2010, Alksne et al., 1993, Synetos et al., 1996).

The flies we obtained in our laboratory bear a lysine to arginine substitution at K60 in RPS23, which was first identified in *E. coli* RPS12 K42R (together with RPS12 K42N, K42Q and -K42T) to be hyperaccurate due to its streptomycin resistance (Gorini and Kataja, 1964a, Funatsu and Wittmann, 1972). These mutations are in the *rpsL* gene (encoding for RPS23) in a region, KQPNSA, with a highly conserved amino acid sequence among evolutionary distant organisms, and interestingly, it has been observed that some organisms, such as *S. enterica*, *E. amylovora*, *M. tuberculosis*, *T. thermophilus* and *X. oryzae* naturally acquire streptomycin resistance by introducing a Lys to Arg mutation in this region (Bjorkman et al., 1999, Bjorkman et al., 1998, Chiou and Jones, 1995, Dobner et al., 1997, Gregory et al., 2001, Zhang et al., 2015, Finken et al., 1993). The first translation fidelity characterization of these mutations showed that in *E. coli* K42R mutants, although protected against streptomycin, did not have reduced stop codon readthrough nor misincorporation (Bohman et al., 1984). Bohman et al. measured nonsense errors using an assay in which the *lacZ* gene (coding β-galactosidase) and the *lacI* gene (coding a β-galactosidase-repressor) are separated by a UGA stop codon and
hence stop codon readthrough can be measured by the activity of the enzyme. Misincorporation was measured by a cell-free poly (U)-directed phenylalanine to leucine misincorporation assay, in which purified ribosomes are mixed with synthetic mRNA and other reagents necessary for poly (U) directed translation of the radio-labelled Leu and its near-cognate Phe (Bohman et al., 1984). Nevertheless, the experiments by Bohman et al. showed that the K42N mutation did not have effects on accuracy either; however, later studies found that the same mutation in Salmonella Typhimurium and Bacillus subtilis decreases the level of errors quite significantly (Bjorkman et al., 1999, Inaoka et al., 2001). In both these studies, nonsense suppression was also measured by the lacI-lacZ fusion gene β-galactosidase assay, but Inaoka et al., measured not only UGA but also UAA and UAG stop codons; in addition, Inaoka et al. measured misincorporation in the cell-free poly (U)-directed Phe to Leu assay.

Interestingly, in the experiments by Inaoka et al. they found that the K56R mutation (the equivalent of K42R for B. subtilis), had no difference with the wild-type for UAG and UAA stop codons readthrough or misincorporation but it had higher rates of UGA readthrough (Inaoka et al., 2001). Furthermore, based on these bacterial studies, the same accuracy mutations were introduced in yeast on a plasmid. The modeled mutations, RPS23 (formerly referred as RPS28 in yeast) K62T, K62N and K62Q showed increased translational accuracy whereas, unexpectedly, K62R showed it to be decreased as measured by the lacZ-based β-galactosidase UGA stop codon readthrough assay (Alksne et al., 1993, Anthony and Liebman, 1995, Synetos et al., 1996).

The differences in accuracy amongst these rpsL mutants have been proposed to be due to the way these amino acids interact with streptomycin and the 16S rRNA (18S rRNA in eukaryotes). Streptomycin binds the phosphate backbone of 16S rRNA at four sites, through salt bridges and hydrogen bonds, and it also contacts RPS12 K42; the hyperaccurate mutations K42N, K42T or K42Q destabilize these bonds reducing the affinity for streptomycin and, moreover, when the antibiotic is not present they also destabilize the contacts with helices 18 and 27 of 16S rRNA, making the ribosome a harder proof-reader and hence hyperaccurate (Carter et al., 2000, Kalapala et al., 2010, Sharma et al., 2007). Similarly, it has been proposed that K42R also disrupts the hydrogen bonds between RPS12 and streptomycin, conferring resistance, but it does not disrupt the salt bridges with the 16S rRNA when streptomycin is not present and hence it does not increase accuracy (Kalapala et al., 2010, Carter et al., 2001).

Unexpectedly, when we studied the level of stop codon readthrough in our heterozygous mutant RPS23 K60R flies, they had significantly lower rates of this type of error at both young and old ages than the control WT flies. This lysine to arginine mutation is often
considered hypoaccurate because of the results by Bohman et al. in *E. coli* and by the Liebman and Alksne laboratories in yeast (Bohman et al., 1984, Alksne et al., 1993, Synetos et al., 1996); however, it is important to remark that there is another study showing K42R is an accurate mutant in *E. coli* (Chumpolkulwong et al., 2004). In that study, K42R had misincorporation rates that were significantly lower compared to the wild-type control, albeit higher than the rest of K42 mutants tested, suggesting K42R is a mild accuracy mutation. Both Bohman et al. and Chumpolkulwong et al. used the Leu to Phe poly(U)-directed misincorporation system; however, these two studies used different media, M9 minimal medium or 2x YT (yeast and tryptone) respectively (Bohman et al., 1984, Chumpolkulwong et al., 2004). This could be a critical difference, as our studies clearly demonstrate that K60R mutants are longer-lived on high yeast content food (corresponding accuracy measurements on different food types are underway). We can hypothesize that this media richer in yeast leads to increased levels of available protein and essential sterols and that in this plentiful status there is an increased energy supply, which will be favourable to hyperaccurate mutants that have lower levels of translation (Davey et al., 2012). In addition, our experiments were done with tissue extracts that had minimal processing (just lysing with a and adding the luciferases’ substrates), whereas the Leu to Phe poly(U)-directed misincorporation assay is based on the use of highly purified ribosomes in addition to artificial buffers, templates and translation-associated factors, which may not fully replicate the natural cell environment.

The discrepancies of accuracy between bacteria, yeast and flies could also be due to variances in the adjacent regions of KQPNSA between organisms that make the lysine to arginine mutation to have contrasting effects in each organism. Also, as it has been explained, the contacts made by RPS12/RPS23 with rRNA 16S (prokaryotes) or 18S (eukaryotes) are essential to translational accuracy, and these could also vary between organisms. Remarkably, the flies we tested for stop codon readthrough were heterozygous for RPS23 K60R and reared under standard conditions, in which they were longer-lived than the controls. However, the homozygous K60R flies were not longer-lived under these same standard conditions, which could mean that either the homozygous mutation results in an even more extreme hyperaccurate phenotype that becomes detrimental or that, the homozygous K60R mutation leads to hyperaccuracy such as their yeast counterparts. Here, it should be noted, that despite the contrary effects on stop codon readthrough by RPS23 K60R in yeast and flies, one of our results agrees with the results by Alksne et al. it is the fact that heterozygous female K60R flies had their lifespan unaffected by paromomycin but the homozygous K60R flies were sensitive to this aminoglycoside (Alksne et al., 1993). Therefore, to test if the homozygous are extreme...
hyperaccurates or hypoaccurates, we are making a double mutant of the translation fidelity reporter flies and the RPS23 K60R flies, so we can have homozygous flies to test.

We have not been able to measure the level of misincorporation in our flies yet, because as it has been discussed before, the bioluminescence values are too close to the background level due to the heat shock promoter that we were using. For this reason, we have changed the promoter for the stronger ubiquitin promoter and we are planning to cross this flies to the RPS23 K60R flies to also study this type of error, since us and other laboratories have shown that misincorporation and stop codon readthrough can be uncoupled and have diverse correlations with longevity (Ke et al., 2017)

5.4.2. The level of stop codon readthrough increases with age in *Drosophila*

One of the most noteworthy results obtained in this thesis is that we found that translation errors increase with age in flies. When we measured the levels of stop codon readthrough in WT flies of 10 or 60 days of age, we saw that there was a significant rise in this kind of error in the older flies. This is a remarkable result as, to the best of our knowledge, stop codon readthrough has not been measured in an aged metazoan organism yet. After L. Orgel’s ‘Error catastrophe’ theory of ageing in 1963 (Orgel, 1963), there were multiple attempts to study the level of translation errors in aged samples; however, the experiments in aged worms and flies or senescent fibroblasts did not show any accumulation of translation errors (Parker et al., 1981, Vanfleteren and De Vreese, 1994, Harley et al., 1980). The available technique at that time to measure translation errors was the 2D gel electrophoresis footprint, where errors are detected as ‘stuttering’ of protein spots, which was criticized by some scientists to not be sensitive enough (it only accounts for errors that change the polarity of the proteins) and because it requires artificial procedures and purifications that do not replicate the cell environment (Gallant et al., 1997, Holliday, 1997). In addition, it has been argued that in older studies they used extremely low concentrations of Mg$^{2+}$, which weakens the near-cognate codon recognition in comparison to the cognate codon recognition, leading to artificially elevated levels of accuracy (Wohlgemuth et al., 2010). For example, all the experiments carried by the Kurland laboratory follow a protocol in which the concentration of Mg$^{2+}$ is 5 mM (Andersson et al., 1982, Bohman et al., 1984, Bouadloun et al., 1983, Fast et al., 1987, Mikkola and Kurland, 1988, Ruusala et al., 1984), but in more recent experiments this concentration has been raised to even 14 mM (Wohlgemuth et al., 2010, Chumpolkulwong et al., 2004). In our experiments, we saw that the level of stop codon readthrough measured *in vivo* was quite
high in the wild-type flies. This is not totally surprising, as *Drosophila* has been reported to have a pervasive readthrough that is hypothesized to provide the flies with new C’ terminal sequences in existing proteins or even new proteins to adapt to stresses (Dunn et al., 2013, Jungreis et al., 2011).

More recently, using a dual-luciferase assay similar to the one used in this report, it has been shown that fibroblasts derived from longer-lived rodent species have more accurate translation than shorter-lived rodent species, and that reduced misincorporation caused by misreading of the first and second codon correlate positively with the species maximum lifespan (Ke et al., 2017, Ke et al., 2018, Azpurua et al., 2013). Interestingly, another recent study in yeast, showed that although inducing translation errors via paromomycin via a similar error-inducing drug or nourseothricin supplementation and RPS2 mutations (SUP38 and SUP38-5) significantly decreased the lifespan of *S. cerevisiae*, the basal level of stop codon readthrough and misincorporation (using the same reporters used in our experiments) did not change in control chronological ageing cells (von der Haar et al., 2017). These differences observed between aged yeast and aged flies could be explained by the multicellular organisms accumulating more errors than unicellular organisms, which could be produced by less efficient protein turnover rates or proteostasis systems. Another remark is that the heterozygous RPS23 K60R flies, which are longer lived in this condition, had the same level of stop codon readthrough in young and old ages; namely, their translation accuracy was improved compared to the WT flies. Thus, it could be that in some aged organisms the damaged proteins produced by translation errors are maintained to safe levels through costly protein quality control systems, such as autophagy, proteasomal degradation or chaperone-mediated refolding, but in others, these systems cannot efficiently cope. Therefore, improving translation accuracy could result in less pressure to the protein quality control system and better invested cellular energy; this can be inferred from comparisons between longer-lived organisms within the same species, as our WT and RPS23 K60R flies, or studies such as the one by Ke et al. in rodent fibroblasts (Ke et al., 2017). Ke et al. (2017) did not see a correlation between longevity and stop codon readthrough; nonetheless, it could be that the heterozygous K60R flies have also improved mistranslation. In fact, the results obtained in S2R+ cells treated with different drugs (discussed in Chapter 4.3.), show that in *Drosophila* it is drugs that extend lifespan also improve both stop codon readthrough and misincorporation. Thus, we have already generated flies that have the luciferase-based misincorporation reporters under a stronger promoter, we have used a reporter that has an H245R mutation in the active site of firefly, meaning that a CGC (Arg) misincorporation to CAU or CAC (His) has to
happen to render the luciferase active again. In addition, to test if there the proteostasis systems are under less pressure in our RPS23 K60R flies, we are planning to check the levels of autophagy, which we can do by immunoblotting against Atg8-I and Atg8-II or p62. In addition, we are planning to study the refolding capacity in these mutants by using a refolding-luciferase based assay.

### 5.4.3. K60R delayed development

We aimed to characterize the phenotype of our RPS23 K60R mutant, and since minute mutants are developmentally delayed (Marygold et al., 2007, Dunn, 1937) we looked at their developmental rates, quantified by the time in which adult flies emerged from the pupae. We saw that at 25°C under standard 1 SYA food, the flies with the K60R mutation were delayed when the mutation is homozygous o heterozygous, and this holds true for both females and males. The delay in developmental rate seen in minute mutants varies in degree depending on the haploinsufficiency and it has been shown to vary at from 0.7 days to 3.5 days at 25°C (Schultz, 1929, Bridges et al., 1923, Dunn, 1937). A more recent study has suggested that the developmental delay, amongst other phenotypes, seen in minutes are due to a signal sent by impaired ribosomal biogenesis, possibly through RPS12 (Drosophila’s nomenclature), that elevates the expression of Xrp1 transcription factor (Lee et al., 2018). This developmental delay attributable to a reduction of translation has also been recently reported in longer-lived C. elegans (Dalton and Curran, 2018). Moreover, hyperaccurate mutants also have slower growth rates, which have been linked to slower translation elongation rates and less efficient translation (Ruusala et al., 1984, Tubulekas and Hughes, 1993, Nagel and Chan, 2006, Gregory et al., 2001).

In addition, we looked at fecundity by monitoring the number of eggs laid by the mutants and the controls, and we also studied the effects of these mutations and the environment in larval development, by calculating the pupae: eggs and the flies: pupae ratios. We saw that at 25°C under standard 1 SYA food the number of eggs was reduced in the homozygous K60R flies but not in the heterozygous flies. This means that the detrimental effect on fecundity is restored in the heterozygous state. However, heterozygous RPS23 K60R flies had a lower ratio of pupae: eggs than the control and the RPS23 K60R PvuII flies. This difference in RPS23 K60R flies as a result of the silent PvuII restriction site could be due to several reasons; first, the modification in the codon we selected could be having a detrimental effect because of different codon usages that can alter the speed or efficiency of the translation of this specific leucine, but this is unlikely, since both CTC (the original codon) and
CTG (the one introduced to give the PvuII restriction site) have extremely similar theoretical and experimental codon optimality measurements (i.e. CTC scores 1 and CTG 0.98) (Acevedo et al., 2018, Shields et al., 1988). Another possible cause of the variances between these two mutants is the breeding process of these flies; for the RPS23 K60R flies we have 6 independent families that were backcrossed independently into the wDah background and are kept separate, whereas for RPS23 K60R PvuII we only have two. Thus, if there is any spontaneous mutation or CRISPR off-target mutation in any other locus in one of the two families, it will have a very strong effect in the phenotype of the RPS23 K60R PvuII experimental flies (which are generated by mixing both families). For this reason, we are now testing the lifespan and healthspan of every individual line.

Since very early studies in *Drosophila*, it has been known that many mutations and their phenotypes are more altered by external conditions than the wild-type, and accordingly, we decided to see if temperature and diet could vary the developmental delay and fecundity status of the RPS23 K60R mutant (Dunn, 1937, Bridges et al., 1923). The homozygous RPS23 K60R flies at 30°C had a shorter developmental delay than at 25°C. In the heterozygous RPS23 K60 flies there was a very slight delay compared to the controls. Furthermore, at 30°C, the number of eggs, the pupae: egg or the flies: pupae ratios were not altered in comparison to the controls by neither the homozygous nor the heterozygous K60R substitution; therefore, the accuracy flies were less affected at this temperature. This means that, although the homozygous were less affected at higher temperatures than at the optimal 25°C, this was even less pronounced in the heterozygotes and conversely, at 18°C the delay was exacerbated. In literature, it has been reported that there is a variation between the delays in adult emergence of minutes too: the minute mutants that both minute mutants and hyperaccurate mutants are often also more affected in their growth rates at lower temperatures (Sinclair and Kaufman, 1984, Isono et al., 1976, Sakka et al., 1987). This could be due to the slower and reduced translation rates seen in colder temperatures affecting mutants that have reduced protein synthesis even more, and on the contrary, higher temperatures that lead to higher rates of translation being better tolerated in the hyperaccurate mutants.

In addition, we checked how a poorer 0.5 SYA diet could affect the developmental delay of RPS23 K60R. We saw that both homozygotes and heterozygotes females and males were slightly less delayed than at the standard 1 SYA diet. This is in accordance with what has been seen in hyperaccurate *E. coli* mutants, whose growth rates were less inhibited in poor media than in rich media (Mikkola and Kurland, 1988). They also saw that neither translation errors nor elongation rates depended on the media and that hence bacteria were
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

compensating for the loss of efficiency in poorer media through other mechanisms. Moreover, although the controls, the homozygous K60R and heterozygous K60R laid similar amounts of eggs in this poorer diet, the ratio pupae: eggs was significantly reduced in both homozygous and heterozygous RPS23 K60R flies compared to the controls. This is not caused by mating or fertility problems, as the same flies were used for assays at 1 and 2 SYA food where such a severe developmental problem was not observed. This means that despite fecundity not being affected by this diet in the accuracy mutants, the K60R larvae were hypersensitive to the poor yeast content and could not reach pupation as well as the controls; nevertheless, the fewer flies that reached adulthood emerged with a shorter delay compared to the controls than when reared at standard conditions. This could be due to the higher translation requirements of larvae, which could not be met by the lower efficiency of translation by ribosomes with an RPS23 K60R mutation in addition to the lowering of protein synthesis caused by nutrient deprivation. Codon usage bias is a phenomenon that occurs across all taxa, and it is characterized by the preferential use of certain synonymous codons over others and it has been related to an evolutionary advantage towards higher translation speed, with extreme bias happening in the translation of genes that require higher speed (Brandis and Hughes, 2016). Interestingly, it has been found that Drosophila has the highest codon usage bias in larvae, which require the highest de novo protein synthesis (Vicario et al., 2008). Furthermore, our results are in accordance to the explanation given by Mikkola and Kurland, who argued that the compensation to the reduced efficiency by the hyperaccurate bacteria in poorer media would be through a global increase in protein synthesis that would exceed the requirements (Mikkola and Kurland, 1988). As a result, maybe the RPS23 K60R larvae invest ample energy in compensating for the lower efficiency and that is why most of them cannot reach pupation, but the ones that do are not so developmentally delayed. We will be able to explore this theory when we perform immunoblots against phospho-4EBP and phospho-S6K in these flies and larvae.

Finally, we tested the effect of the error-inducing drug paromomycin. Interestingly, the developmental delay was intensified in females and males with the homozygous RPS23 K60R mutation. This had already been observed with the yeast equivalent RPS23 K62R mutants, and other hyperaccurate mutants, which were hypersensitive to paromomycin and their growth was even further reduced (Synetos et al., 1996, Panopoulos et al., 2004). As expected, in the heterozygous K60R flies, the developmental rates were much less affected by paromomycin than in the homozygous flies. Usually, a detrimental effect by paromomycin is considered to be due to a cumulative effect of translation errors, and although it is possible that the
The role of decreased protein synthesis in delaying ageing  

Martínez Miguel, V.E.  

homozygous RPS23 K60R flies are hypoaccurate, this is an unlikely result due to heterozygous K60R flies having shown to be hyperaccurate. Thus, another possibility is that paromomycin is inhibiting translocation, as it was found to be by Tsai et al., and that this together with the reduced efficiency of K60R flies is inhibiting growth (Tsai et al., 2013).

In addition, the pupae: egg ratio was reduced when paromomycin was supplied in both homozygous and heterozygous K60R flies but not in the K60R PvuII homozygous flies. Again, this means that the K60R larvae are hypersensitive to paromomycin, independently of being homozygous or heterozygous for the mutation. A reason why there are some differences in the effect of paromomycin on the K60R and K60R PvuII flies could be that the K60R PvuII flies having developed a suppressor mutation that is affecting the binding of paromomycin to the ribosome or to 18S rRNA. Although the levels of stop codon readthrough were measured in both sets of mutants, the developmental assay for the flies treated with paromomycin was performed two months after the level of translation fidelity had been tested, therefore this suppressor mutations could have arisen. Therefore, we are planning to repeat these results with more flies to see if these effects replicate.

5.4.4. K60R can extend lifespan and improve healthspan in a condition-dependent manner

The main aim of this thesis was to study how altering translation fidelity affects ageing, so our RPS23 K60R flies can give us an insight into the link between translation accuracy and lifespan. Under standard conditions, 25°C and 1 SYA food, we saw that the homozygous K60R female flies lived the same than the control, whereas the lifespan of the males was shorter; however, the lifespan of both females and males K60R PvuII was shorter than the control. Therefore, the silent PvuII mutation or the breeding circumstances of this mutant can influence the lifespan of these flies. It is also interesting that there is a sexually dimorphic effect of the homozygous K60R mutation since males were shorter lived, but females were not. We can hypothesize that this sexually dimorphic phenotype could be a result of different translation and accuracy requirements for each sex; if homozygous K60R mutants are extremely hyperaccurate (even more than the heterozygotes) this will be accompanied by a less efficient translation, meaning that males cannot cope with reduced translation. On the other hand, if these homozygous RPS23 K60R flies are hypoaccurate, the errors might be affecting more the male flies than the females. We will find out which of these two is the correct hypothesis when we test the level of translation fidelity in the homozygous mutants. Curiously, this sexually dimorphic effect was also seen in the heterozygous flies under standard conditions.
conditions: K60R and K60R PvuII female flies were long-lived whereas the males lived the same than the controls. This is important, because the fidelity data we have is for the heterozygous flies in this standard 25°C 1 SYA condition, and it showed that both K60R and K60R PvuII female flies are more accurate than the control which means that hyperaccuracy leads to improved lifespan, at least, in females.

The sexually dimorphic effect of lifespan-extending interventions has been previously reported; dietary and caloric restriction lead to a more pronounced effect on the female lifespan (Nakagawa et al., 2012), this has been explained by the different energetic cost of reproduction in each sex, being higher in female flies (Partridge et al., 2005, Maklakov et al., 2009). This has been reinforced by the observation that caloric restriction leads to reduced fecundity, which has been explained as a shift of energy resources allocated to somatic maintenance rather than reproduction (Chapman et al., 1998, Partridge et al., 2005). Nevertheless, this sexually dimorphic effect can be reversed when the lifespan is extended not by dietary restriction but by lowering the level of dietary protein, even if fecundity is still more reduced in females than in males (Jensen et al., 2015). It should be clarified at this point too that the lifespan extension seen in our female heterozygous RPS23 K60R flies is not due to a reduction in fertility, as the number of eggs laid by these flies was the same as the WT; however, the homozygous K60R flies laid significantly fewer eggs than the controls and their lifespan was reduced.

Another interesting remark is that, as it has been discussed in the Chapter 3.3. section, in yeasts, humans and Drosophila, RPS23 is naturally hydroxylated in the proline belonging to the highly conserved KQPNSA region (Loenarz et al., 2014, Katz et al., 2014, Singleton et al., 2014). This hydroxylation leads to newly synthesized RPS23 forming a complex with Nro1 (not found in metazoa) and Ofd1 (Sudestada1 in Drosophila) leading to the nuclear localization of the r-protein and its subsequent incorporation in the 40S ribosomal subunit (Clasen et al., 2017). Therefore, there is a possibility that our mutation at K60 could be affecting this hydroxylation and hence leading to a reduction in ribosomal biogenesis and therefore a lifespan extension through a reduction of protein synthesis. For this, it would be interesting to study ribosomal biogenesis by either measuring the levels of different rRNAs through RT-qPCR or northern blots (Sanchez et al., 2016). It would also be informative to see the polysome profiling of these flies where the proportion of 40S, 60S and 80S can be studied (Chasse et al., 2017).
We also assessed the healthspan of these flies by measuring the decline in their locomotor behaviours under standard conditions and we saw that both homozygous and heterozygous K60R flies performed better than the controls. Therefore, this mutation leads to improved locomotor behaviour, which is coupled with improved lifespan in the heterozygous K60R flies, but it is not with the homozygous flies. This improvement in negative geotaxis could be linked to the extreme sensitivity of the neurological system to translational errors (Kapur and Ackerman, 2018). Increases in frameshifting, nonsense mutations and missense suppression have been linked to neurological diseases in patients and model systems as a consequence of mutations in aminoacyl-tRNA synthetases, elongation factors and ribosomal proteins (de Ligt et al., 2012, Inui et al., 2016, Lam et al., 2016, Lopes et al., 2016, Nakajima et al., 2018, Nakajima et al., 2015, Paolini et al., 2017, Sekiguchi et al., 2018, Nakayama et al., 2017, Brooks et al., 2014, Klauck et al., 2006, Thevenon et al., 2015). Additionally, there could be a link between neurological age-related diseases or the natural decline in cognitive capacity and translational fidelity, but it remains unexplored.

In addition, we looked at the effect of higher temperatures on the lifespan of these flies, and we saw that both female and male homozygous K60R were unaffected this time. This shows that, as we deduced from the developmental assay, at 30°C, this mutation does not affect the flies negatively, even males. Importantly, the heterozygous K60R and K60R PvuII female flies and the K60R male flies were markedly longer lived than the control at 30°C. Again, we see an effect of the silent PvuII mutation, but only in the male flies, which could again be explained by the rearing conditions of these flies or by suppressor mutations having spontaneously arisen. Even more interesting is how at elevated temperatures hyperaccuracy has a protective effect even in males. This again supports the hypothesis that male flies have less capacity to cope with lower translation efficiency and that only in conditions where translation is higher, then, hyperaccuracy can be beneficial. In fact, accuracy and thermoresistance are tightly linked because both hyperaccuracy and slower elongation speeds produce reduced levels of misfolding, which is particularly protective under heat shock (Gingold and Pilpel, 2011, Lee et al., 2006, Siller et al., 2010, Hekman et al., 2012, Hawer et al., 2018, Crombie et al., 1992), and hypoaccuracy can lead to heat sensitivity (Kimata and Kohno, 1994, Hawer et al., 2018). Furthermore, if the hyperaccurate mutants spend less energy correcting and degrading mistranslated polypeptides, more energy can be spent in chaperones and the heat-shock response (Gingold and Pilpel, 2011). Nevertheless, the homozygous K60R flies did not live longer at elevated temperatures, which could mean that either the translation efficiency is so low owing to hyperaccuracy, that it cannot suffice the organism when its
requirement for protein synthesis is higher, or that the homozygous state of RPS23 K60R renders translation inaccurate, but we would expect this to have a detrimental effect at higher temperatures.

We also tested the effect of the mutation in female flies fed a poor or a rich diet because the translation efficiency requirements could vary in flies, although older studies in bacteria show that there is no difference in the levels of translation accuracy in richer or poorer diets (Mikkola and Kurland, 1988). We saw that the homozygous K60R female flies lived shorter than the control under the poor 0.5 SYA diet, but contrarily the lived longer than the control under the richer 2 SYA food. The heterozygous RPS23 K60R flies, however, lived the same as the controls and K60R PvuII lived slightly shorter than the controls in the 0.5 SYA food and longer than the controls in 2 SYA food. Again, in the poorer conditions there is an exacerbated detrimental effect on the homozygous flies, this could be due to the same reason as K60R flies lived longer at higher temperatures; in a richer medium, there is a higher level of protein synthesis, and therefore any effect of accuracy will be more accentuated than when translation is lower. On the contrary, it has been shown that the main response to nutrient deprivation is a decrease in translation, by both degrading ribosomal proteins and halting translation through mTOR and the general amino acid control pathway (GAAC) (Spriggs et al., 2010, Davey et al., 2012); thus, if the homozygous K60R flies are extremely hyperaccurate, their lower levels of protein synthesis might not suffice the organism under nutrient deprivation. On the other hand, it has been shown that hypoaccurate E. coli mutants were unable to respond to the stringent response because of a reduction in the level of deacylated tRNAs, which are effectors of this response (Bullwinkle and Ibba, 2016). Thus, if the K60R homozygous mutants are hypoaccurate they would be misincorporating amino acids and reducing their deacetylated tRNA pools too.

These shifts of lifespan behaviour depending on food are not unusual, some good examples are flies with overexpressed dFOXO, which have only extended lifespan under richer diets but not in DR-like diets or chico null mutants (Drosophila’s insulin receptor substrate) which under restricted diets live shorter than the controls but under richer diets their lifespan is extended (Clancy et al., 2002, Clancy et al., 2001, Min et al., 2008). Furthermore, it has been seen that NSUN5, which methylates 25S rRNA in a conserved region, increases stop codon readthrough and extends Drosophila’s lifespan in poorer media but in richer diets it can be even detrimental (Schosserer et al., 2015). Remarkably, the food that in their study was considered as ‘poor’ corresponded to media containing 22 g/l sugar 18 g/l yeast and 80 g/l corn (our poorest food, 0.5 SYA contained 50 g/l sugar and 50 g/l yeast) and the ‘rich’ media
was more similar contained 60 g/l sugar, 100 g/l yeast and 2 g/l peptone (our 1 SYA food contains 50 g/l sugar and 100 g/l yeast). Therefore, this is exactly the opposite that we see in our hyperaccurate flies, meaning that maybe the balance between translation efficiency and translation accuracy must be shifted depending on the environmental conditions to improve lifespan (Fig. 5.10.).

![Diagram showing the relationship between translation load and fitness](image)

**Figure 5.10.** Schematic representation of the accuracy requirements for the best fitness depending on the translation load. In normal conditions, for example, when nutrients are neither scarce nor abundant and the temperature is optimal for normal metabolism, the translation load is ‘medium’ and to have the best fitness accuracy should also be in a medium range. However, when translation is naturally lower owing to, for example, scarce nutrients or lower temperatures, it is better to have lower translation accuracy to obtain longer lifespans. In the opposite direction, if the translation is high because of elevated temperature or ad libitum nutrients and rich diets, hyperaccuracy is beneficial. Therefore, evolution will favour a level of accuracy that keeps the organism the fittest depending on the environment.

Finally, we tested the resistance of these flies to the error-inducing drug paromomycin by assessing the survival of the female homozygous and heterozygous RPS23 K60R and WT flies on this antibiotic. We saw that homozygous RPS23 K60R flies were very sensitive to this drug, but heterozygous were unaffected. This replicates the effects of the drug on the developmental delay too, in which homozygous larvae were very sensitive to this drug. Even though resistance to another error-inducing drug, streptomycin is usually an indicator of increased accuracy, this is not the case with paromomycin. In fact, a large screen of omnisuppressor (i.e. hypoaccurate mutants) showed that these could be from resistant to hypersensitive to paromomycin and that this phenotype was totally independent of the degree
of accuracy (Wakem and Sherman, 1990). This was further explored in yeast with a mutation in the 25S rRNA (C2658U, termed rdn5), that is hypoaccurate but resistant to paromomycin and that achieves these two phenotypes by disrupting the sarcin/ricin loop of the 25S rRNA and hence its interactions with RPS23, eEF2 and paromomycin (Panopoulos et al., 2004, Liu and Liebman, 1996). Thus, it has been proposed that the rdn5 mutation alters the normal interaction between 25S rRNA and eEF2 and hence it prevents paromomycin from accelerating the accommodation and GTP hydrolysis steps of translocation (Panopoulos et al., 2004). Moreover, paromomycin and ram mutations have been shown to exploit the thermodynamics of the initial selection stage of elongation (lowering the necessary energy for discrimination), whereas the restrictive mutations act through the proofreading phase of elongation (increasing rejection) (Zhang et al., 2018, Zaher and Green, 2010). This difference between the effect of paromomycin and streptomycin is also highlighted by the fact that streptomycin directly binds prokaryotic K42 whereas paromomycin does not (Panecka et al., 2014). Furthermore, paromomycin interacts with 18S rRNA, and it has been shown that the susceptibility to this drug and its misreading effects are influenced by this interaction (Fan-Minogue and Bedwell, 2008). Therefore, the RPS23 K60R mutation (which directly interacts with 18S rRNA in C912 (E. coli nomenclature) in flies could be rendering the structure of the decoding centre more susceptible to paromomycin, independently of the higher accuracy of the K60R mutants under standard conditions. In fact, a study in extreme thermophile archaea, including S. solfataricus which we found using Ensembl BAST that it has arginine in the homologous position of our mutation, are hyperaccurate and unaffected by streptomycin but sensitive to paromomycin (Londei et al., 1988)
Chapter 6: General discussion
An evolutionarily conserved mechanism of lifespan extension is the reduction of protein synthesis, either by downregulating mTOR, by using translation inhibitors or by genetically downregulating translation factors or ribosomal proteins (Hansen et al., 2007, Curran and Ruvkun, 2007, Hamilton et al., 2005, Henderson et al., 2006, Kaeberlein et al., 2005, Steffen et al., 2008b, Steffen et al., 2012, Kapahi et al., 2004, Pan et al., 2007, Rogers et al., 2011, Zid et al., 2009). In this thesis, I aimed to study if downregulation of translation improves longevity through an increase in translation fidelity. For this purpose, first, I carried out a focused RNAi longevity screen, in which I found that downregulating in adult flies eIF4E, eEF2 and RPS23 could extend their lifespan and health span. Then, I studied the effect of several drugs on translation fidelity and I saw a correlation between reduced mistranslation and improved ageing. Lastly, I studied how directly altering translation could affect longevity and I found that introducing an amino acid substitution in the highly conserved RPS23 resulted in developmental delay and a condition-dependent lifespan extension.

One of the main parts of this thesis was to find if downregulating translation by RNAi can extend lifespan as it does in C. elegans and yeast (Syntichaki et al., 2007b, Steffen et al., 2008b, Hansen et al., 2007, Curran and Ruvkun, 2007, Hamilton et al., 2005, Pan et al., 2007). Successfully, we found several translation-associated proteins that can extend lifespan in flies when RNAi is used in the metabolic tissue, despite seeing that under the ubiquitous act-GS driver, most of the RNAi lines were shorter-lived, signifying the importance of appropriately downregulating translation in Drosophila. Nevertheless, we focused on eIF4E, eEF2 and RPS23, which consistently extended lifespan when downregulated in the metabolic tissue. As it has been discussed, there are many reasons why they can be long-lived, and unfortunately, we have not been able to corroborate yet that the main cause of this longevity effect is improved fidelity. Nevertheless, there are possible links between these proteins and improved fidelity. Downregulating elf4E or elf4G had totally opposite effects, the first extending lifespan and health span the second shortening them. These effects can be caused by a plethora of mechanisms, ranging from the suppression of Cap-independent mechanisms in elf4G RNAi flies that are still allowed in elf4 RNAi flies and consequently regulate the expression of genes involved in stress-resistance and longevity (Ramirez-Valle et al., 2008, Hernandez et al., 2004, Marr et al., 2007, Howard and Rogers, 2014b) to the differential translation of mRNAs with certain structures (Zid et al., 2009, Acevedo et al., 2018) or decreased ribosome stalling (Tuller et al., 2010). Nevertheless, if following our hypothesis, downregulating elf4E improves translation accuracy, this will happen through indirect ways; lowering elf4E could be triggering a factor, such as Ctk1, that increases translation fidelity and regulates canonical translation.
through its interaction with initiation factors that have not been identified yet (Coordes et al., 2015, Rother and Strasser, 2007). Another possibility is that downregulating eIF4E but not eIF4G could be leading to improved PABP1 function, which can promote translation errors when overexpressed and that interestingly, is naturally downregulated in elderly (Cosson et al., 2002, Roque et al., 2015, Anvar et al., 2013).

Similarly, eEF2 RNAi could be extending lifespan through several mechanisms that we have not pinpointed yet. One of these could be that eEF2 triggers the differential translation of certain genes that can improve longevity, by for example altering gene expression or promoting the translation of mRNAs with weaker mRNA Kozak sequences (Acevedo et al., 2018). Lowering eEF2 could also improve folding capacity by slowing down elongation speed (Rodnina et al., 2017, Siller et al., 2010), as for example cycloheximide, an elongation inhibitor can do (Meriin et al., 2012a, Meriin et al., 2012b), in addition, protein folding could be enhanced by a decrease is mistranslation. This is feasible since eEF2 has been directly implicated in the generation of frameshifting errors, both by antibiotics (Dinman et al., 1997, Harger et al., 2001) and by deletion of a highly conserved histidine to diphthamide modification (Hawer et al., 2018, Liu et al., 2012, Ortiz et al., 2006, Uthman et al., 2013)

Likewise, slower elongation speed is one of the main trade-offs of translational fidelity (Wohlgemuth et al., 2010), meaning that tuning speed can also be a way of tuning fidelity. Importantly, rapamycin, which improved misincorporation and stop codon readthrough in our experiments and others, has been proposed to do so by a slowing of elongation through eEF2K (Conn and Qian, 2013).

The last factor that when downregulated extended lifespan in our screen was RPS23. The most apparent cause behind the longevity effect of this RNAi line could be due to a global downregulation of translation by impairing ribosome biogenesis. This is supported by depletion of RPS23 leads reduced ribosomal biogenesis in humans (Fumagalli et al., 2009) and that none of the minute mutants has been found to be caused by RPS23 haploinsufficiency suggests that the importance of this protein is such that ribosomes do not form without it (Marygold et al., 2007, Genuth and Barna, 2018); thus, S106>RPS23 most likely have lower levels of global translation Nevertheless, this still does not explain how downregulating protein synthesis can extend lifespan. Steffen et al. explained the extended lifespan of their yeast with deleted ribosomal proteins as the cause of GCN4 activation (Steffen et al., 2008b). In addition, it has been proposed that decreasing translation, the most energetically expensive cellular process, provides the cell with energy that can be spent in other repair mechanisms (Kaeberlein and Kennedy, 2008).
Furthermore, recently RPS23 has been also proposed to be a sensor of nutrient availability that affects the regulation of the hypoxic response and sterol metabolism (Clasen et al., 2017). When RPS23 levels are down, Ofd1 is free to sequester the Sre1 transcription factor, leading to a shift in lipid metabolism that matches nutrient deprivation. Thus, RPS23 downregulation could be a way of mimicking dietary restriction. Interestingly, the hydroxylation of RPS23 by Ofd1 (Sudestada1 in *Drosophila*) is a safe keeper of translation fidelity and it happens at P62 (*Drosophila’s* nomenclature) in yeast, flies and humans (Katz et al., 2014, Loenarz et al., 2014, Singleton et al., 2014). Remarkably, this residue is the proximity of K60, which is the amino acid that we engineered by CRISPR/Cas9, reinforcing the importance of this protein in translation accuracy. Interestingly, when paromomycin was given to S1106>RPS23 RNAi flies, they were not longer-lived anymore, and this treatment had a greater effect on this mutant than on the controls, which suggests that the lifespan extension probably occurs through an improvement in translation fidelity.

RPS23 has been a central protein in this thesis, as it forms part of the accuracy centre and it has been widely characterized to be involved in translation accuracy (Anthony and Liebman, 1995, Bohman et al., 1984, Chumpolkulwong et al., 2004, Finken et al., 1993, Funatsu and Wittmann, 1972, Inaoka et al., 2001, Panecka et al., 2014, Sharma et al., 2007, Synetos et al., 1996, Zaher and Green, 2010, Gorini, 1966, Gorini, 1969). We were able to introduce a Lys→Arg substitution at K60 in this protein, which according to some studies should have led to hypoaccuracy (Bohman et al., 1984, Alksne et al., 1993), but to others to no effect or to hyperaccuracy (Chumpolkulwong et al., 2004, Inaoka et al., 2001). This indicates that the effect of this mutation on accuracy is highly condition-dependent and affected by the heterozygous and homozygous states. Interestingly, when we tested the level of stop codon readthrough, we saw that this was reduced in the mutant flies compared to the wild types; as it has been discussed in more detail in Chapter 5.3., this could be caused by structural modifications that alter the interactions of this residue between organisms, or just because of the different experimental procedures used. Nonetheless, here it should be remarked that RPS12 K42R mutation (*E. coli* nomenclature) was considered (and it can still be, depending on the organism/context) to be hyperaccurate because it confers streptomycin resistance in a variety of prokaryotes (Gorini and Kataja, 1964b, Gregory et al., 2001, Bjorkman et al., 1999, Chiou and Jones, 1995, Dobner et al., 1997, Finken et al., 1993, Zhang et al., 2015), but it did not against paromomycin in *S. cerevisiae* (Alksne et al., 1993, Synetos et al., 1996) and neither in our flies. In fact, homozygous RPS23 K60R were sensitive to this aminoglycoside, both at their development and in the survival analysis, but heterozygous were unresponsive. However, it is
important to mention that paromomycin, on the contrary to streptomycin, does not interact directly with K60 and that not only interacts with the interface between RPS23 and 18S RNA, as streptomycin does with prokaryotic RPS12 K42 and 16S RNA, but it also induces the closed conformation of the decoding site by activating the activity of GTPase through binding 23S rRNA in helix 69 (Rodnina et al., 2017, Kalapala et al., 2010). This implies that maybe the K60R eukaryotic mutants are hyperaccurate but hypersensitive to this aminoglycoside, or that on the other hand, their hyperaccuracy is not strong enough to compensate for the error-inducing effects of paromomycin.

Importantly, we saw that the heterozygous RPS23 K60R flies were longer lived and had improved locomotor behaviour than the wild-type under standard rearing conditions, on rich 2 SYA food or at 30°C, which implies that increasing translation fidelity (at least stop codon readthrough) can extend lifespan under certain environmental conditions. This is also in accordance to our data in S2R+ cells, which has shown a correlation between lifespan-extending drugs (i.e. rapamycin, trametinib and Torin 1) and reduced stop codon readthrough and misincorporation (at the second codon). Although this is slightly in disagreement with the studies by Ke et al. who saw a correlation between increased longevity and reduced misincorporation (at the first and second codons), but not stop codon readthrough in rodents (Ke et al., 2017), it does not mean that in Drosophila, or even insects, improving stop codon readthrough together with misincorporation is a mechanisms behind healthier ageing. Furthermore, another indication that if this type of error can be ameliorated, an improvement in ageing can be achieved, is that the mutant K60R flies had the same level of stop codon readthrough when measured in young and old flies, whereas the wild-type flies had a significant age-dependent increase of this type of error. This, in addition, is one of the most exciting results obtained in this thesis as the level of mistranslation has not been appropriately measured in an aged metazoan organism yet (Ke et al., 2018). Interestingly too, the level of stop codon readthrough that we detected in vitro was much lower than what was obtained in vivo, which could mean that the effects of any fidelity-improving interventions can have an exponential effect on the multicellular organism. Finally, when we combine the results of the screen for drugs that improve fidelity in vitro and lifespan in vivo and the results obtained in the measurements of fidelity in vivo wild-type flies, we can conclude that we could use the translation fidelity assay in S2R+ cells to screen for new lifespan-extending drugs and their optimal concentrations.

At this point, we can say that an improvement in translation can lead to extended lifespan; however, it can also have detrimental consequences, as both homozygous and
heterozygous RPS23 K60R mutants were developmentally delayed compared to the wild-types especially under certain rearing conditions, and in addition, the homozygous flies were longer-lived under 2 SYA but shorter-lived when fed a poor 0.5 SYA diet. This implies that translation accuracy can be beneficial under certain circumstances, e.g. elevated temperatures and richer diets, but it is detrimental in others, e.g. under nutrient starvation or colder temperature. These food-dependent contradictory effects can be attributed to the different requirements for translation efficiency. Translation is the most energetically expensive process in the cell, taking up to 50% of the cell’s energy (Sonenberg and Hinnebusch, 2009); therefore, when nutrients are scarce, the cell downregulates global protein synthesis through mTOR and the general amino acid control pathway (GAAC) (Spriggs et al., 2010). Therefore, if our flies on the 0.5 SYA diet have translation downregulated through these pathways and in addition they are translating less efficiently due to hyperaccuracy, their fitness will be compromised. This is also in accordance of the results by Schosserer et al., who saw that C. elegans and flies were longer lived with the hypoaccurate NSUN5 mutation under very poor diets but shorter lived in normal or richer diets (Schosserer et al., 2015). On the other hand, under a richer diet, the surplus energy being generated will compensate for the reduced translation efficiency and the benefits of hyperaccuracy will be exerted. These benefits include a better proteome that leads to better folding and less aggregation, removing some pressure from the protein quality control system (Komar et al., 1999, Tsai et al., 2008, Pedersen, 1984). Similarly, at higher temperatures, the stability of the proteome is reduced (Mohler and Ibba, 2017, Akerfelt et al., 2010); therefore, if translation is more accurate, the quality of the proteins will be better and hence the quality control systems will have more capacity to take care of the proteome (Kaushik and Cuervo, 2015). In fact, it has been shown that hypoaccurate mutants are more sensitive to hot temperature whereas hyperaccurate are more sensitive to cold (Isono et al., 1976, Bennoun et al., 1980, Hawer et al., 2018). Furthermore, the longest-lived animal, Arctica islandica, has been found to have an extremely stable proteome with higher resistance to stress-induced aggregation and unfolding, and it would be interesting to measure translation accuracy in this species (Treaster et al., 2014).

In the wild, these conditions of ad libitum nutrients and very rich diets are not usually encountered and therefore, evolution has not favoured the incorporation of the hyperaccurate amino acid substitutions in most species. It is actually known that the maximum possible accuracy is not achieved in nature because of a phenomenon known as the ‘trade-off’ between accuracy, efficiency and energy expenditure (Wohlgemuth et al., 2010). For example, discriminating for a stronger binding or a more frequent rearrangement would lead to higher
accuracy but at the expense of efficiency, and this has not been selected for in most organisms, as these individuals would be quickly outcompeted (Prabhakar et al., 2017). In fact, molecular evolutionary simulations have concluded that the selection for translation accuracy occurs when misfolding promotes a direct fitness cost and hence it is required to prevent toxic misfolded products (Drummond and Wilke, 2008). This could also be why we, and others, have seen thermophile archaea such as A. fulgidus, P. aerophilum, T. pendens, A. pernix, H. butylicus, S. solfataricus, C. korarchaeum cryptofilum and N. equitans and 4% of all bacterial species have Lys→Arg because this could be conferring an advantage towards protein damage by higher temperatures (Panecka et al., 2014). Despite being certain evidence of the improved accuracy of some extreme thermophilic archaea (Londei et al., 1988), the overall fidelity of these species is largely unexplored (de Koning et al., 2010). Nevertheless, there is plenty of evidence showing that in evolution across organisms, there has been a selection for more accurate translation and less misfolding (Warnecke and Hurst, 2010), less frameshifting (Farabaugh and Bjork, 1999) and fewer nonsense errors (Gilchrist et al., 2009). In addition, slower speed is beneficial when more complex secondary structures are required and to aid in co-translational folding, and indeed rarer codons that slow down elongation rates are used in proteins with these requirements (Komar et al., 1999, Tsai et al., 2008, Pedersen, 1984). Recently, it has also been shown that a hypoaccurate E. coli mutant with defective tyr-tRNA editing site was not able to respond to nutrient deprivation (Bullwinkle and Ibba, 2016).

Nevertheless, extreme hyperaccuracy has not been generally selected for, not only because of the unavoidable slowness trade-off that this would mean but because sometimes mistranslation is beneficial (Ribas de Pouplana et al., 2014). In a groundbreaking study, it was shown that HeLa cells, under oxidative stress, increase Met-misacylation that protects the cells due ROS-protective capacity of this amino acid (Netzer et al., 2009); later, it was found that this Met-misacylation occurs also in bacteria and yeast (Jones et al., 2011, Wiltrout et al., 2012). Another benefit from mistranslation is found in the pathogenic Candida albicans, which shows an especially high serine/leucine ambiguity, which has been proposed to be a way of providing this fungus with genome-wide rearrangements that can favor its pathogenicity and phenotypic diversity (Bezerra et al., 2013, Gomes et al., 2007, Miranda et al., 2013, Sarkany et al., 2014). In addition, it has been demonstrated that varied species of Drosophila have amino acid coding sequences following stop codons, meaning that readthrough is necessary for their translation (Dunn et al., 2013, Jungreis et al., 2011). This readthrough might probably be a mechanism to regulate the expression of genes that are necessary to adapt under stress conditions or produce new C-terminal extensions that provide the protein with novel functions.
Dunn et al., 2013). Therefore, to adapt to the environment, accuracy must be finely tuned, and either improved or impaired fidelity can be beneficial depending on the situation. In situations where competition is high, energy levels are low, or translation has been halted, mistranslation can be preferred; however, when nutrients are plentiful, translation is upregulated, or the stability of the proteome is imperative, misfolding imposes a special risk, then hyperaccuracy will be beneficial (Fig. X). If we think about human health, in most developed countries, food is plentiful and there is a trend towards richer diets, with obesity is a burden to socioeconomic states that has reached epidemic proportions (Wang and Ren, 2018, Hinnig et al., 2018); therefore, any anti-ageing drug that is designed to target these societies will have to be studied taking into account the effect on translation accuracy.

Figure 6.1. There is an inherent trade-off between speed and accuracy, and this has to be balanced depending on the physiological requirements. There are environmental conditions that favour an improvement in accuracy whereas there are others that favour an improvement in speed, so depending on these, and the organism will benefit from its effects or will be harmed by them.
6.2 Future work

Although we have been able to answer some questions regarding the role of protein translation and translation accuracy in ageing, there are still some important ones that remain unanswered. The main inquiry after the RNAi focused longevity screen is if these lines have improved fidelity. For this, we are making a double mutant of flies carrying the S106-Gal4 driver and the luciferase-based fidelity reporters, so we can cross them to our RNAi lines (Nicholson et al., 2008, Salas-Marco and Bedwell, 2005). In addition, now that we have set up and corroborated the efficiency of RNAi on S2R+ cells, we are planning to also test how different concentrations of dsRNA against our proteins of interest can affect translation in vitro. Furthermore, we would like to know if downregulating these translation-associate proteins also reduces the level of global translation, and for this purpose we are finishing optimizing the puromycin-based assay to measure the level of de novo protein synthesis, both in vivo and in vitro (Deliu et al., 2017). We are also planning to study the polysome profiles of these flies, which would inform us of the status of the translatome; this technique is based on the separation of polysome-associated mRNAs from free-mRNAs in a sucrose gradient, which makes it ideal for studying the total levels of translation (i.e. including UTRs) (Chasse et al., 2017). Nonetheless, if this does not work, we will measure translation by 35S-Methonine, which consists of labelling cells with the 35S-methionine radioisotope, which gets incorporated in polypeptides and allows for its quantification in SDS-PAGE (Pollard, 1994). Finally, to understand the mechanisms behind the lifespan extension of these flies, we have thought of several assays that we can perform. We could analyze the level of cap-independent IRES initiation by using a bicistronic dual-luciferase assay, similar to the one we have used to study translation fidelity, that has Renilla and firefly separated by an IRES sequence (Jackson, 2013, Van Eden et al., 2004). We could use this assay both by crossing the reporters to the flies, which would take longer as double mutants with the S106 driver are necessary, or by using it in the S2R+ cells treated with dsRNA against our proteins of interest. It would be very interesting to uncover in the long-lived flies having downregulation of eIF4E, eEF2 and RPS23, which mRNAs are being translated. To this end, it would be very informative to perform ribosome profiling (Ingolia, 2016), a technique that provides a snapshot of proteins being synthetized. This could both reveal which proteins might be accountable for the longevity, as well as it would indicate some mechanistic about translation.

We have been able to design a screen for anti-ageing drugs; thus, we are planning to test for other drugs implicated in translation and their optimal concentrations for longevity to see if they also extend lifespan in flies. Furthermore, we are planning to test if the
concentrations of rapamycin that extend lifespan in *Drosophila* also improve translation fidelity *in vivo*, as we have shown it does in the S2R+ cells.

Finally, although we have found that lowering stop codon readthrough can extend lifespan in flies, we still need to find what happens with misincorporation in the RPS23 K60R mutants. For this, we are going use the luciferase-based misincorporation reporter under the stronger ubiquitin promoter. We are also already generating a double mutant of the two luciferase-based fidelity reporters and RPS23 K60R so we can study the translation errors in the homozygous mutants. Moreover, we are planning to study both the stop codon readthrough in the accuracy mutants and the wild-types under different diets and temperatures. This will give us an insight not only of the environment can alter the fidelity of the RPS23 K60R mutants, but also how different environmental conditions affect accuracy in wild-type flies. Moreover, it will be important to see how translation efficiency is affected in the hyperaccurate flies, since there is an inherent trade-off between speed and accuracy (Wohlgemuth et al., 2010). This can be studied by using polisome profiling, and even better ribosome profiling, since this technique allows for a detailed measurement of translation almost at the nucleotide level (King and Gerber, 2016). This procedure is based on the treatment with RNase (which degrades any unprotected mRNA) of cell lysates to allow the subsequent deep-sequencing of ribosome-protected RNA fragments (Ingolia et al., 2009). This technique requires also total mRNA extractions and sequencing done in parallel so they can be normalized (King and Gerber, 2016). It is also important to note that this technique can provide insightful information about the state of both initiating ribosomes (if treated with lactimidomycin) or elongating ribosomes (treated with cycloheximide or harringtonine, termed ‘run-off’) (Ingolia et al., 2011). A new high-throughput technique called SunRISE allows for elongation rate to be measured in single-cells under their normal physiological conditions (Arguello et al., 2018). This is achieved by using puromycin and detecting it by flow cytometry or immunoblotting together with run-off ribosome profiling.

Importantly, the mechanism of how increased translation fidelity improves ageing remains unknown. One of the hypothesis is that this is due to the lower misfolding and aggregation. For this we can cross our RPS23 K60R flies to *Drosophila* models of protein misfolding disorders and study if their phenotypes are ameliorated. Many of these models take advantage of the expression in the eye of the disease-associated proteins (e.g., Huntingtin, Aβ, Tau, Atx) making a clear rough-eye phenotype when the proteins are misfolded or aggregated (Rincon-Limas et al., 2012). Furthermore, there is a refolding assay based on the refolding of firefly luciferase that we could also do both *in vivo* and *in vitro*. In this assay, the luciferase is
denatured by heat and progressive measurements of its activity are taken, which indicate if the enzyme has refolded or not (Michels et al., 1995). Moreover, we want to study the physiological status of these flies since this can indicate if they are altering certain mechanisms involved in protein synthesis; we will assess the levels of phosphorylation of eIF2α as an indication of the activation of GCN2 (i.e. effector of GAAC) and S6K and 4EBP (i.e. effectors of mTOR) (Saxton and Sabatini, 2017, Vlanti et al., 2013).
References


ARUM, O., BONKOWSKI, M. S., ROCHA, J. S. & BARTKE, A. 2009. The growth hormone receptor gene-disrupted mouse fails to respond to an intermittent fasting diet. Aging Cell, 8, 756-60.


BANKS, A. S., MCALLISTER, F. E., CAMPOREZ, J. P., ZUSHIN, P. J., JURCZAK, M. J., LAZNIK-
BOGOSLAVSKI, D., SHULMAN, G. I., GYGI, S. P. & SPIEGELMAN, B. M. 2015. An
ERK/Cdk5 axis controls the diabetogenic actions of PPARgamma. Nature, 517, 391-5.


Ribosome recycling depends on a mechanistic link between the FeS cluster domain
and a conformational switch of the twin-ATPase ABCE1. Proc Natl Acad Sci U S A, 108,
3228-33.

Tool to Target Aging. Cell Metab, 23, 1071-81.

Sci, 62, 1071-81.

BASS, T. M., WEINKOVE, D., HOUTHOOFD, K., GEMS, D. & PARTRIDGE, L. 2007b. Effects of
resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. Mech
Ageing Dev, 128, 546-52.


BECKER, T., FRANKENBERG, S., WICKLES, S., SHOEMAKER, C. J., ANGER, A. M., ARMACHE, J. P.,
SIEBER, H., UNGEWICKELL, C., BERNINGHAUSEN, O., DABERKOW, I., KARCHER, A.,
THOMM, M., HOPFNER, K. P., GREEN, R. & BECKMANN, R. 2012. Structural basis of

BECKMAN, K. B. & AMES, B. N. 1998. The free radical theory of aging matures. Physiol Rev, 78,
547-81.

BELIN, S., BEGHIN, A., SOLANO-GONZALEZ, E., BEZIN, L., BRUNET-MANQUAT, S., TEXTORIS, J.,
PRATS, A. C., MERTANI, H. C., DUMONTET, C. & DIAZ, J. J. 2009. Dysregulation of
ribosome biogenesis and translational capacity is associated with tumor progression of

BELL, G. 1984. Evolutionary and Nonevolutionary Theories of Senescence. American Naturalist,
124, 600-603.

BELL, J. 1844. On Regimen and Longevity: Comprising Materia Alimentaria, National Dietetic
Usages, and the Influence of Civilization on Health and the Duration of Life. Med Chir

BEN-SAHRA, I. & MANNING, B. D. 2017. mTORC1 signaling and the metabolic control of cell


BENEDETTI, M. G., FOSTER, A. L., VANTIPALLI, M. C., WHITE, M. P., SAMPAYO, J. N., GILL, M. S.,
OLSEN, A. & LITHGOW, G. J. 2008. Compounds that confer thermal stress resistance
and extended lifespan. Experimental Gerontology, 43, 882-891.

nuclear and chloroplast mutants of photosynthesis in chlamydomonas. Genetics, 95,
39-47.

BERG, J. M., TYMOCKZO, J. L. & STRYER, L. 2002. Protein Synthesis Requires the Translation of
Nucleotide Sequences Into Amino Acid Sequences.

BEZERRA, A. R., SIMOES, J., LEE, W., RUNG, J., WEIL, T., GUT, I. G., GUT, M., BAYES, M.,
RIZZETTO, L., CAVALIERI, D., GIOVANNINI, G., BOZZA, S., ROMANI, L., KAPUSHESKY, M.,
MOURA, G. R. & SANTOS, M. A. 2013. Reversion of a fungal genetic code alteration
links proteome instability with genomic and phenotypic diversification. Proc Natl Acad
Sci U S A, 110, 11079-84.
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


DINMAN, J. D., RUIZ-ECHEVARRIA, M. J., CZAPLINSKI, K. & PELTZ, S. W. 1997. Peptidyl-transferase inhibitors have antiviral properties by altering programmed -1 ribosomal
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

patients of a family with neurodegeneration accompanied by iron deposition in the brain. *Neurobiol Aging*, 38, 216 e11-216 e18.


258
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing


MEIGEN, J. & HOFFMANNSEGG, J. 1830. [*Diptera manuscript and drawing collection*].


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


PEARL, R. 1928. The rate of living, S.l., Univ. of London Press.

The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


272
The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


ROSENBERGER, R. 1982. STREPTOMYCIN-INDUCED PROTEIN ERROR PROPAGATION APPEARS TO LEAD TO CELL-DEATH IN ESCHERICHIA-COLI. J IRCS MEDICAL SCIENCE-BIOCHEMISTRY, 10, 874-875.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

275


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


STANFIELD, I., JONES, K. M., KUSHNIROV, V. V., DAGKESAMANSKAYA, A. R., POZNYAKOVSKI, A. I., PAUSHKIN, S. V., NIERRAS, C. R., COX, B. S., TER-AVANESYAN, M. D. & TUIE, M. F. 1995. The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in Saccharomyces cerevisiae. EMBO J, 14, 4365-73.


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.

280


The role of decreased protein synthesis in delaying ageing

Martínez Miguel, V.E.


supplementation does not improve metabolic function in nonobese women with normal glucose tolerance. *Cell Metab.*, 16, 658-64.


