Pharmacogenetics of ageing and neurodegeneration

Thesis

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Gower Street, London. 26th of September 2014.

Declaration

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

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Jorge Ivan Castillo Quan

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"As I give thought to the matter, I find four causes for the apparent misery of old age; first, it withdraws us from active accomplishments; second, it renders the body less powerful; third, it deprives us of almost all forms of enjoyment; fourth, it stands not far from death."

Marcus Tullius Cicero

I dedicate this work to my beautiful family:
my father Jorge Ivan Castillo Riverol,
my mother Ana Maria Quan Hulse, and
my sister Stacey Marie Castillo Quan

You are my strong fortress and inspiration.

I would also like to dedicate this work to the pillars of our family

My great grandparents:
Fabian Castillot
Bartola Castillot (76 years)
Marcelo Riverolt (92 years)
Franscisca Riverolt (94 years)
Alfonso Quant (73 years)
Estella Quant (101 years)
Ernesto Hulset (72 years)
Dionicia Hulset (92 yeas)

My grandparents:
Romeo Castillo (76 years)
Herlinda Castillo (76 years)
Ignacio Quan (80 years)
Norma Quan (75 years)

Age brought you wisdom and gave me the opportunity
to share special moments with you (though not with all).
I appreciate your genes, but overall
I am thankful for your love and support.

To my extensive family and friends.

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Contents

Abstract

Genetic manipulations and dietary restriction in model organisms have proven that lifespan extension is achievable. Moreover these same interventions can protect against age-related diseases and improve general healthiness. Therefore, current efforts are being put forward to identify drugs that mimic healthy lifespan. Lithium has been documented to be able to extend the lifespan of the worm *Caenorhabditis elegans*, reduce mortality in *Drosophila* and a recent report has suggested that lithium concentrations in drinking water correlate with reduced mortality for all causes in a human population. The main objective of the project presented here was to determine the anti-ageing properties of lithium using the fruit fly and to determine the mechanism by which lithium exerts its broad health benefits and anti-ageing properties. My results showed that lithium extended lifespan independent of sex and genetic background. Lithium treated flies were also resistant to multiple stressors and showed reduced triglyceride levels. Lithium did not modify fecundity and it further extended lifespan of DR flies. The pro-longevity effects seemed to also be independent of the nutrient sensing network as it could further extend lifespan of flies with reduced signalling though the insulin/IGF-1 and the mechanistic target of rapamycin (mTOR) network. I performed transcriptional and translational microarrays in lithium treated flies and found out that lithium up-regulated a transcriptional response to stress regulated by the transcription factor cap'n'collar/NRF-2 and down-regulated functional categories implicated in mitochondrial complex I. By performing epistasis experiments I found out that lithium and shaggy, the fly homologue of GSK-3, regulate ageing by acting in the same molecular pathway. However, shaggy seemed to modulate ageing in a tissue-specific manner The combination of lithium and genetic manipulations of shaggy revealed a very complex regulation of ageing and neurodegeneration.

I also explored the nature of neurodegeneration induced by over-expression of $A13_{1\_42}$ and found out that some of the changes induced by $A13_{142}$ impact general physiology and not only neurons. For example, our results suggest that altered metabolism is a prominent feature of the toxicity of $Ar3_{1\text{-}42}$ in *Drosophila.*

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Abbreviations

4E-BP	Eukaryotic initiation 4E binding protein
(3IRK0	(3-cells insulin receptor knockout
A01-42	Amyloid beta 1-42
actGS	Actin-GeneSwitch
atg	Autophagy related gene
AD	Alzheimer's disease
Akt	see PKB
AL	Autophagic-lysosomal
AMP/ATP	Adenosine monophosphatase/Adenosine triphosphatase
AMPK	AMP-activated protein kinase
APOE	Apolipoprotein E
BPD	Bipolar disorder
CncC	Cap'n'collar C
CNS	Central nervous system
daGS	Daughterless-GeneSwitch
DN	Dominant negative
DR	Dietary restriction
EAAs	Essential amino acids
eIF	Eukaryotic initiation factor
elavGS	Embryonic lethal abnormal vision-GeneSwitch
ELISA	Enzyme-linked immunoabsorbent assay
ER	Endoplastic reticulum
FGF	Fibroblast growth factor
FIRKO	Fat insulin receptor knock out (white adipose tissue)
FOXO	FOrkhead boX subgroup 0 transcription factor
GCN2	General control-non depressible 2
GH	Growth hormone
GSK-3	Glycogen Synthase Kinase 3
H202	Hydrogen peroxide
HD	Huntington's disease
HDL	High density lipoprotein
HIF-1a	Hypoxia inducible factor la
HSP	Heat shock protein
IGF	Insulin-like Growth Factor
IGFBP	IGF binding protein
IIS	Insulin/IGF-1 signalling
ILPs	Insulin-like peptides
IMPase	Inosito1-1(or 4)-monophastase
InR	Insulin receptor
IPP	Inositol-1,4 biphosphate 1-phosphatase
IRS	Insulin receptor substrate
KD	Kinase dead
LC3-I/II	Microtubule associated proteinA 1A/1B-light chain 3
LiC1	Lithium chloride
LIRKO	Liver insulin receptor knockout

LKB1	Liver kinase B1
M	Molar
MIRKO	muscle insulin receptor knockout
mL	mililitre
mM	milimolar
MNCs	Median neurosecretory cells
mTOR	mechanistic Target of Rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NaC1	Sodium chloride
NIRKO	Neuronal insulin receptor knockout
NRF-2	Nuclear factor erythroid-related factor 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDK	Phosphoinositide-dependent kinase-1
PGC-1a	Peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PI3K	Phosphatidylinositol 3-OH Kinase
PKB	Protein kinase B or Akt
PN	Proteostasis Network
PQ	Paraquat
PTEN	Phosphatase and tensin homologue
RCR	Respiratory control ratio
Rheb	Ras homolog enriched in brain
RNAi	RiboNucleic Acid interferemce
RT-qPCR	Real Time quantitative PCR
ROS	Reactive oxygen species
RU/RU486	Mifepristone
S6K	S6 kinase
S9A	Substitution of Serine 9 to Alanine
S9E	Substitution of Serine 9 to Glutamic acid
SEM	Standard error of the mean
Sgg	Shaggy
SOD	Superoxide dismutase
SY	Sugar/yeast
TBST	Tris-buffered saline with Tween 20
TF-EB	Transcription Factor-EB
Tris	Tris(hydroxymethyl)aminomethane
TSC	Tuberous Sclerosis Complex
UAS	Upstream activating sequence
UN	United Nations
UPR	Unfolded protein response
UPS	Ubiquitin proteasomal system
vLDL	Very low density lipoprotein
$_{w^{1118}}$	White 1118 flies
$_{w^{Dah}}$	White Dahomey flies

Chapter 1

Introduction: Genetics and Pharmacology of Ageing

"No scientist is admired for failing in the attempt to solve problems that lie beyond his competence. The most he can hope for is the kindly contempt earned by the Utopian politician. If politics is the art of the possible, research is surely the art of the soluble. Both are immensely practical-minded affairs."
Peter B. Medawar[1]

Ageing is a universal deleterious trait. Even when lifespan differences across taxa can vary by as much as 1,000,000 fold, an increase in mortality and decrease in fecundity with age is observable (Kirkwood and Austad, 2000). These deleterious traits are used by evolutionary biology to define ageing (Partridge and Barton, 1993). However, it should be mentioned that it is apparent that some organisms show negligible senescence, which represents the minor changes in mortality over extended periods of time documented in some organisms (Finch, 2009). An example of this negligible senescence was reported after a 4 year follow up study that described that the Hydra did not show age-related mortality or reproduction decline (Martinez, 1998). However, most organisms seem to age, this is particularly evident when organisms are taken from the wild and studied under laboratory conditions (Hayflick, 2000a). This might suggest that ageing can be a laboratory artefact of comfort and perhaps unique to humans. From an evolutionary perspective ageing in the wild is rare given that most organisms seem to succumb to external hazards like temperature changes, famine, predation, etc., before the establishment of functional decline (Comfort, 1961; Hayflick, 2000a; Kirkwood and Austad, 2000). These arguments have their origin, as I will describe bellow, in the seminal discussions by Peter Medawar and Alex Comfort, both scientist at University College London (Nussey et al., 2013).

"Whether animals *can*, or cannot, reveal an innate deterioration is almost literally a domestic problem; the *fact* is that under the exactions of natural life they do not do so.

[1] Quote taken from (Medawar, 1999).

They simply do not live that long." Peter Medawar (italics in original).[2]

However, a recent evaluation of long-term field studies, indeed showed evidence that senescence is encountered in nature (Nussey et al., 2013). The universality of ageing has made it interesting-enough that its field of study ranges from philosophy to sociology to biology and even economics. The study of the biology of ageing or biogerontology is fairly recent, at least the part of the field experimentally testing the mechanisms underlying the ageing process. Even more recent is the field of interventional biogerontology where ageing researchers actively explore for ways to improve health at older ages and prolong lifespan in model organisms (Partridge, 2010). However, the pursuit of youthfulness is not new, my mentor Linda Partridge often exemplifies this by reminding us of Robert Boyle's to-do list (Figure 1.1). Robert Boyle wrote a list of things he hoped could be achieved through scientific research and at the very top of his wish list he wrote "The Prolongation of Life" and "The Recovery of Youth" (Henderson, 2010).

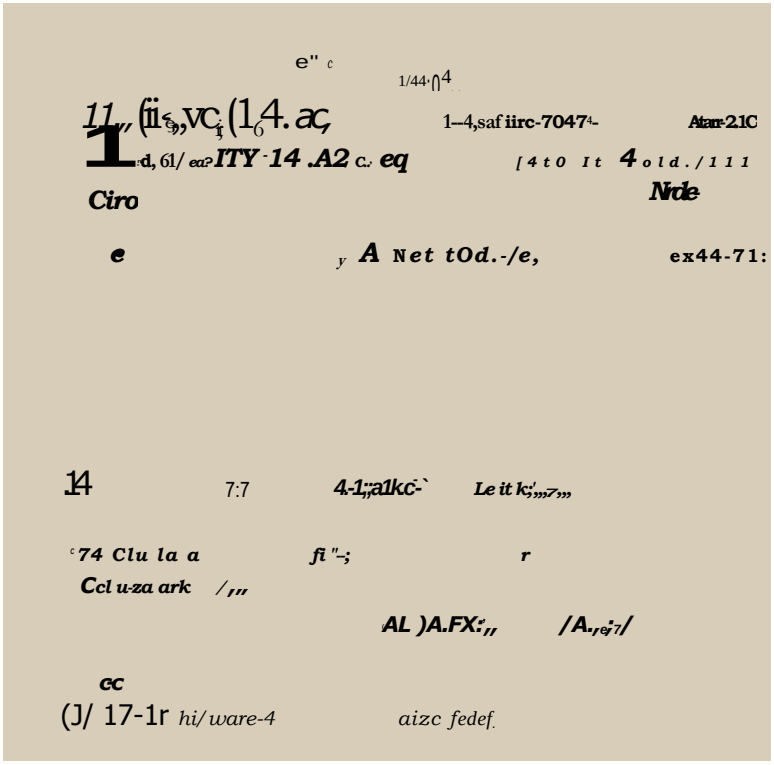


Figure 1.1. Robert Boyle, FRS wrote this to-do list. The first two items read as follow: The Prolongation of Life (1st line) and, The Recovery of Youth, or at least some of the Marks of it, as new Teeth, new Hair colour'd as in youth (2nd and 3rd lines). For the complete list and transcript see (Henderson, 2010)

In the reminder of this chapter I will explore what is ageing, or at least how different trains of thought have defined it, and most importantly, I will also describe the mechanisms thought to underlie the ageing process, i.e., how do we age. These types of questions have transformed biology, not only because of what we think and get to know

[2] **Quote extracted from (Nussey et al., 2013).**

about life, but also because of what we, as a species, have been able to achieve with this knowledge. Simple questions are usually the beginning of great discoveries. For example, Schriidinger's book 'What is life?' has been credited by both Francis Crick and James Watson, co-discoverer's of the structure of DNA, as an inspiration for embarking on the quest that finalised (our should instead say 'started') in the description of the DNA structure, opening a new era in the field of molecular biology (Watson, 2000). Although Schriidinger's exploration of the idea of genetic flow of information was not novel, even at its time, it inspired some of the greatest minds to engage in the quest of the identification of the DNA architecture (Schrodinger, 1967). The discovery of DNA has also inspired and transformed other fields. The relevance of genetic information and signalling mechanisms became central for neuroscience, especially for the fields of learning and memory, when it was discovered that signalling events between neurons and the synthesis of proteins is central for the reinforcement of synaptic communication (Kandel, 2006). The concept of signalling events is now central to the understanding of biological mechanisms governing the ageing process. These signalling pathways (particularly the nutrient-sensing network, discussed later in this chapter) were identified following the discovery of single genes capable of modifying lifespan and healthspan (Kenyon, 2010; Partridge, 2010). Genetics and signalling pathways regulating gene expression have become central to the study of the biology of ageing, not only in model organisms where I carry out my research, but also in human populations, especially those that have been fortunate to live through advanced-age.

1.1 Gerontology: socioeconomic and biologic principles

Gerontology refers to the integral study of ageing, is a broad specialty as it covers social, psychological and biological aspects of ageing. Gerontology should not be confused with geriatrics, which refers to a sub-specialty of Internal and Family Medicine that focuses on medical aspects (mainly pathologies) of older people (Fillit et al., 2010). In the context of this dissertation, I will only consider ageing in its biological terms; however it is important to note that ageing can also be studied from other perspectives. Human ageing is multidimensional, as it comprises not only its biology, but also psychological and social aspects. These broader aspects of gerontology keep the research we do in biogerontology oriented towards its biomedical implications.

As I will describe in the next section, life expectancy has been increasing steadily since the second half of the last century in most parts of the world; this has occurred with no intervention of biogerontology. However, it has uncovered that alongside the great achievement of longer lifespans, our older populations are experiencing the devastating effects of age-related diseases. This has made the idea of living longer not only unattractive to most people, but perhaps even thoughtless 'selfish' and irresponsible. Though biogerontologists have been able to prolong lifespan of model organisms (as Boyle wished), there is no clear evidence to suggest that this will translate into increased lifespan in humans. Moreover, most if not all research in model organisms has shown that interventions that extend lifespan in the laboratory also significantly extend healthspan, as measured by their increased locomotor capacity, resistance to environmental stressors and to ageing-related diseases. The goal of biogerontology is first and foremost to promote healthspan during ageing. We do not aim to extend the decrepit moribund period of life. However, extending healthspan is likely to increase lifespan as interventions identified in model organisms suggest (Hayflick, 2000a; Juengst et al., 2003).

Although the ethical implications of ageing research escape the scope of this dissertation, it is of great relevance to realise that our research has direct implication to human health. This not only obliges us towards careful scientific practice, but to also be wary of the perception of our findings by the general public. This does not mean that scientific findings in biogerontology (or in any field) should be kept from the general public, but to be cautious of not overindulging our findings in the aim of securing funding or attracting media attention. Our responsibility as scientists should not end with the publication of our results, but we should be accountable for making sure that the public is aware of our results and its implications first-hand. Is not easy to translate research from model organisms like yeast and flies to humans, but it has become apparent in recent years that the mass media easily makes careless correlations, potentially damaging the perception of our field (Le Bourg, 2000; Juengst et al., 2003). We should be aiming to avoid miscommunication between the scientific finding and the general public.

1.1.1 Epidemiological data of human longevity

For most part of our history (99.9% of it) our average life expectancy has fluctuated between 20 and 30 years of age, based on estimations from skeletal remains (Hayflick, 2000a; Klein, 2000). However, just in the first 70 years of the last century, life expectancy

increased by 27 years, and the remainder of the century saw a 6-year increase (Hayflick, 2000a). This has had two major consequences, first the 'greying' of our population and, second, uncovering the consequences of chronic diseases like diabetes, cancer and Alzheimer's disease (Hayflick, 2000b).

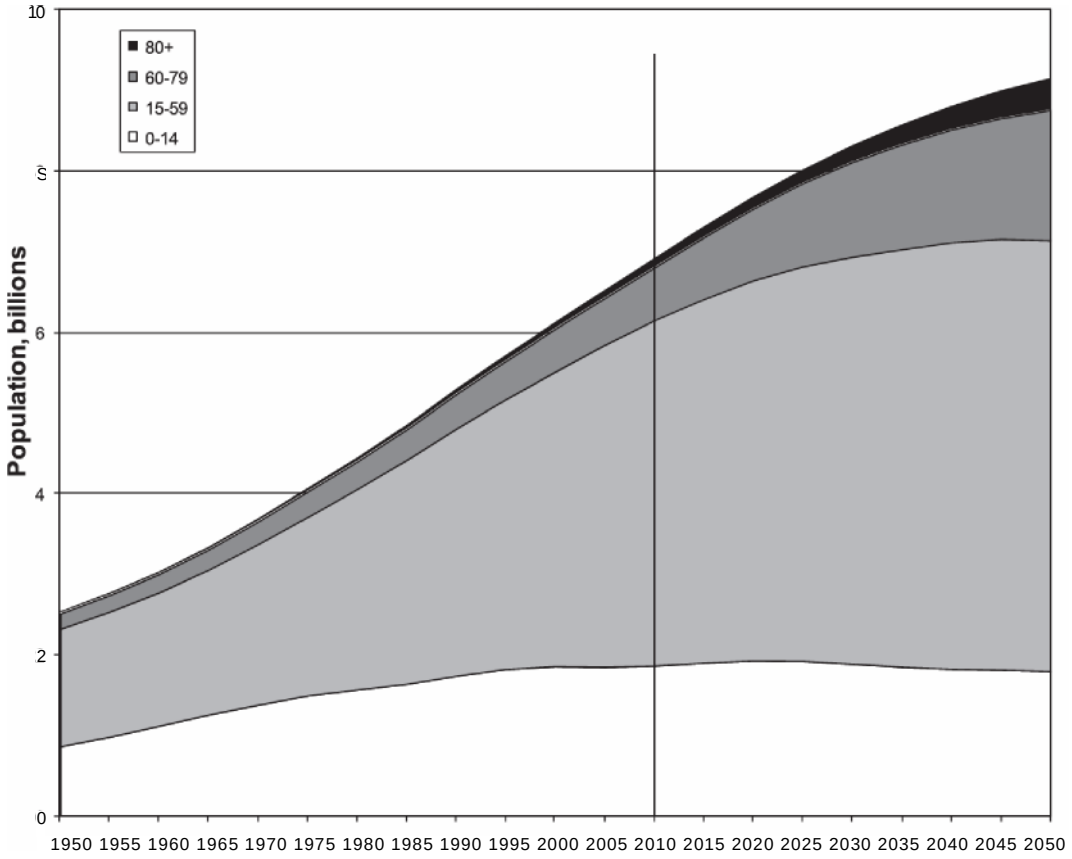


Figure 1.2 World population by age group according to the UN, 2009. Taken from (Bloom et al., 2011)

The world's population is estimated to be above 7 billion (Bloom, 2011; Lee, 2011). Approximations of population growth do not seem to favour a significant deceleration in the future (Figure 1.2). The Division of the Department of Economic and Social Affairs of the United Nations (UN) estimates the world population will reach 9.3 billion in 2050 and 10.1 billion in 2100 (Bloom, 2011). Importantly, almost the entire population growth (97%) between now and 2050 will occur in the less developed regions of the world, with 38% taking place in the least developed countries. The two most populated countries by this time will be India and China, with estimated populations of 1.69 and 1.30 billion respectively.

According to the UN Population Division currently 800 million people are over the age of 60 years and comprise 11% of the world's population. By 2050 this percentage will double to 22%, meaning 2 billion people worldwide will be over the age of 60 years (Bloom, 2011). By 2050 Japan, currently housing one of world's oldest populations (31% being over the age of 60 years) and with the greatest life expectancy (83 years), will drop out of the top ten of most populated countries. However, by 2050 42% of the Japanese population will be over the age of 60 years (Bloom, 2011; Sanderson and Scherbov, 2005).

Increased population ageing is influenced by three main events: migration, increased life expectancy, and decreased birth rate. However, two important epidemiological events from the 20th century also account for the world's current longevity. First, the so-called epidemiological transition resulted in the reduction of early death due to improved perinatal care, vaccination and control of infectious diseases through antibiotics (Manton, 2010; Timiras, 2003). The second event comprised the reduction in deaths due to age-related diseases, this has been more progressive (having started in the 1970s to 1980s), but has had a clear impact on mortality at older ages (Timiras, 2003). The greatest reduction in mortality has been in diseases such as ischemic heart disease and stroke, especially in developed countries such as the United States of America (Alzheimer's Association, 2012).

In spite of the morbidity compression, the prevalence of ageing diseases, as the population continues to grow, has increased. Diseases of old age have become a central issue of public health in developed countries (Figure 1.3). Moreover, as developing economies transition to economical stability they encounter the worst of the two worlds, the diseases of the developing world (increased at-birth mortality, infections, etc.) and also the diseases of ageing from the established economies.

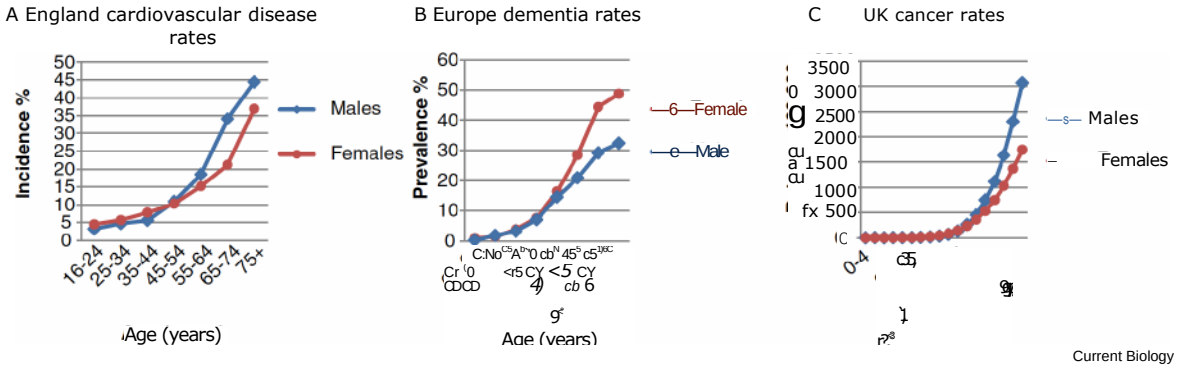


Figure 1.3 Ageing and Disease. (A) Incidence of cardiovascular disease in England, 2006; (B) Prevalence of dementia in countries from the European Union, 2006; (C) Age-specific mortality rate per 100,000 population, UK. Taken from (Niccoli and Partridge, 2012)

Thus, understanding of the biological principles that govern the ageing process should become an integral part of the public health effort to tackle the increasing burden of ageing related pathologies.

1.1.2 Ageing in Biology and Medicine

Ageing is a stochastic process that occurs after reproduction maturity and is the consequence of the diminished capacity of organisms to maintain molecular fidelity. The reduced capacity of the organism to sustain homeostasis often leads to age-related pathologies. Hence, the ageing process, or the decline in homeostatic capacity, is the major risk factor for chronic age-related diseases. The ensuing increase in systemic molecular disorder ends up in death (Hayflick, 2000a). Hayflick has suggested that no one actually dies of the diseases found in their death certificates. This is likely to be accurate since if a patient was admitted to hospital for renal failure secondary to diabetes mellitus, and in the course of treatment developed hyperkalaemia (high levels of circulating potassium) with hypocalcaemia (low circulating levels of calcium), the patient would probably die of cardiac arrest secondary to cardiac arrhythmia. In the death certificate the medic validating cause of death would probably write in order of appearance (suggesting the order of progression from primary to immediate cause of death): type II diabetes mellitus, end-stage renal failure secondary to diabetic nephropathy, cardiac arrhythmia secondary to electrolyte imbalance and cardiopulmonary arrest. In this scenario neither the primary cause of the disease (the origin of the insulin resistance), nor the ultimate dysfunction that led to cardiac arrest would be properly diagnosed. Our understanding of the molecular mechanisms behind disease and cause of death need to improve dramatically. Hayflick has emphasized that even if we were to fmd cures for three of the major killers of our time (namely cardiovascular disease, cancer and stroke), we would only increase life expectancy by about 15 years. And then we would probably find out what is the ultimate cause of death when no disease is present, i.e., what leads to death when the capacity of the organism to cope with molecular disorder ceases (Hayflick, 2000a).

In spite of the efforts of many biogerontologist to classify ageing as a disease, as a health care professional, I have not encountered convincing arguments. Gems argues that ageing should be considered as a disease or syndrome because it is characterised by a broad-spectrum of age-related pathologies that lead to death. Additionally he argues that

no one dies of ageing (Gems, 2014). Even for those that age 'gracefully' functional decline

is imminent. Hence, ageing is associated with deterioration and therefore is undesirable. In the same vein is the consideration that this decline should be treated, thus enabling the concept of anti-ageing medicine (Gems, 2014). One cannot "treat" any other thing that is not a disease. Although treating ageing sounds rather logical, I wonder if the train of thought followed by Gems would hold when analysed from the perspective of developmental biology and embryology. Alterations during development also cause syndromes and disease, most of which lead to death due to severity. The main difference between diseases during ageing and development is that during the former functional decline allows for a broad spectrum and greater incidence of pathology than during development in which the system is trying to maximise fitness.

Treatments for diseases are regulated by agencies that approve and regulate their use. As of now, anti-ageing products are not regulated and are classified as supplements, unless they are actual treatments for disease that have been repurposed. If ageing was to be considered a disease, then all anti-ageing interventions would be under the scrutiny of appropriate agencies ensuring efficacy and safety (Juengst et al., 2003). This in my opinion calls action into changing the regulation of products with nutritional relevance (this would not only include supplements, but also junk food and drinks). I consider that what needs to change is our view of interventions to regulate the ageing process and general health instead of the classification of ageing *per se*. Focusing into health preservation and prevention of disease would probably lead to a better regulation of any product with either harmful or beneficial effects for health. Medicine as a field needs to transition more efficiently into disease prevention. This transition would stimulate a reorganisation of the medical community and the way health during ageing is assessed. The ageing process would not only be an aspect of geriatric medicine, but of general medical practice and family physicians. Geriatricians would, however, continue to be the specialists in treating the diseases of ageing.

The clear overlap between biogerontology and geriatrics should require constant communication between geriatricians and gerontologists. In one-way or another some knowledge should be shared. Geriatricians are required to understand the basics of the ageing process and its social and psychological implications, while biogerontologists should keep in mind that retardation of the ageing process in model organisms is rather pointless unless these interventions, from an evolutionary point of view, are applicable to human health (Fillit et al., 2010).

1.1.3 Measurements of Ageing

Our understanding of the biological processes that govern ageing has rapidly advanced in the last 30 years. This can be attributed to three main events. Firstly, the use of simple organisms in the laboratory has unveiled signalling and molecular pathways that are translatable to mammalian systems, confirming evolutionary conservation. Secondly, the field has successfully adapted methods for genetic manipulation in model organisms, and also methods to interrogate the genome, proteome and metabolome are gradually being integrated into biogerontology both for ageing research in model organisms and human ageing. This is allowing us to understand the changes that occur during ageing and the molecular basis of interventions that potentially retard the process (Soltow et al., 2010). Third, we now understand better how ageing affects function during ageing. However, this is perhaps the point that requires further experimentation and development. We need to determine clear biomarkers of ageing (Fontana et al., 2014). Furthermore, although we can measure relatively easily lifespan in yeast, worms, flies and mice, we do not completely understand cause of death, especially in invertebrates. Research into the events leading to death should unravel some insight as to how ageing culminates and the point of no return in the adaptive process.

Several concepts are, however, indispensable for the study of ageing and should be introduced. Demographical parameters like mortality, birth rate and fertility shape several important aspects of human ageing and specifically population ageing. For the purpose of this dissertation only life expectancy is considered as it can be regarded as quasi-equivalent of survival curves, which are a key form of ageing assessment in *Drosophila*. Life expectancy refers to the total years remaining at the moment at which the study is being conducted, i.e., the average number of years a person is expected to live (Hayflick, 2000a; Timiras, 2003). Life expectancy has been increasing linearly for over 165 years, and there is no indication that we are approaching the limit of human lifespan (Christensen et al., 2009; Oeppen and Vaupel, 2002). This steady-state increase in life expectancy, has led to the suggestion that most babies born since 2000 will celebrate their 100th birthday if born in countries with a long history of longevity (Christensen et al., 2009). Of course the increase in life expectancy has been accompanied by an increase in diseases of old-age (Bloom, 2011; Christensen et al., 2009). The presence of disease is termed morbidity which regularly leads to increased mortality i.e., deaths.

Lifespan can be defined as the duration of life of an individual or organism in a particular environment and/or under specific circumstances (Timiras, 2003). For the

purpose of this dissertation lifespan will be defined as a measure of the duration of life of a group of flies under certain pharmacologic and/or genetic manipulations. The easiest and most direct way to assess treatments that increase longevity is to measure if an intervention (environmental or pharmacological), genetic manipulation (gene mutation, over-expression or down-regulation) or background (genetic strain or cytoplasmic background, the latter referring to the *Wolbachia* status of flies) can prolong the survival of a group of flies subjected to the treatment in question (Partridge et al., 2011). For the purpose of assessing pro-longevity success, median and maximum lifespan are often measured (as in the following chapters). Median lifespan corresponds to the time at which 50% of the population remains alive. On the other hand, maximum lifespan corresponds to the time at which the last 10% of the population remains alive (Mair and Dillin, 2008).

1.1.4 Ageing in model organisms including *Drosophila*

Evolutionary-less complex organisms like *C. elegans* and *Drosophila* offer several advantages for the study of ageing because of their relative short lifespan, knowledge of their complete genomes and their well characterized biology. However, in order for ageing in these species to be relevant for mammals, and especially to humans, interventions to specific cellular pathways have to be evolutionary conserved (Partridge, 2001). All model organisms have advantages and disadvantages for the study of ageing and ageing-related diseases. For example, yeast cells are incredibly cheap, easily manipulated genetically, show age-associated changes in cellular organelles, and can be studied for their chronological and replicative lifespan. While the chronological lifespan refers to their capacity to maintain vitality (integrity of the cell wall or capacity to form a colony) in a non-dividing state, their replicative lifespan refers to the number of times they can divide, which has been calculated to be a mean of between 20 to 30 generations (Blagosklonny and Hall, 2009; Denoth Lippuner et al., 2014). However, the lack of complex interaction derived of multi-cellularity is a major disadvantage of yeast cells. Hormonal regulation of ageing cannot be studied as they lack the appropriate signalling pathways (Alic and Partridge, 2011). In contrast, the nematode worm *Caenorhabditis elegans is* still cheap, amenable to genetic manipulation and with a relatively short life cycle (adult mean lifespan is between 2 to 3 weeks at 25°C), yet it possesses complex organised tissues.

Additionally, *C. elegans* is transparent which allows examination of their internal structures. However, as a *`Drosophilisr* the trait I wish flies had was the ability to survive freezing/thawing. *C. elegans* can be stored at -80°C, which coupled to their relatively short lifespans makes them an interesting model for ageing research. Another interesting feature of *C. elegans* is the simplicity and efficiency of RNA interference for knocking down gene expression. Worms fed genetically transformed bacteria expressing double stranded RNA (dsRNA) complimentary to the gene of interest show very good knock-downs. This makes *C. elegans* a very powerful genetic tool for genetic screens. Moreover, *C. elegans* shares 40% of its genetic code with humans. However, amongst others I highlight two disadvantages of using *C. elegans* as a model organism. First, there are no females, most worms in a given population are hermaphrodites, whit a very low percentage of males (0.05%), which complicates the study of sex-specific effects. Second, though they posses a semi-organised nervous system (with precisely 302 neurons), there is no brain-like organisation, which limits their use and interpretation for the study of the CNS and diseases within this structure (Finch and Ruvkun, 2001; Fire, 2007; Hull and Timmons, 2004; Markaki and Tavernarakis, 2010; Vanfleteren and Braeckman, 1999; Walker et al., 2005).

In the case of mammalian models, the preferred system has been the mouse since it has a shorter lifespan in comparison to primates (3 years on average for mice and over 30 years in the case of rhesus monkeys), and genetic tools are more readily available. Though ageing studies in non-human primates have been instrumental to further our understanding of the relationship between diet and ageing (Colman et al., 2009, 2014; Mattison et al., 2012; Roth et al., 2004). In general mammalian models are difficult to maintain, very expensive and their life cycles make ageing research practically unrealistic for a doctoral dissertation. Preparation for studies in these animals requires careful examination and sample size estimation, as the cost of keeping them is often a limiting factor for big sample sizes. However, ageing studies in mammalian models are required to prove evolutionary conservation of interventions tested in less complex organisms (Swindell, 2012; Weindruch et al., 1986; Yuan et al., 2011).

1.1.4.1 Drosophila: a brief historical perspective

Drosophila has been a key model organism for the study of genetic inheritance, development, behaviour, disease and aging. Early in the 1900s William Ernest Castle

introduced the used of *Drosophila melanogaster* as a genetic tool at Harvard University. Castle influenced many others to incorporate *Drosophila* in their research as a viable model organism. It was only after the experiments carried out by Thomas Hunt Morgan and his students at Columbia University in New York that *Drosophila* acquired certain popularity in genetics. Morgan discovered in 1910 the sex-linked *white* eye mutation. This seminal discovery is still used in the day-to-day handling of flies as a genetic marker. It is important to notice that the *white* gene encodes for red, which is the wild type eye colour. Morgan's team not only paved the way for the use of *Drosophila* as a genetic tool, but also made important contributions to the general field of genetics. For example, Morgan's student Calvin Bridges proved the chromosome theory of inheritance, Alfred Sturtevant generated the first chromosome map as an undergraduate student, and Herman Muller demonstrated the mutagenicity of X rays. After an intense era of genetics research at the beginning of the 19th century, *Drosophila* genetics was overshadowed by the use of viruses and bacteria until early in the 1970's when attention shifted to the control of development and behaviour in more complex organisms. Ever since *Drosophila* has remained instrumental for the understanding of complex processes like behaviour, ageing and disease (Hartwell et al., 2011; Kenney and Borisy, 2009; Kohler, 1993; Stephenson, 2013).

1.1.4.2 Drosophila melanogaster: natural history and genetics

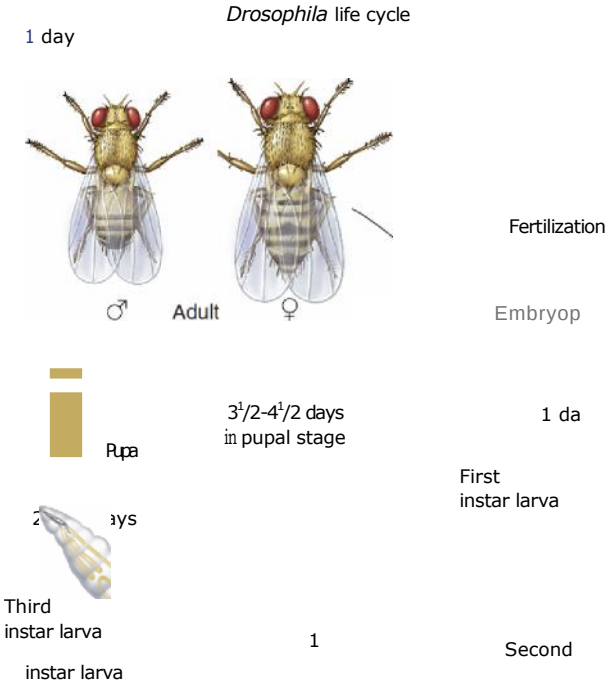


Figure 1.4 *Drosophila* life cycle. Fertilised females can store sperm in storage organs called spermatheca and seminal receptacle. They fertilise their eggs just before laying the embryo. The embryo completes its development within 24 hours and 'hatches' as a first instar larvae and progresses up to third instar in a process called molting. During these phases the main larval functions are eating and growing. Molting is controlled by the hormone ecdysone. Just before pupariation third instar larvae usually crawl up the sides of their housing bottles and undergo metamorphosis inside the protective pupal case. During this four-day period most larval tissues are replaced to adult-like tissues. After flies emerge from their pupa their wings expand, their exoskeleton hardens and becomes pigmented. Taken from (Hartwell et al., 2011).

The genus *Drosophila* of the family Drosophilidae is made up of over 900 species, which inhabit almost every corner of the world, except for the coldest tundras and the driest deserts. *Drosophila,* particularly *D. melanogaster* has been called the great hitchhiker among *Drosophilas.* It seems that wherever we have gone (we being the greatest travellers among primates), *Drosophila* has followed us (Kohler, 1993). For example, I have taken our Dahomey (see Chapter 2 for more details about our w^{pah} flies) flies to Uganda and Tanzania in an attempt to introduce the use of *Drosophila* in biomedical research in Africa. Students attending our courses have then taken them back to their universities across the continent (Yusuf et al., 2014). Interestingly our $w^{p}a^{h}$ flies are originally from Dahomey (today Republic of Benin) in West Africa. It can be said that these flies are returning home. Recently *Drosophila* has even gone to space and there is great interest as to how the anti-gravity environment changes their physiology, including for example cardiac function and ageing (Anthony et al., 1996; Marco et al., 1996, 2003). The name *Drosophila* means, "dew lover" as a moist temperate climate offers the best conditions for their survival. Although they are called fruit flies, they should be instead called yeast flies, as this is their preferred food source. *D. melanogaster is* often found in decaying vegetation or fruits that support yeast colonies (Kohler, 1993). At 25°C their developmental life cycle is approximately 10 days from egg to adult fly (Figure 1.4). Under laboratory conditions *Drosophila* can live between two to three months. Female flies liver longer than male flies.

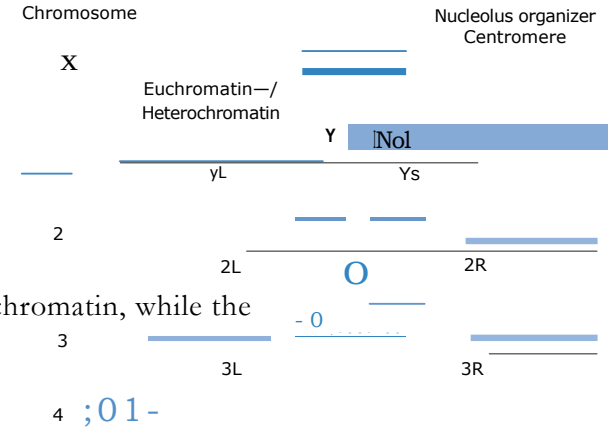


Figure 15 *Drosophila* chromosome complement. Dark blue areas represent heterochromatin, while the light blue area represents euchromatin. L and S in superscript refer to the long and short arms of chromosomes. Taken from (Hartwell et al., 2011).

Drosophila has an haploid genome with four chromosomes numbered 1 to 4 (Figure 1.5). Chromosome 1 is the acrocentric X chromosome, while the other chromosomes (2 to 4) are autosomes (2 and 3 are metacentric, while 4 is acrocentric and is

only about 2% of the major autosomes). Unlike in mammals sex is determined by the ratio of X chromosomes to the number of autosomes. Additionally, each cell decides whether to be male or female. The Y chromosome is required for male fertility but is not involved in sex determination. By convention the Y chromosome is not given a number. The Y chromosome is almost entirely heterocromatin and it probably only carries genes involved in the physiology of male germ line (seven or eight genes). Chromosomes 2, 3 and the X chromosome possess large blocks (a quarter of the length of the chromosome) of heterochromatin near their centromere (Hartwell et al., 2011).

The *Drosophila* genome is about 5% the size of the human genome and it contains approximately 13, 600 genes (Adams et al., 2000). Besides the fact that the entire genome has been sequenced *Drosophila* shares approximately 60% of its genetic code with humans and it has been identified that 75% of genes related to human disease have a counterpart in flies, making them an attractive tool to model human diseases (Chien et al., 2002; Reiter and Bier, 2002; Reiter et al., 2001). Additionally several genetic tools like the GAL4/UAS gene expression system (see Chapter 2 for a detailed description) have made *Drosophila* extremely amenable to genetic manipulation. Its low cost, rapid turnover, and complex behaviour have made it an ideal model for the study of ageing and disease (Lessing and Bonini, 2009; Muqit and Feany, 2002; Reiter, 2005; Whitworth et al., 2006).

1.2 Biology of Ageing: the origins of the ageing process

When one considers the triumph of the developmental and growth processes that lead to reproductive fitness it becomes rather remarkable that organisms fail to mainly maintain in good shape the integrity of cells and tissues. This was in so many words a thought that the prominent evolutionary biologist George Williams had about the ageing process. In the context of the great energetic and programmed nature of development it does seem surprising that organisms are incapable of repair and preservation (Kirkwood, 2005). This has led to numerous currents of thought that have speculated why nature has allowed such a decline. There are a number of biological and evolutionary theories that attempt to explain the origins and causes of ageing (Medvedev, 1990). It is likely that components of these theories might contribute in varying degrees to the explanation behind the ageing process (Jin, 2010). These theories can be categorised into evolutionary, molecular and cellular theories of ageing (Weinert and Timiras, 2003). In the context of this dissertation

the molecular and cellular theories are categorised as 'mechanistic' theories of ageing. The latter attempt to explain what causes ageing, i.e. how do we age, while the evolutionary theories try to explain the origin of the ageing process, why ageing exists.

1.2.1 Evolutionary Theories of Ageing

Some of the theories proposed to explain the origin of the ageing process have the unforgiving flaw of assuming that ageing already exists. For example, the altruistic theory of ageing proposes that ageing exists for the benefit of the species. According to this theory ageing gets rid of older individuals that would consume resources of the young ones, those that would contribute to the fitness of the population. By removing the old and decrepit that do not contribute to the continuation of the species, nature makes way to the young and fit ones (Kirkwood and Austad, 2000). However, fitness decline is secondary to ageing, if ageing did not exist then the old ones would still be able to contribute to the perpetuation of the species. Therefore, the assumption that ageing exists does not help to explain why ageing exists. Moreover, a recent theory of ageing has proposed that longer lifespans in the post-reproductive period in women, are determinants of the successful reproduction and survival of their offspring and also of the survival of their grandchildren (Hawkes and Smith, 2009; Landenperd et al., 2004). This has been called the "grandmother theory of ageing". While the validity of this theory is contested, it does not explain why do we age, it might merely hint as to why we live as long as we do, if anything.

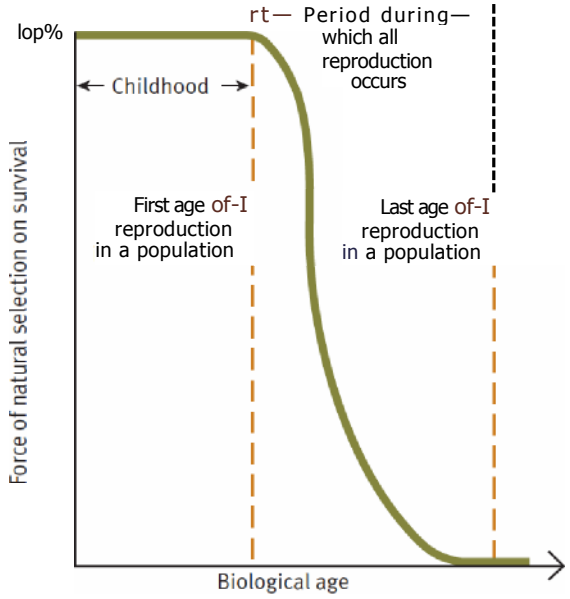


Figure 1.6 The force of natural selection declines with age. Mutations arising during childhood before the onset of reproductive fitness are under strong natural selection. Mutations whose phenotypes manifest after sexual maturity are under less selection pressure. Therefore, late-acting mutations are allowed to pass on to the next generation. Taken from (Rose, 1999).

Perhaps the greatest contribution to the understanding of the evolution of ageing came from the seminal observation that as individuals age, the force of natural selection diminishes (Figure 1.6). John Burdon Sanderson (J.B.S.) Haldane made this observation when studying the age of onset of genetic diseases, particularly Huntington's disease (HD) (Haldane, 1941). The question here was, why disease-causing genes have not been removed by natural selection if they are deleterious to the species? However, as Haldane described, diseases that present later in life are under less selection than those that present early in life. An example commonly given is the comparative prevalence of progeroid syndromes (low prevalence), which present fairly early in life, and the prevalence of HD (higher prevalence than progerias), which presents later in life. Patients with progeria show manifestations of the disease very early in life, limiting their reproductive capacity. On the other hand, most forms of HD onset after the genes have been passed to the next generation, perpetuating the existence of the disease (Rose, 1999). Thus, Haldane hypothesised that ageing occurs as the result of late-acting deleterious mutations. These mutations would be under low or no natural selection (Haldane, 1941). Where do these mutations come from?

Peter Medawar rightly discussed that mutations are always happening in populations, most of these mutations will have no or little impact on development and reproductive fitness. However, if they did affect these traits they would be removed from the population by not allowing them to pass on to the next generation. Mutations that do not affect traits that manifest before reproductive capacity and success are achieved will persist. As these mutations, which are not under natural selection, continue to accumulate, they will become prominent in the population. Medawar proposed that the accumulation of late-acting deleterious mutations over the evolutionary history of populations has resulted in what we call now ageing (Medawar, 1957). Earlier such effects would have not been manifested, as predation and other external hazards would have not allowed for the functional decline and manifestation of these mutations to occur. However, as organisms have learnt to eliminate or control their environment the consequences of the accumulation of late-acting mutations have become very apparent. This theory is called the mutation accumulation theory of ageing.

George Williams took a complementary approach to that of Medawar. However, Williams focused on mutations that would be favoured by evolution because of their contribution to reproductive fitness, rather than in the accumulation of late-acting deleterious mutations (Kirkwood and Austad, 2000; Weinert and Timiras, 2003). Williams

proposed that mutations that would ensure reproductive capacity will be favoured by natural selection even if they pose a disadvantage later in life (Williams, 1957). As natural selection drives perpetuation of the species, these mutations would remain in the population allowing the appearance of ageing (ageing being the negative side effect) (Weinert and Timiras, 2003; Williams, 1957). Williams's proposal is called the antagonistic pleiotropic theory of ageing to highlight the dual early benefit and later negative effect of such mutations. Thomas Kirkwood proposed a specific case of antagonisict pleiotropy in a theory that has focused on the disposable soma. Kirkwood assumes that resources are limited and given a choice organisms should allocate those resources in either somatic maintenance or reproduction. When resources are plentiful natural selection will favour reproduction and energy will be invested in the germ line. However, when resources become limiting, nature will favour somatic maintenance until resources become available after which investment in reproduction will resume. Kirkwood's interpretation of the antagonistic pleiotropy theory of ageing proposes that ageing arises because of the sacrifice of somatic maintenance in favour of reproduction. As limited resources are allocated to repair the accumulation of cellular and molecular damage, the soma becomes disposable and senesces (Kirkwood, 2000, 2005; Kirkwood and Austad, 2000). Two problems arise from this theory however. Firstly, it only explains why we live up to a certain age and secondly its principle contribution has been to describe why long-lived mutants show compromised reproduction (Weinert and Timiras, 2003). However, reduced fecundity and longevity have been recently uncoupled (Grandison et al., 2009a).

More recently Mikhail Blagosklonny has proposed another variation of the antagonistic pleiotropy theory of ageing. In contrast to Kirkwood who assumes that ageing is driven by the accumulation of unrepaired damage (Kirkwood, 2005; Kirkwood and Austad, 2000), Blagosklonny has discussed that ageing arises from the continuation of developmental programmes (Blagosklonny, 2008). Blagoskonny's theory brings centre stage the knowledge we have of how certain pathways regulate ageing to the origins of the ageing process. His theory has been called the hyperfunction theory of ageing to highlight that the driving force behind ageing is the perpetuation of processes started during development (Blagosklonny, 2013a; Gems and De la Guardia, 2013; Gems and Partridge, 2013). During development, genetic pathways in charge of biomass synthesis and accumulation are set up, however as the organism ages these programmes are not switched

off and they will continue operating, in a way they hyperfunction as their main purpose

was not to drive ageing. Blagosklonny's theory is, in a way, centred in the mechanistic target of rapamycin (mTOR) pathway (Blagosklonny, 2008, 2013b, 2013c). As I will discuss later, this pathway is very important for development and growth as it promotes protein synthesis and prevents degradation (Jacinto and Hall, 2003; Jia et al., 2004; Wei et al., 2013). In *Drosophila* for example, down-regulation of this pathway reduces reproductive capacity, but it extends lifespan (Bjedov et al., 2010). Hence it highlights the nature of pleiotropic functions of known cellular processes. Blagosklonny also tackles the idea that ageing is programmed. The biogerontology community has long discussed the possibility that certain genes function is to drive the ageing process, hence the existence of a programme (Austad, 2004). Such genes have not been found yet. By moving the drivers of ageing to developmental programmes, Blagosklonny proposes that ageing is not programmed but quasi-programmed. Ageing is not encoded in our genomes, but it happens because processes controlled during development do not have an off switch (Blagosklonny, 2008, 2012b).

1.2.2 Mechanistic Theories of Ageing

Many theories as to how we age have been proposed. Most of these theories have in common the generation of damage. For example, the error catastrophe theory of ageing proposes that the accumulation of defects arising in the regulation of transcription and translation lead to a catastrophic error that ultimately limits vitality. Similarly, it has been proposed that accumulation and changes in protein structures impairs cellular function ultimately limiting tissue and organism physiology (Weinert and Timiras, 2003). This theory features prominently in the collapse of proteostasis seen in many neurodegenerative diseases like HD, Alzheimer and Parkisnon's disease. I will discuss proteostasis and ageing in the subsequent section.

The most prominent and also the most debatable proposed reason for ageing is damage accumulation, secondary to free radicals. The free radical theory of ageing proposes that highly reactive free radicals are generated from oxidative metabolism, resulting in damage to different macromolecules (Weinert and Timiras, 2003). This theory has been regarded as the most promising explanation for the ageing process. However, very recently there has been a surge of conflicting data showing that damage accumulation does not have to be prevented to retard ageing, and that in some cases generation of

reactive oxygen species (ROS) correlate with increased healthy ageing. Indeed generation of ROS is sometimes necessary for lifespan extension in invertebrates (Doonan et al., 2008; Blagosklonny, 2008). I will discuss the role of ROS and stress resistance in subsequent sections.

Recently nine hallmarks of ageing were described that account for processes that are thought to drive the ageing process, adaptive responses to damage accumulation, and the hallmarks that translate into phenotypes (Figure 1.7) (Lopez-Otin et al., 2013). According to the primary hallmarks the processes that drive the ageing process are genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis. I will discuss these hallmarks in more detail in the subsequent section. These hallmarks were defined in an attempt to provide a framework to conduct research and target the scientific effort. This approach followed from the one taken by the cancer biology community (Hanahan and Weinberg, 2000, 2011). Targeted research into the regulation and interconnectivity of these hallmarks should provide a clearer picture as to why we age, what are the processes regulating the ageing process and how to target them to improve lifespan and healthspan (Lopez-Otin et al., 2013).

Our understanding of the processes driving ageing is still in its infancy. Therefore, it is quite remarkable that in spite of lacking an absolute explanation of why and how we age, we have been able to successfully intervene in the ageing process. Recent data has shown that, as with all biological processes, our genes play a significant role. The importance of the success of these interventions, beyond their pro-longevity effects, resides in the theoretical insight they provide to explain the origin and drivers of ageing.

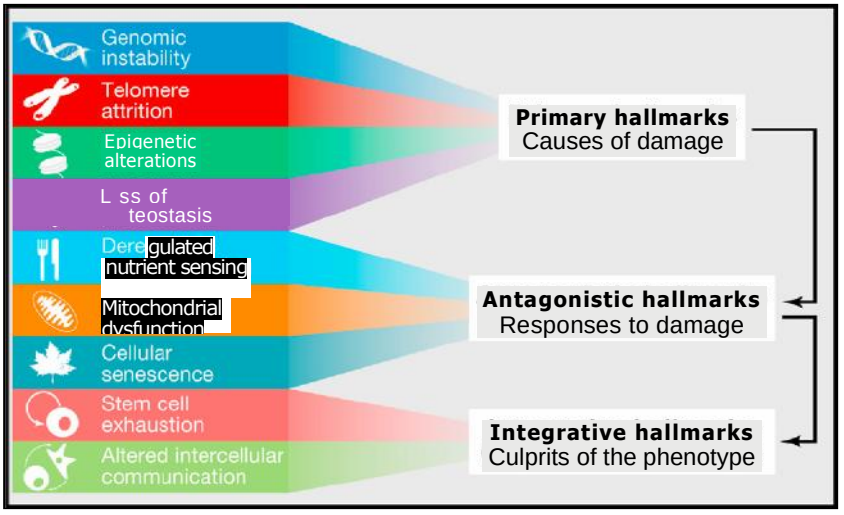


Figure 1.7 The nine hallmarks of ageing. These hallmarks highlight process that cause damage and should drive the ageing process, cellular and organismal processes that respond to damage accumulation and the hallmarks that are likely to drive the manifestation of ageing. Taken from (Lopez-Otin et al., 2013).

1.3 Molecular Basis of the Ageing Process

1.3.1 Dysfunctional molecular fidelity as a driver of ageing

Proper relay of information is instrumental for appropriate cellular function. Being able to suitably dictate functions from the nucleus to specific cellular compartments ensures survival at the cellular level. In turn, the nucleus should be able to adapt to the cellular needs that cells encounter in the ever-changing environment by appropriately activating or silencing its vast genomic code. Dysfunctional adaptive capacity renders cells, tissues and entire organisms unfit for survival. Additionally, as time passes and the adaptive capacity declines, damage accumulates (Garinis et al., 2008; Hoeijmakers, 2009; van de Ven et al., 2007).

No cellular compartment is void of the deleterious effects of damage, whether inflicted by intrinsic or extrinsic factors. Yet repair mechanisms are normally capable of dealing with damage. The relay system that allows information to flow from genes to proteins, and the genome itself, are prone to damage accumulation. Damage to the genome is a universal feature of ageing (it can be of several kinds including point mutations, chromosomal rearrangements, shortening of telomeres, translocations, etc.) and when the repair systems are overwhelmed phenotypes of accelerated ageing manifest (Hoeijmakers, 2009). For example, Werner syndrome is a progeroid syndrome that arises from mutations in the WRN ATP-dependent helicase, important in DNA metabolism. Cells lacking WRN are defective in DNA repair, particularly double-strand breaks. They also present telomere attrition and senescence (limited replication capacity due to cellular arrest). Patients carrying mutation in WRN start manifesting alterations between 10 and 20 years of age. The phenotypes include early greying and hair loss, bilateral cataracts, osteoporosis, atherosclerosis and neoplasm among others. Other diseases like Cockayne syndrome, tricothiodystrophy and ataxia telangiectasia also lead to progeroid phenotypes. All of these diseases are linked to deficient DNA repair, highlighting the relevance of appropriate DNA repair in survival and ageing (Burtner and Kennedy, 2010; Hoeijmakers, 2009). Moreover, it was recently shown that preservation of genomic integrity by ensuring faithful chromosomal segregation extends lifespan in mice and protect against cancer (Baker et al., 2013).

Another important part of cellular repair is the maintenance of telomeres. Telomere attrition is a hallmark of ageing as extensive work has shown that telomeres shorten upon each cellular division (Bernardes de Jesus and Blasco, 2013). Cancer cells have overcome

this problem by over-expressing telomerase enzyme to extend chromosome ends. Though telomere-shortening correlates with ageing there had always been concern about the therapeutic value of over-expressing enzymes preventing telomere attrition as this could potentially lead to cancer. However, it was recently demonstrated that telomerase activation can delay ageing and, importantly, this was achieved without increasing cancer incidence in mutant mice (Bernardes de Jesus et al., 2012). Overall, these examples clearly demonstrate the relevance of DNA damage prevention (or its repair) and genome integrity for ageing. Furthermore, the fine balance between stem cell maintenance and tumor suppression should be cautiously considered (Serrano and Blasco, 2007).

Protein homeostasis or proteostasis referrers to the adequate balance between synthesis, folding, trafficking and secretion, and degradation of proteins (Labbadia and Morimoto, 2014; Morimoto and Cuervo, 2014). All cells are equipped with a set of exquisitely regulated processes for protein quality control and organelle surveillance called the proteostasis network (PN) (Labbadia and Morimoto, 2014; Shore and Ruvkun, 2013; Taylor and Dillin, 2011). The PN includes control of protein translation, molecular chaperones, the ubiquitin-proteasomal system (UPS) and the autophagy-lysosomal (AL) system. These processes, which also include the ones regulating protein trafficking and secretion, are coupled with an array of stress-regulated pathways that ensure appropriate response under physiological and, more importantly, under pathologic conditions (Labbadia and Morimoto, 2014; Taylor et al., 2014). Proteostasis and the regulation of the PN are a hallmark of ageing because when proteolysis is dampened lifespan is shortened, while stimulation either genetically or pharmacologically extends lifespan. Every major lifespan-extending intervention has at least one proteostatic process downstream. Unfortunately with age the rate of damage accumulation increases, this can be ascertained by the amount of accumulated damaged cells and by the rate of mistakes in folding and appropriate quality surveyance performed. Proteostasis collapse is likely to be an early event during ageing (Labbadia and Morimoto, 2014; LOpez-Otin et al., 2013).

Protein synthesis is a tightly regulated process consisting of three phases. In the initiation phase the mRNA is recruited to the ribosome by a host of translation initiation factors (particularly from the groups of eukaryotic initiation factor 4 (eIF4) and eIF2) that will interact with the mRNA and the ribosome. During the elongation phase the mRNA is translated into a newly synthesised protein, after which it is released from the ribosome in the termination phase (Browne and Proud, 2002; Scheper et al., 2006). Protein translation has been shown to be under the control of the growth and nutrient-sensing network (see

below) and required for lifespan extension under dietary restriction. Interestingly, though interventions to target the initiation phase of translation are the most widely studied for pathology and longevity, activity of the elongation phase is inversely related with translation fidelity (Browne and Proud, 2002). It has been calculated that approximately 15% of translated proteins in *Escherichia coli* will contain amino acid mis-incorporations (Drummond and Wilke, 2009; Ogle and Ramakrishnan, 2005; Parker, 1989). Mistranslated proteins are likely to be aberrantly folded and aggregate. This will be particularly toxic for post mitotic long-lived cells like neurons. Hence, interventions to reduce protein translation could be beneficial for ageing and disease (Proud, 2002; Sherman and Qian, 2013; Taylor and Dillin, 2011). During the conversion of genes to proteins, numerous enzymes involved in replication, transcription, translation and folding are involved in safeguarding the fidelity of the information. When this information is corrupt, cells opt to either correct the problem, or, when the error or damage surpasses the repair capacity, target for degradation (Ben-Gedalya and Cohen, 2012; Taylor and Dillin, 2011). Even under physiological conditions, proteins are constantly being synthesised and destroyed. This is a continuous process, and although it could be conceived as energetically costly, it minimizes damage to proteins in the hazardous environment of the intracellular compartment (Martinez-Vicente et al., 2005; Wong and Cuervo, 2010). Mis-folding and protein accumulation have detrimental consequences for cell physiology and this is clearly manifested in aggregation-prone neurodegenerative diseases like Alzheimer's and Parkinson's disease (Ben-Gedalya and Cohen, 2012; Wong and Cuervo, 2010).

After protein synthesis, the newly synthesised peptide requires adequate conformational integrity to function properly. For this the protein will require to fold to obtain its appropriate three-dimensional structure (sometimes quaternary) assisted by molecular chaperones which will prevent mis-folding (Valastyan and Lindquist, 2014). Proteins can start the folding process at the same time as they are synthesised (co-translational folding), others are folded in the cytoplasm and endoplasmic reticulum (ER) (Dobson, 2004; Wolff et al., 2014). Co-translational folding is assisted by a subset of heat shock proteins (HSP40 and HSP70). Mis-folded and potentially toxic proteins are assisted by HSP104 for disassembly, refolding and potential aggregation into less toxic species (Taylor and Dillin, 2011; Wolff et al., 2014). These and other HSPs are under the transcriptional regulation of the heat shock factor-1 (HSF1). Several of them are transcriptionally up-regulated under conditions of reduced signalling through the nutrient sensing network and can extend lifespan when over-expressed in worms and flies (Hsu et

al., 2003; Morley and Morimoto, 2004; Morrow et al., 2004; Murphy et al., 2003; Tower, 2009).

To safeguard the proteome the PN is coupled with several stress responses including the heat shock response, the ER unfolded protein response (UPR) and the mitochondrial UPR (UPR^{mt}) (Labbadia and Morimoto, 2014; Taylor et al., 2014). During ageing the appropriate induction of these stress-response pathways is dampened making them driving forces of proteostasis collapse (Haigis and Yankner, 2010; Taylor et al., 2014).

In spite of the processes ensuring molecular fidelity, some proteins escape quality control or accumulate damage over time. To minimise the toxic effects of aggregated proteins, cells use the UPS and AL system for degradation. Old and damaged proteins are marked and targeted by molecular chaperones for degradation by the proteasome. This process in comparison to degradation by autophagy is highly selective as proteins are tagged by E3-ligase-mediated ubiquitylation of which those with lysine residue 48 (K48)-linked polyubiquitin are directed to the proteasome (Labbadia and Morimoto, 2014; Taylor and Dillin, 2011). Proteasomal function downstream of the growth factors and nutrient sensing regulate lifespan in yeast and worms (Carrano et al., 2009; Kruegel et al., 2011; Liu et al., 2011; Vilchez et al., 2012).

Alternatively cells can make use of autophagy for a less targeted degradation process. Autophagy can refer to the bulk engulfing of cellular material called macroautophagy, a more targeted approach called chaperone-mediated autophagy (CMA) and microautophagy. (Cuervo, 2008; Levine and Kroemer, 2008; Martinez-Vicente et al., 2005). When I use the term autophagy I will be referring to macroautophagy as this is the most studied form of autophagy in biogerontology. Autophagy refers to the self-eating process that uses the lysosome for degradation of proteins, cellular organelles and even pathogens (Choi et al., 2013; Kroemer et al., 2010). Autophagy ensures survival under starvation conditions as it recycles cellular material within the cells to allow continual cellular function. The evolutionary conservation of the machinery controlling the process is exquisite and denotes the relevance of the process across taxa. Just after birth mammals require appropriate autophagy function for survival, inability to induce autophagy during the first few hours of life limits lifespan (Efeyan et al., 2012). The function and induction capacity of autophagy declines with age, yet the underlying mechanism is poorly understood (Cuervo, 2008). In *Drosophila* loss of the autophagy gene atg7, required for autophagy induction, reduces lifespan, sensitises against starvation and the redox cycler

paraquat, leads to accumulation of ubiquitylated proteins and impairs locomotor ability (Juhdsz et al., 2007). Similarly, in mammals, loss of atg7 in neurons reduces median lifespan of mice to below 10 weeks (lifespan can be up to 3 years), leads to the accumulation of polyubiquitylated proteins, cell death in cerebral cortex and cerebellum and the concomitant behavioural defects of neurodegeneration (Komatsu et al., 2006). A similar scenario is also observed in mice deficient for atg5 in neurons (Hara et al., 2006).

Autophagy is regulated by the nutrient sensor mTOR and also by mTOR independent mechanisms (Ravikumar et al., 2010). As I will discuss later autophagy seems to be an important process downstream of many genetic and environmental interventions that increase healthy ageing (Cuervo, 2008; Vellai et al., 2009). For example, dietary restriction (DR), the most successful intervention to delay ageing across taxa, is thought to confer its benefits, partly through induction of autophagy (Bergamini et al., 2003; Cavallini et al., 2001).

1.3.2 Mitochondrial Dysfunction and Mitohormesis

As mentioned earlier, one of the proposed players driving ageing is the accumulation of oxidative modifications by free radicals or ROS. The damage accumulated by ROS was thought to be an initiating event leading to cellular senescence and aging (Harman, 1956; Muller et al., 2007). This view has been changing in recent years as the evidence against the free radical theory of ageing mounts up (Blagosklonny, 2008; Doonan et al., 2008; Gems and De la Guardia, 2013; Hekimi et al., 2011; Kawagishi and Finkel, 2014; Ristow, 2014; Stuart et al., 2014). Studies in model organisms have shown that lifespan extension can co-exist with damage accumulation (or without reducing damage), reduction in damage and stress-resistance can be uncoupled from lifespan extension and that toxin-induced mitochondrial stress (with ROS generators) can extend lifespan (Cabreiro et al., 2011; Doonan et al., 2008; Frankowski et al., 2013; Van Raamsdonk and Hekimi, 2009; Schmeisser et al., 2013a; Valentini et al., 2012; Yang and Hekimi, 2010).

Dysfunctional mitochondria are regarded as pathogenic markers of several clinical entities including neurodegenerative diseases (Schapira, 2008; Schapira and Gegg, 2011). For example, inhibition of mitochondrial complex I in dopaminergic neurons leads to parkinsonism in humans and rodents (Schapira, 2010). The knowledge of toxin-induced complex I inhibition has often been used to generate non-protein aggregation Parkinsonian models in flies and rodents (Bayersdorfer et al., 2010; Blesa et al., 2012). In contrast, it has

been reported that mild reductions of the function of several of the mitochondria' respiratory complexes (I, III, IV and ATPase) in worms and flies extends lifespan and protects against paraquat-induced oxidative stress (Copeland et al., 2009; Dillin et al., 2002; Lapointe and Hekimi, 2008; Lapointe et al., 2012). Moreover, a recent report showed that the degree of mito-nuclear imbalance determines the longevity phenotype (Houtkooper et al., 2013). The role of the mitochondria in ageing is continuously being explored. Recently it was identified that over-expression of the mitochondrial biogenesis transcription factor peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1a), particularly in the gut stem cells of *Drosophila*, resulted in extended lifespan, preservation of the function of the mitochondria' respiratory chain and the gut microarchitecture integrity (Rera et al., 2011). Altered function or activity of PGC-la has been implicated in a plethora of age-related diseases including diabetes, obesity, Alzheimer's and Parkinson's disease (Castillo-Quan, 2011, 2012; Robinson et al., 2013; Spiegelman, 2013).

These rather contrasting, and sometimes conflicting, results showing that reduced function of mitochondria' components leads to disease in some cases and healthy lifespan extension in others, can perhaps be reconciled in terms of degree and appropriate stress response. When cells encounter a stressful situation, for example reduced mitochondria' respiration, they are able to mount up a stress response. Either the degree or duration of the stressful situation regulates the stress response. When the stress is low or brief and cells are capable of activating repair processes, survival is ensured. However, if the stress is to strong or last for longer than what the energetic capacity of the organism allows, or the cell is unable to appropriately respond it will die. It can be proposed that, for example, the observed mitochondrial complex I inhibition in PD patients, and in long lived organisms vary in duration and intensity, and this influences the capacity of cells and organisms to appropriately respond to this stressful event. However, we are still far from understanding the role of mitochondria in ageing.

Genetic or pharmacological manipulations of the respiratory chain are thought to induce oxidative stress and UPR^{mt} (Houtkooper et al., 2013; Schmeisser et al., 2013a, 2013b; Yang and Hekimi, 2010). The stress caused by the abnormal mitochondria activates an oxidative and xenobiotic response transcriptionally orchestrated by SKN-1 in worms, cap'n'collar (cnc) in flies and the nuclear factor erythroid-2 related factor (NRF)-2 in mammals (Mattson, 2008a; Rattan, 2001). Induction of a small amount of stress has been associated with lifespan extension in several reports. The underlying mechanisms

seem to be the up-regulation of cellular repair processes. Stress-response pathways increase lifespan perhaps by re-allocating energy resources in somatic maintenance, i.e., investing in the control of molecular fidelity.

The lifespan and healthspan benefits derived from sublethal exposure to toxins or stressors have been called hormesis (Calabrese, 2013; Gems and Partridge, 2008; Lithgow, 2001; Mattson, 2008b). Hormesis (a term taken from toxicology) refers to the biphasic response observed during the study of drugs and compounds. In pharmacology and toxicology small amounts of a toxin will provide a stimulatory or beneficial effect, while increasing the concentration will lead to inhibition or, in the case of ageing, lifespan reduction (Calabrese et al., 2007; Rattan, 2004; Ristow, 2014; Ristow and Schmeisser, 2011). To date several toxins have been tested for their ability to modulate lifespan in model organisms and lower doses confer benefits while high doses become progressively detrimental (Schmeisser et al., 2013a; Yang and Hekimi, 2010). The hormetic response is not limited to toxins. For example, the positive effects of food restriction are thought to elicit a stress-adaptive mechanism that increases cellular maintenance and repair (Masoro, 2007; Schmeisser et al., 2013b; Schulz et al., 2007). Similarly, gradual adaptation to heat stress can confer longevity in worms, and resistance to a subsequent heat shock (Epel and Lithgow, 2014; Lithgow, 2001). The underlying mechanism could be the progressive up-regulation of defence processes that are then already in place for protection when challenged with a stronger stressor (priming). The hormetic response can also account for the positive lifespan effects of phytochemical and compounds isolated from plants (including fruits and vegetables). Plants also respond to their environment by up-regulating stress-response mechanisms. Molecules produced during the stress-phase are thought to induce a hormetic response in the organisms consuming the plant. This is xenohormesis (Goldberg et al., 2010; Howitz and Sinclair, 2008; Surh, 2011); while when the stressor-signal is induced within the mitochondria is called mitohormesis (Ristow, 2014; Ristow and Zarse, 2010; Yun and Finkel, 2014).

1.3.3 Dietary Restriction and the Nutrient Sensing Network

1.3.3.1 Dietary restriction (DR)

To date DR is the most successful environmental intervention to extend lifespan in organisms ranging from yeast to mammals (Fontana et al., 2010; Piper and Partridge, 2007). First observed in rodents at the beginning of last century (McCay et al., 1935;

Osborne et al., 1917), DR has shown extraordinary evolutionary conservation. Different forms of DR can prolong healthy lifespan from the unicellular yeast to non-human primates and have even shown to improve health in humans (Fontana et al., 2010).

DR is still ill-defined, but it generally refers to the reduction of food intake without malnourishment (Katewa and Kapahi, 2010; Piper and Partridge, 2007). DR protocols include caloric restriction where total caloric intake per day is modulated irrespective of the source of calories (lipids, carbohydrates or proteins). In spite of its wide use in the literature, nowadays we understand that certain components of the diet impose greater effects on survival and longevity than others, revealing the complex nature of the interaction of diet with ageing (Piper et al., 2005, 2011). Moreover, multiple DR protocols exist in different species making it virtually impossible to compare studies that report lifespan extension employing completely different approaches even when using the same species. For example, DR in *C. elegans* can be performed by dilution of the bacteria in which it is co-cultured, eliminating the bacteria alltogether (axenic liquid media which contains nutrients, but lacks *E. coli),* or by mutations that affect the neuromuscular regulation of pharyngeal pumping (anorexia-induced DR), etc. It is worth mentioning that different DR regimes have varying degrees of impact on fecundity, metabolism and lifespan (Mair and Dillin, 2008; Piper and Bartke, 2008). While anorexia-induced DR by mutations in the *eat2* gene extend median lifespan by 46%, axenic media extends lifespan by up to 85% (the biggest DR effect in *C. elegans)* (Mair and Dillin, 2008). Unless different *eat2* mutant alelles with different degrees of pharyngeal pumping are used, these two examples of DR protocols in *C. elegans* only allows for DR to be tested as a discrete rather than a continous variable. Using DR as a continous variable is very important and informative when assesing the molecular mechanisms of DR (the interaction of a particular gene with the diet) or the effect of a compound (a potential DR-mimetic). In rodents, though the effects of DR can be studied as a continuos variable (different values of caloric restriction, days of of intermittent fasting, etc), only one DR regime is usually employed (Cerqueira and Kowaltowski, 2010; Longo and Mattson, 2014). Using only one DR regime against the *ad libitum* condition complicates the analysis of the interaction of DR with other interventions.

DR studies in *Drosophila* are performed a bit differently than in rodents and *C. elegans.* Instead of reducing the food provided, the diet is manipulated by dilution or reduction of specific components and then provided *ad libitum* (Bass et al., 2007; Tatar et al., 2014). *Drosophila* diet used in ageing studies is usually comprised of a carbohydrate

source (usually sucrose) and yeast as a source of amino acids, vitamins, minerals, cholesterol, and essential fatty acids held together by the combination of water and agar (Bass et al., 2007; Skorupa et al., 2008). Some studies also add cornmeal and dextrose to the diet (Min et al., 2008; Zid et al., 2009). In *Drosophila* it has been shown that modifying the carbohydrate content or the amount of water in the diet has minimal effects on lifespan, comparatively to the effects conferred by modifying the yeast component of the medium (Grandison et al., 2009b; Mair et al., 2005; Piper et al., 2010; Skorupa et al., 2008). Therefore, to evaluate food restriction as a continuous variable, the yeast component is modified from 0-200 grams (or more) per litre keeping the carbohydrate component constant. At least 4 to 5 yeast concentrations are tested. Lifespan increases gradually up to a maximum after which further increases in the yeast concentration become detrimental for lifespan. Plotting the median or maximum lifespan values always depicts a tent-shaped curve. The yeast concentration that confers the biggest lifespan extension is considered the optimal and therefore the DR condition. Values on either side of the DR condition limit lifespan by either malnourishment (left hand side of the DR tent) or over-nourishment (right hand side) (Bass et al., 2007; Grandison et al., 2009b; Metaxakis and Partridge, 2013). When a molecular target is being tested as a mechanism for lifespan extension under DR, the interaction of the different dietary regimes with the gene/intervention of interest reveals whether the non-dietary intervention acts in the same molecular pathway. In a hypothetical scenario where the effect of over-expressing the transcription factor involved in lysosomal biogenesis (transcription factor EB (TF-EB) or mitochondrial biogenesis PGC-1 a were tested as the molecular mechanism for lifespan extension under DR, one of the three scenarios can occur. First, a true DR mechanism would show a right-shifted DR tent, where the lifespan benefits would only be observed at the higher end of the yeast concentration (Figure 1.8A). Given that the intervention is already maximised for lifespan, food restriction would not be able to further extend lifespan and is likely to reduce it. Second, the intervention is able to increase lifespan under conditions maximised for DR, but to a minimum extent in comparison to the effect under over-nourishment (Figure 1.8B). Perhaps in this scenario the intervention tested and DR have converged molecularly, but not enough to right-shift the tent. Thirdly, the intervention in question would extend lifespan irrespective of the food condition tested (Figure 1.8C). This would be an unlikely molecular mechanism of DR as the effects seem additive (Mair and Dillin, 2008).

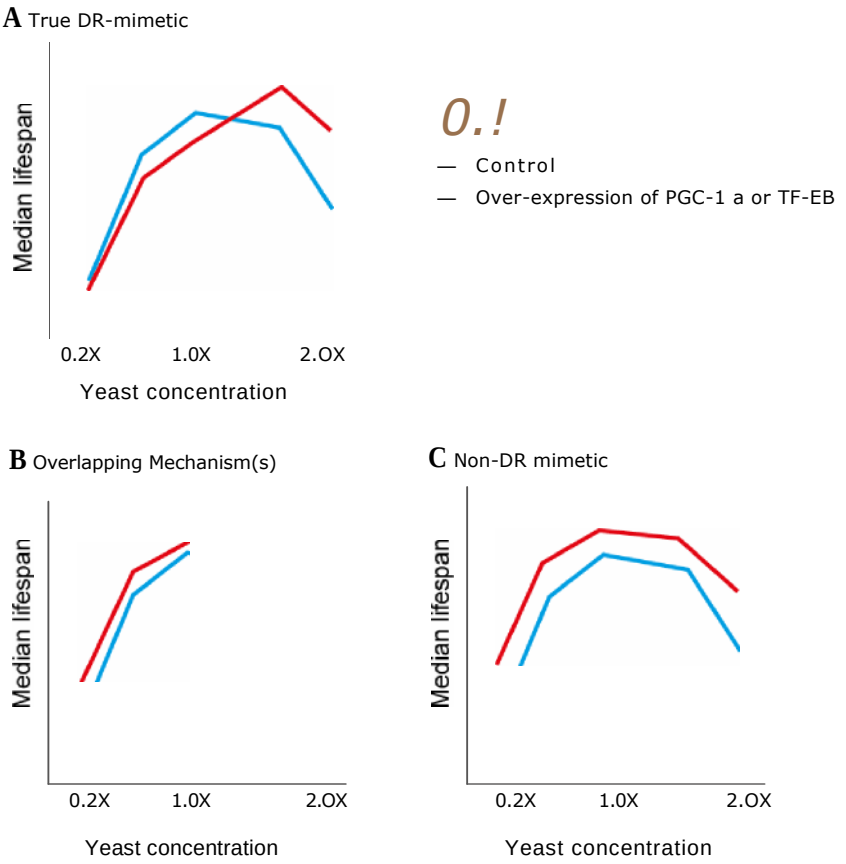


Figure 1.8 Testing a genetic pro-longevity intervention and its relationship with DR. The effect of over-expressing the mitochondria' biogenesis transcription factor PGC-la or the lysosomal biogenesis transcription factor TF-EB. (A) In this case the effect is of a true DR-mimetic, (B) the mechanisms are overlapping, but in (C) they are completely independent.

As we uncover the effects of specific nutrients, targeted protocols for evaluting interactions of non-dietary interventions will emerge. So far, we have known for a while that within proteins, certain amino acids regulate lifespan with greater effects (Grandison et al., 2009a; Piper et al., 2014). Methionine restriction extends lifespan of yeast (Johnson and Johnson, 2014; Ruckenstuhl et al., 2014; Wu et al., 2013), flies (Grandison et al., 2009a; Lee et al., 2014), mice (Miller et al., 2005), and rats (Orentreich et al., 1993). Moreover, methionine restriction in yeast cells, and mouse and human fibroblasts has been reported to confer resistance to several cytotoxic stressors (Johnson and Johnson, 2014). In mice, methionine restrcition leads to reduced body weight, improved glucose and lipid metabolism, increased expression of the starvation hormone fibroblast growth factor-21 (FGF-21) (in blood and hepatocytes), PGC-la and the branch of the UPR that responds to aminoacids (GCN-2-eIF2a) (Ables et al., 2012; Lees et al., 2014). The authors speculated that the reduction in circulating methionine could drive the expression of FGF-21 downstream of PGC- 1 a and GCN-2 (general control non-derepresible 2) (Lees et al., 2014). Interestingly, over-expression of FGF-21 in hepatocytes (which increases circulating levels by 5-10 fold) increased median lifespan of male and female mice by

36%. The authors identified that over-expression of FGF-21 reduced the risk of death by 65% in males and 88% in females (Zhang et al., 2012). Interestingly, these animals did not show compensatory over-feeding as elicited by methione restriction (Lees et al., 2014; Zhang et al., 2012). Additionally methione restricted mice show reduced circulating levels of insulin, IGF-1 and thyroid hormone (Miller et al., 2005).

The widespread health benefits of DR have encouraged biogerontologists to try and understand the molecular mechanisms behind the effects of DR. Research of the pathways responsive to dietary macromolecules, carbohydrates and proteins have unveiled that ageing is plastic, evolutionary conserved and can be targeted pharmacologically (Fontana et al., 2010; Kenyon, 2010).

1.3.3.2 Insulin/IGF-1 signalling pathway in ageing

Two nutrient signalling pathways have been implicated in the ageing process. The first evidence of the role of the insulin signalling pathway in ageing came from a mutagenic study performed in *C. elegans* by Klass showing the fundamental principle that lifespan can be modified genetically (Klass, 1983). Gary Ruvkun's group found that the long-lived mutation induced in Klass's experiments was in a gene which encodes for the worm phosphatidylinositol-3-OH kinase (PI3K), *age-1* (Finch and Ruvkun, 2001; Johnson, 2013). Several other groups extended the work to show that down-regulation of the insulin/IGF-1 signalling (IIS) pathway extended lifespan (Finch and Ruvkun, 2001; Kenyon, 2006, 2011).

In this section I will describe the components of the IIS pathway in *C. elegans*, *Drosophila* and mammals. Interestingly, hormonal regulation between these organisms is highly conserved (Figure 1.9). I will also do this by describing the components of the pathway from ligand(s) to nucleus highlighting functions those that have been shown to regulate ageing.

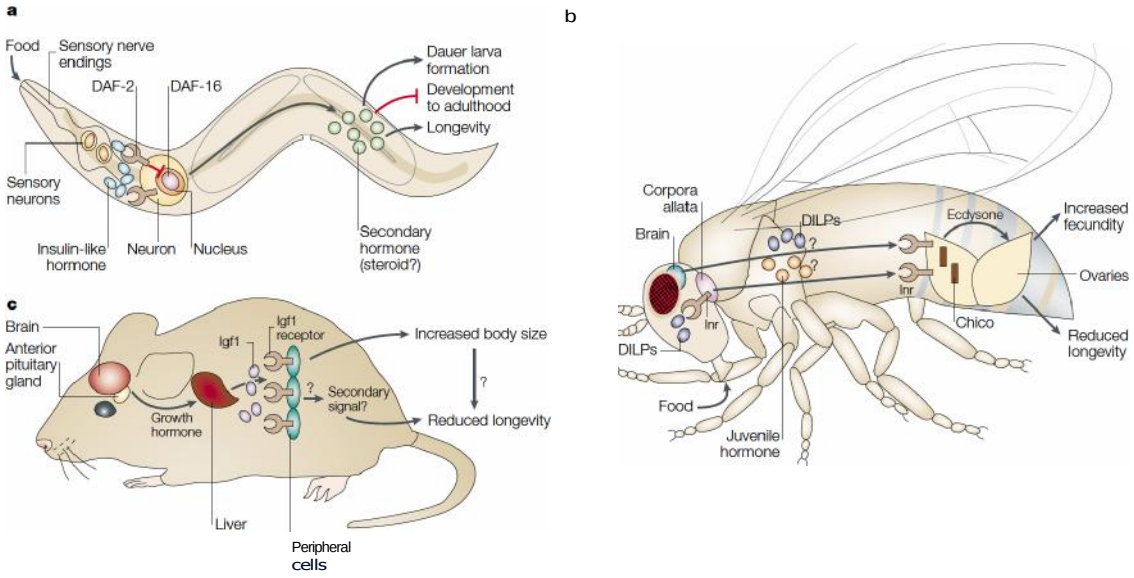


Figure 1.9 Hormonal regulation is highly conserved between *C. elegans, Drosophila* and mice. These cartoons show the high degree of evolutionary conservation between (a) C. *elegans*, (b) *Drosophila* and (c) mice for the components of the IIS pathway. Taken from (Partridge and Gems, 2002).

Ligands

C. elegans has ~40 insulin-like peptides expressed primarily in neurons, though a few are also expressed in the intestine (Figure 1.10). Some of these insulin-like molecules act as agonists of the worm insulin receptor daf2, while others act as antagonists (Kaletsky and Murphy, 2010). Given the different functional nature of these signals it is not entirely surprising that they are differentially regulated at the transcriptional level in response to reduced IIS signal. Ins-7, one of these peptides, is repressed in animals lacking daf2; when ins-7 is knocked down by RNAi, worms are significantly long-lived in comparison to their respective controls (Murphy et al., 2003). *Drosophila* has 8 insulin-like peptides (dILPs). In adult physiology and the regulation of ageing, the most important ones seem to be dILPs 2, 3 and 5 synthesised in the median-neurosecretory cells (MNCs) in the fly brain, and dILP 6 produced in the fat body (the fly equivalent of the liver/adipose tissue). Ablation of the MNCs, or genetic deletion of dILPs 2, 3 and 5 extend lifespan in *Drosophila* (Broughton et al., 2008, 2005; Griinke et al., 2010). On the other hand, over-expression of dilp6 in the fat body extends lifespan (Bai et al., 2012). In mammals, insulin is produced by pancreatic I3-cells, whereas Insulin-like Growth Factor-1 (IGF1) and 2 are mainly produced in the liver, but synthesized in almost all tissues (Kaplan and Cohen, 2007). It is important to note that IGFs are synthesized in the liver in response to Growth Hormone (GH) released from the pituitary gland in the brain. Reducing the level of GH or the elements that regulate its production, and the IGFs show some overlap to promote

longevity (Bartke, 2008; Brown-Borg et al., 1996) and show similar phenotypical characteristics (Bartke et al., 2013). Reduced levels of insulin (hypoinsulinemia) lead to high glucose levels, which clinically translates into type I diabetes mellitus, commonly due to autoimmune destruction of the insulin producing 0-pancreatic cells. On the other hand, type II diabetes follows peripheral insulin resistance with a compensatory hypertrophy of (3 cells to synthesize more insulin and lead to hyperinsulinemia (Castillo-Quan et al., 2010). Both ends of this spectrum, lower or higher circulating levels of insulin, are pathological. Therefore, speculations lurked over the relevance of the finding in invertebrates that reducing insulin signalling *per se* could extend healthy lifespan (see below).

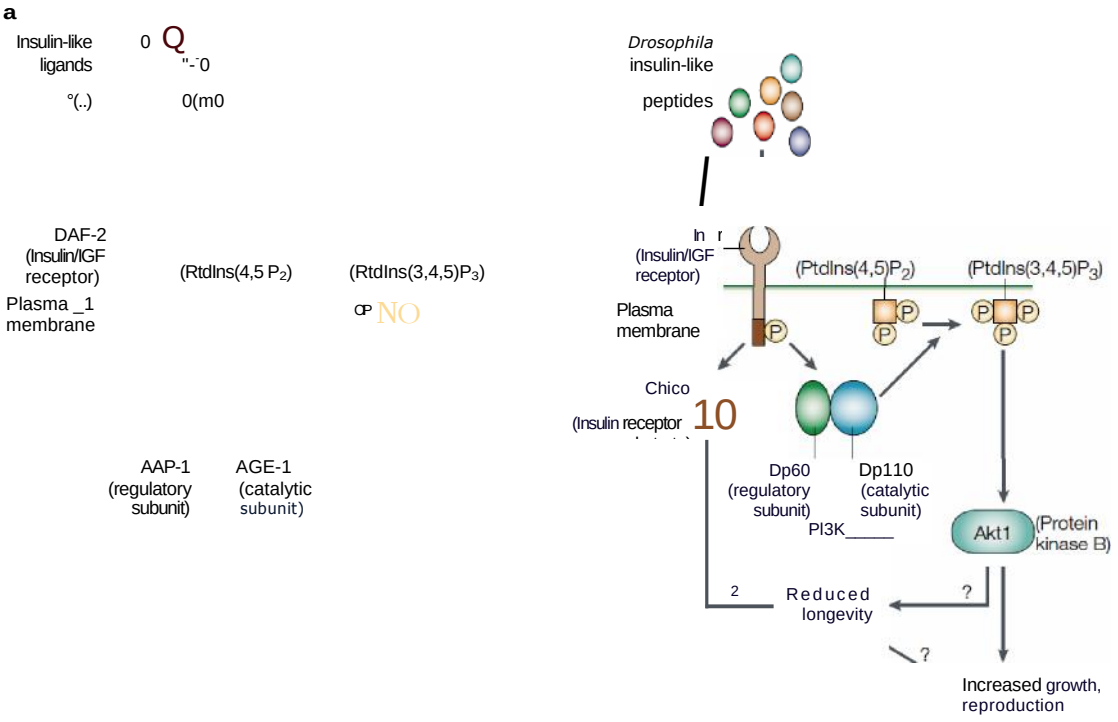
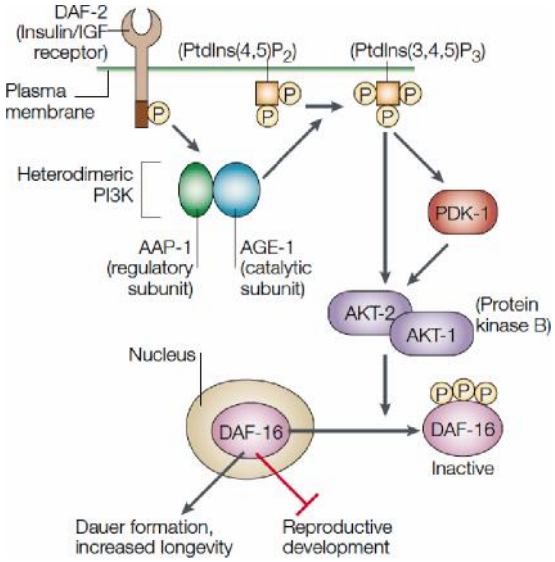


Figure 1.10 IIS pathway in C. *elegans* and *Drosophila*. (a) shows a schematic diagram of the IIS pathway in *C. elegans*. The *Drosophila* IIS pathway is shown in (b). Please note the lack of the transcription factor FOXO in the fly diagram that should be immediately downstream of Akt (as DAF-16 in the worm). At the time this diagram was produced the requirement of FOXO for lifespan extension under IIS down-regulation in the fly had not been shown experimentally. Taken from (Partridge and Gems, 2002).

Ligand levels can be regulated at several different stages, such as their synthesis, post-translational processing, secretion, or binding to partner proteins, which can modulate their circulation or bioavailability in the systemic environment. IGFs circulate bound to IGF binding proteins (IFGBP) of which 6 have been identified. IGFBPs protect IGFs from degradation and also compete with them for the IGF receptors (IGFR) (Kaplan and Cohen,



2007). Since IGFBPs can potentially down-regulate the IGF signalling pathway, over-expressing them could lead to lifespan extension. For instance, increased expression of the dILP-binding protein ImpL2, a homologue of IGFBP7, extends lifespan in *Drosophila* by reducing signalling through dILP2 and dILP5 (Alic et al., 2011a). No mammalian study has described the effects of loss or over-expression of any of these IIS binding partners on the ageing process. However, mammalian IFGBPs are subject to protease degradation. Pregnancy-associated plasma protein A (PAPP-A), is a metalloproteinase involved in IGFBPs degradation, in particular of IGFBP4. Loss of PAPP-A leads to a 38% lifespan extension of both male (33%) and female (41%) mice (Conover and Bale, 2007).

Receptor

Next the ligand binds to the insulin/IGF receptor at the plasma membrane surface. Whereas the invertebrate model organisms have an abundance of different insulin-like ligands, both *C. elegans* and *Drosophila* have only a single receptor (DAF-2 in worms and dInR in flies; Figure 1.10). The situation in mammals is more complex with multiple receptors existing, including the insulin receptor (IR; spliced in two isoforms), IGF receptors (IGF-1R and IGF-2R), and heterodimers IR-IGF-1R. Furthermore, insulin can bind and activate the heterodimers and the IGF-1R, though the affinity is 100 and 1000-fold weaker, respectively (Buck and Mulvihill, 2011; Jensen and De Meyts, 2009).

In the context of ageing, many long-lived models act at the level of the insulin/IGF receptor. Indeed, the first systematic description of lifespan extension due to a single gene mutation was a worm *daf-2* mutant (Kenyon et al., 1993). Subsequently, it was shown that a dInR mutant allele with reduced kinase activity showed increased lifespan (Tatar et al., 2001). Our group later showed that over-expression of a dominant-negative form of dInR makes flies long-lived (Ikeya et al., 2009). In mice, IR and IGF-1R are necessary for early development and growth. Mice lacking either of them die 4 days postnatal, or at birth, respectively (Accili et al., 1996; Liu et al., 1993). Given this it was surprising when the first long-lived IIS mammalian mutant described to be long-lived was a heterozygous null IGF-1R KO mice that lives 26% longer than controls when both sexes are pooled; 33% longer for females and 16% for males (Holzenberger et al., 2003), although subsequent work at San Antonio by Arlan Richardson's team largely failed to confirm this (Bokov et al., 2011) Almost simultaneously it was published that mice lacking the insulin receptor in adipose tissue (FIRKO) show extended lifespan, reduced insulin levels, and resistance to age-related loss of glucose homeostasis and diet-induced obesity (Bliiher et al., 2003).

Binding of the ligand to its receptor induces autophosphorylation and subsequent recruitment and phosphorylation of its substrate.

Tissue-specific effects of HS down-regulation

It is very interesting that the FIRKO mice are currently, the only tissue-specific deletion of the IR shown to be long-lived. Other IR tissue-specific KOs like liver (LIRKO), muscle (MIRKO) and neuron-specific (NIRKO) develop insulin resistance and/or diabetes along with several other metabolic alterations; even I3-pancreatic cell-specific IR-KO mice ((3IRKO) develop metabolic alterations making them unlikely to be long-lived (Bruning, 2000; Briining et al., 1998; Kulkarni et al., 1999; Michael et al., 2000). This tissue specificity is also highlighted by the fact that mice lacking IGF-1R specifically in the (3-pancreatic cells also develop insulin resistance (Kulkarni et al., 2002), while mice heterozygous for the IGF-1R in the central nervous system live longer (Kappeler et al., 2008).

Intracellular cascade

The first component of the intracellular IIS cascade is the insulin receptor substrate (IRS), which interacts with the activated insulin receptor and relays the signal to other proteins to convey the message to the nucleus. Unlike mammals that posses three or four IRS proteins, *Drosophila* only has one IRS called *chico* (small in Spanish for the phenotype they produced when missing). In flies, autophosphorylation of the dInR recruits CHICO and the adaptor SH2B Lnk protein to relay the signal downstream. Female flies carrying a deletion for the IRS *chico* live 48% longer, while the lifespan extension in males is of only 13% (Clancy et al., 2001). Flies lacking Lnk show a more modest yet robust lifespan extension. Lifespan extension by most *Drosophila* insulin mutants is greater in females (and dependent on the cytoplasmic endosymbiont *Wolbachia),* but the longevity effect of Lnk is not only independent of sex, but also of genetic and cytoplasmic background (Ikeya et al., 2009; Partridge et al., 2005; Slack et al., 2010). The *chico* and dInR with reduced kinase activity were the first long-lived mutants reported for *Drosophila* (Clancy et al., 2001; Tatar et al., 2001). Human's possess two widely expressed IRS proteins, IRS1 and IRS2 (also the most studied ones); IRS4 expression is limited to the thymus, brain, kidneys and I3-cells. Rodents on the other hand also express IRS3 in the adipose tissue (White, 2002). In mice, global $Irs1^{-/-}$ null mutants show an extended lifespan. Females are long-lived relative to controls by 32%, while males are

16% longer lived than controls (Selman et al., 2008a, 2011). Importantly, long-lived female IRS1 null mice show delayed onset of ageing pathologies including osteoporosis, cataracts, ulcerative dermatitis, as well as preservation of glucose homeostasis, immunity (T cells), and motor function. Unlike $Irs1^{-/-}$ mice, global $Irs2^{-/-}$ null mice are short-lived (Selman et al., 2008a). The main difference between mice lacking IRS1 or IRS2 might stem from the deficient downstream signalling in the $Irs2^{-/-}$ null mice which show reduced phosphatidylinositol-3-OH kinase (PI3K) activation, which leads to overt diabetes, while Irs1-/- mice develop insulin resistance, but no diabetes (Selman et al., 2008a; Withers et al., 1998). Tissue-specific complexity is also revealed at this level where brain-specific knockout of IRS2 has been reported to extend murine lifespan (Taguchi et al., 2007), though this result has been challenged (Selman et al., 2008b). In light of insufficient PI3K activation leading to shorter lifespan its surprising that the lifespan of flies lacking *chico* is greater in homozygous (48%) than in heterozygotes (36%) (Clancy et al., 2001). However, unlike in mammals, the dInR has an additional 400 amino acids at the C-terminus which allows direct communication between dInR and PI3K (Teleman, 2010). In flies, over-expression of a dominant negative version of the catalytic subunit Dp110 of dPI3K extends lifespan (Slack et al., 2011). Similarly heterozygous inactivation of the p110a of PI3K extends murine lifespan (Foukas et al., 2006).

PI3K activation leads to the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) from phosphatidylinositol-4,5-biphosphate (PIP2), which in turn allows the translocation and activation of Akt (Fayard et al., 2005; Manning and Cantley, 2007). This process is antagonised by the phosphatase PTEN that dephosphorylates lipids produced by PI3K (Manning and Cantley, 2007). Given that PI3K production is essential to activate the downstream player Akt, inhibition of IIS through PTEN seems as a straightforward intervention to extend healthy ageing. In *C. elegans* the lifespan extension by inactivation of the insulin receptor *daf-2* is dependent on appropriate function of the homologue of PTEN daf-18. Loss of function of *daf-18* abolishes the lifespan extension by *daf-2* mutants. Importantly, the inactivation of PTEN leads to reduced lifespan, which corresponds with increased IIS signalling being detrimental (Mihaylova et al., 1999). In flies, over-expression of PTEN leads to lifespan extension (Hwangbo et al., 2004). Recently it was shown that mice carrying extra genomic copies of PTEN are long-lived independent of the protection against cancer; males live 12% and females 16% longer than controls. Furthermore these mice are protected against insulin resistance, steatosis and other markers of metabolic imbalance (Ortega-Molina et al., 2012).

PIP3 production recruits Akt (also referred to as protein kinase B; PKB) and its activators (PDK1 and mTORC2) to the cell membrane where it gets phosphorylated and in turn activated. Akt is a serine/threonine protein kinase, central to cellular proliferation, metabolism and apoptosis. Akt regulates cell survival and metabolism by regulating many downstream targets (Fayard et al., 2005; Manning and Cantley, 2007). In *C. elegans* RNAi-mediated knockdown of Akt extends lifespan (Hamilton et al., 2005). *C. elegans* Akt functions solely to inhibit the nuclear translocation and transcription regulation of the transcription factor FOXO/daf-16 (Kenyon et al., 1993; Lin et al., 2001). Mutants for Akt have not been shown to be long-lived in *Drosophila* (Clancy et al., 2001) and experimental evidence in mice is lacking. In *Drosophila* and mammals, Akt regulates many downstream players involved in metabolism, fecundity, stress-response and survival, among others (Manning and Cantley, 2007; Teleman, 2010). Amongst the downstream Akt targets, the transcription factor FOXO is considered a key player (Giannakou et al., 2007; Slack et al., 2011). The longevity effect of IIS down-regulation requires the FOXO *C. elegans* homologue *daf-16* (Kenyon et al., 1993; Kwon et al., 2010). Similarly in flies, lifespan extension by genetic manipulations that reduce IIS signalling is abolished in flies lacking *dFOXO* (Slack et al., 2011). Furthermore, *daf-16/dFOXO* is required for regular survival as these mutants are shorter lived than controls (Kenyon et al., 1993; Slack et al., 2011). Although most interventions to extend healthy ageing in worms and flies do so by genetic interventions in the whole organism, *daf-16/dFOXO* seems to be required only in some tissues, where it is likely to act non-cell autonomously, although over-expression in the whole worm can extend lifespan (Kwon et al., 2010). Worms lacking *daf-16* abrogate the lifespan extension of *daf-2* mutants, but restoring the expression of *daf-16* only in the intestine increased lifespan by 50-60%, in comparison to the 5-20% lifespan increase when *daf-16* was expressed only in neurons. This suggest that the expression of *daf-16* in the worm intestine is sufficient to significantly extend lifespan, yet one or more tissues should act in concert (Libina et al., 2003). Correspondingly, in *Drosophila* over-expression of *dFOXO* in the adult intestine and fat body (similar to the adipose tissue and liver in mammals) extends lifespan (Giannakou et al., 2004; Hwangbo et al., 2004). Given its role in promoting the health benefits and longevity of IIS down-regulation, much attention has focused in identifying downstream targets.

Nuclear targets/response

In order to better understand the mechanisms of IIS-mediated lifespan extension, recent studies have focused on elucidating the downstream targets of FOXO/daf-16, which has emerged as a master regulator of numerous other transcription factors, therefore explaining the complexity of IIS (Alic et al., 2011b, 2014a; Schuster et al., 2010).

Numerous studies have reported transcriptional signatures for long-lived animals. Pinpointing a specific gene responsible for the lifespan extension is quite difficult and it seems that no individual gene is sufficient to abrogate or reproduce the lifespan extension of reduced IIS (Murphy et al., 2003).

1.3.3.3 Mechanistic target of rapamycin (mTOR) in ageing

The IIS pathway is to carbohydrates and growth factors, what the mTOR pathway is to amino acids and proteins. The mTOR pathway was first identified in yeast and then in mammals (Heitman et al., 1991; Sabatini et al., 1994). mTOR is present in two complexes, mTORC1 and mTORC2 (Figure 1.11) (Wullschleger et al., 2006). Common to both complexes are the mTOR protein, DEP domain containing mTOR-interacting protein (DEPTOR) and mammalian lethal with SEC13 protein 8 (mLST8), which seems to be dispensable for mTORC1, but necessary for complex integrity and catalytic activity of mTORC2 (Guertin and Sabatini, 2009; Kim et al., 2013). mTORC1 is formed, in addition, by regulatory associated protein of mTOR (Raptor) and Akt/PKB substrate 40 kDa (PRAS40). Raptor is central for the function of mTORC1 as it acts as a scaffold for recruiting substrates and it also phosphorylates downstream effectors. It is a primary site for the regulation of mTORC1. Both DEPTOR and PRAS40 are substrates and suppressors of mTORC1 (Guertin and Sabatini, 2009; Kim et al., 2013; Wullschleger et al., 2006). mTORC2 is not usually considered relevant in nutrient sensing, though is necessary for the phosphorylation and activation of Akt downstream of the insulin receptor and other growth factors (Guertin and Sabatini, 2009). mTORC2 consists of Raptor-independent companion of mTOR (Rictor), mSIN1 (MAPKAP1) and Protor (PRR5) (Kim et al., 2013). mTORC2 seems to be relevant for actin polymerization and cell spreading (Loewith et al., 2002; Wullschleger et al., 2006). mTORC1 is sensitive to inhibition by rapamycin, while mTORC2 is not, though it can be inhibited under prolonged exposure to rapamycin in certain cell types (Sarbassov et al., 2006; Zeng et al., 2007).

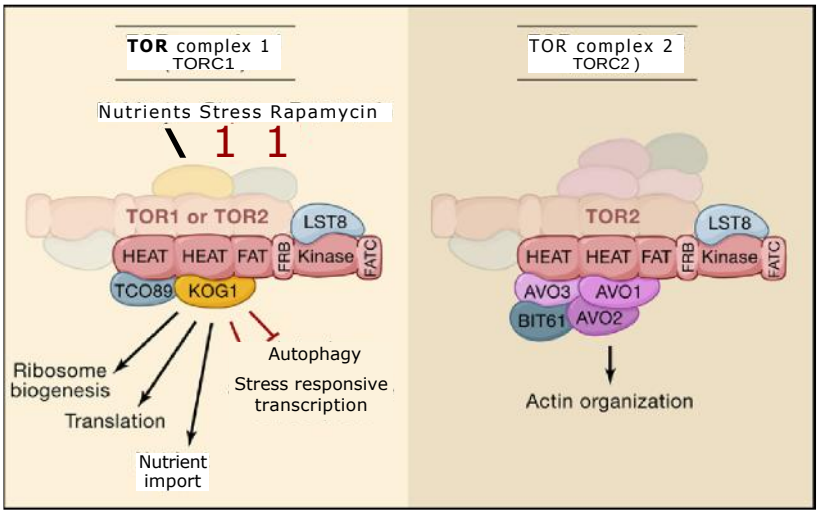


Figure 1.11 The mechanistic target of rapamycin (mTOR) presents in two complexes. mTORC1 and mTORC2 are integrated by different components and regulate diverse molecular processes. Taken from (Wullschleger et al., 2006).

mTOR is a hub for the integration of multiple signals from within and outside the cell (Figure 1.12). It receives information from growth factors through PI3K/Akt and also through the Ras-MAPK pathway (Jewell and Guan, 2013; Kim et al., 2013; Mendoza et al., 2011). Akt phosphorylates (at five sites) and inhibits the GTPase activating protein (GAP) function of tuberous sclerosis complex 2 (TSC2). TSC2 is part of a triple protein complex (TSC-TBC complex, which I will refer to as TSC complex) integrated by TSC1, TSC2 and Tre2-Bub2-Cdc16 (TBC)1 domain family member 7 (TBC1D7), upstream of mTOR (Crino et al., 2006; Dibble et al., 2012). Both TSC1 and TBC1D7 stabilize the complex, while TSC2 has GAP properties, though it has also been speculated that TBC1D7 could have GAP activity (Dibble et al., 2012). Active TSC2 inhibits the function of mTOR by acting on the Ras-homologue expressed in brain (Rheb). TSC2 stimulates the transition of active GTP-bound Rheb to the inactive GDP-bound state. Hence the GAP function of the TSC complex inhibits Rheb which results in mTOR inhibition (Crino et al., 2006). Under conditions of low stimulation by growth signals the TSC complex resides at the lysosome in close proximity to lysosomal-residing Rheb (kept here through a C-terminal farnesyl group which anchors it to the lysosomal membrane) and mTORC1. Akt phosphorylation of TSC2 redistributes the complex away from the lysosome then allowing activation of mTORC1 (Menon et al., 2014). A similar mechanism seems to regulate the inhibition of mTORC1 in the absence of amino acids (Demetriades et al., 2014).

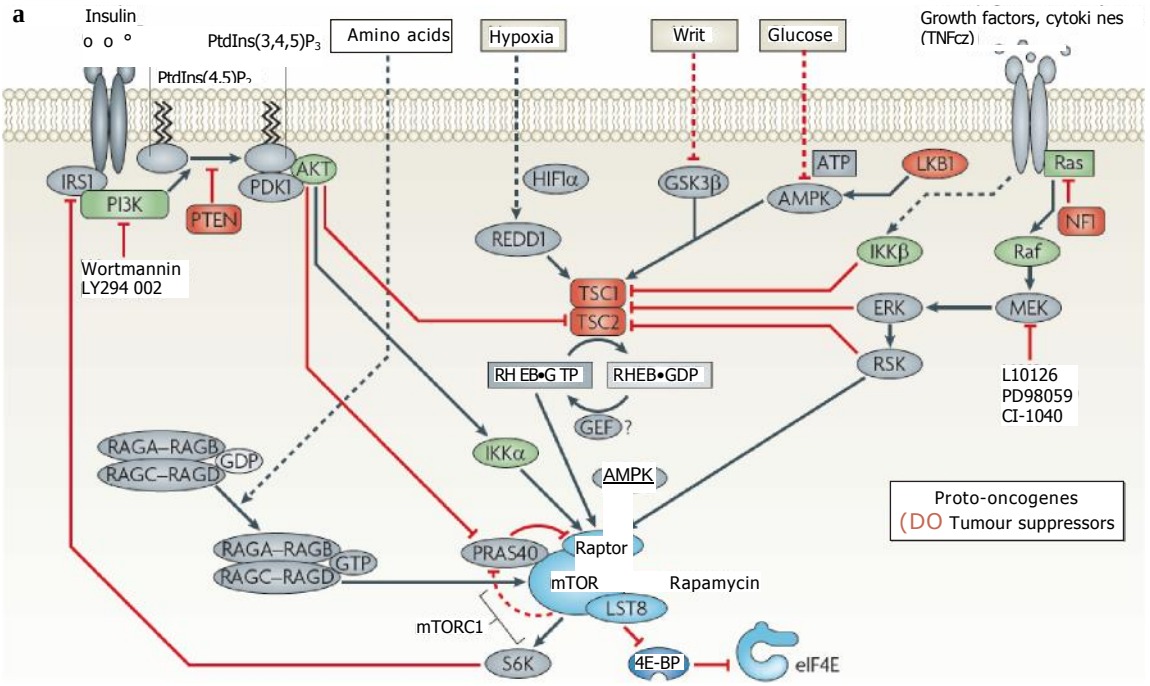


Figure 1.12 mTOR integrates multiple signals from inside an outside the cell. mTORC1 integrates many diverse signals some of which converge at the TSC2 complex. Taken from (Ma and Blenis, 2009).

The TSC complex integrates multiple signals. For example, besides the inhibition of the complex after IIS stimulation through Akt, TSC2 is also inhibited through the Ras-Erk pathway through phosphorylation directly by Erk and, indirectly, through p90 ribosomal S6 kinase (RSK), downstream of Erk (Mendoza et al., 2011; Shaw and Cantley, 2006). This allows independent activation of mTOR by PI3K/Akt and Ras-Erk. Additionally other signalling kinases like GSK-3 can also modulate mTOR signalling. GSK-3 also phosphorylates TSC2, but in turn this phosphorylation activates the GAP activity of the complex, hence inhibiting signalling through mTOR. It is important to note that this activating phosphorylation is dependent on a priming phosphorylation by 5' AMP-activated protein kinase (AMPK) (Inoki et al., 2006). Both Erk and GSK-3 can modulate mTOR independent of the TSC complex. Erkl and Erk2 interact and directly phosphorylate Raptor (Carriere et al., 2011). GSK-3 can directly phosphorylate p70 S6 kinase (S6K) regulating its function (Carriere et al., 2011). Akt can also phosphorylate PRAS40 blocking its inhibitory mTOR regulation (Sancak et al., 2007). Given the central role of mTOR in the integration of multiple cellular inputs, the stratified interaction with other signalling pathways is not surprising.

Though amino acid sensing is a primary function of mTOR it is the least understood process regulating it (Dibble and Manning, 2013; Kim et al., 2013).

Downstream of mTORC1, regulated by amino acids, growth factors or stressors, are S6K, eIF4E binding protein (4E-BP), atgl/ULK, the transcription factor EB (TF-EB), amongst others.

The rate-limiting step for translation initiation is the recruitment of the small ribosomal unit the 5' end of mRNA. For this to occur eIF4E, eIF4G and eIF4A form the eIF4F complex. During conditions of poor stimulation by growth factors or nutrients mTORC1 is poorly activated which results in hypophosphorylated 4E-BP which binds tightly to eIF4E not allowing its interaction with the other members of the eIF4F complex. Stimulation and activation of mTORC 1 kinase activity leads to phosphorylation of 4E-BP resulting in its dissociation from eIF4E which is then is free to interact with the mRNA and recruits eIF4G and eIF4A to initiate translation (Ma and Blenis, 2009; Tee and Blenis, 2005).

Additionally, active S6K or RSK can phosphorylate eIF4B, which is then recruited to the translation pre-initiation complex to increase the RNA helicase activity of eIF4A. Some mRNAs, particularly those involved in growth and proliferation, contain secondary structures in their 5' untranslated regions (UTR), inhibiting the efficient scanning of the start initiation codon. The recruitment of eIF4B to the pre-initiation site (stimulated by S6K) increases the efficiency of eIF4A to unwind the secondary structures allowing their proper translation (Ma and Blenis, 2009).

Evolutionary conserved from yeast to humans, regulation of longevity by mTOR inhibition has attracted scientific and popular interest (Johnson et al., 2013; Laplante and Sabatini, 2012; Stipp, 2012). Genetic modulation of several of the components of the mTOR pathway extend lifespan from yeast to mammals (Kapahi et al., 2004; Lamming et al., 2012; Pyo et al., 2013; Selman et al., 2009; Simonsen et al., 2008). mTOR is regulated by several cellular and environmental cues, including amino acids and growth-related factors (Bjedov and Partridge, 2011; Wullschleger et al., 2006). mTOR signals through two complexes, one of which is sensitive to rapamycin. mTORC1 regulates the activity of its downstream effectors S6K and 4E-BP. Activation of mTORC1 phosphorylates and activates S6K, while it inhibits 4E-BP also through phosphorylation. Both of these proteins are involved in the regulation of protein translation (Bjedov and Partridge, 2011; Chauvin et al., 2014; Thoreen et al., 2012). Specifically targeting protein translation (independent of mTOR) by manipulating key proteins involved in the regulation of protein synthesis has also been shown to extend lifespan in worms (Curran and Ruvkun, 2007; Hansen et al.,

2007; Kapahi et al., 2004; Pan et al., 2007; Syntichaki et al., 2007). Like with IIS down-regulation, inhibition of mTOR is associated with broad health benefits. KO of the downstream player S6K1 extends lifespan of female mice and protects them against age-related locomotor decline, loss of bone mass and gluco-metabolic alterations (Selman et al., 2009).

Another output of mTOR is the regulation of autophagy, the self-eating process by which cells replenish nutrients under starvation conditions (Cuervo, 2008; Madeo et al., 2010; Rubinsztein et al., 2011). Under conditions of mTOR activation, degradation through the proteasome is favoured over autophagy (Zhang et al., 2014). Down-regulation of mTOR is not necessarily linked to inactivation of the proteasome, but it activates autophagy. Activation of autophagy has been reported to extend lifespan in both *Drosophila* and mice (Pyo et al., 2013; Simonsen et al., 2008).

1.4 Diseases of ageing a problem of an ageing population

1.4.1 Geriatric medicine: common problems of old-age

Our interest in understanding the biology of ageing cannot stem too far away from its medical implications. Prolonging the healthspan of yeast, worms or flies is *per se* pointless, except in the eyes of evolution. The great evolutionary conservation in these organisms has highlighted their relevance to biomedical research. Furthermore, the use of these organisms has initiated a new era for research into the biology of ageing (Partridge, 2010). As highlighted at the beginning of this chapter, lifespan extension in the form of life expectancy has been increasing continuously, without the aid of biogerontology research or interventions (Christensen et al., 2009; Oeppen and Vaupel, 2002). Therefore, it is clear that lifespan extension is not a main concern for public health or for geriatrics. As with simple organisms, human ageing is linked to reduced functionality and diminished capacities. Frailty is commonly used in medicine to refer to a syndrome that particularly highlights physical and functional decline without identifiable disease (Fulop et al., 2010). One component of frailty that ageing fruit flies recapitulate is age-related motor decline. Like in humans ageing flies have diminished capacity to fly, speed of movement and climbing capacity (Augustin and Partridge, 2009). Therefore, age-related locomotor decline appears to be a very useful ageing phenotype in flies, probably even more useful

than lifespan (Gargano et al., 2005; Jones et al., 2009).

One of the major problems that the continuous rise in life expectancy has brought to modern societies, and especially to public health, is the increasing prevalence of age-related diseases. Although age-related disorders are not the main cause of mortality worldwide, they are an increasing problem, particularly in developed countries. Cardiovascular disease, diabetes and neurodegeneration are some of the disorders that are becoming a great burden (Bloom, 2011; Marrero et al., 2012). Of these diseases, I am particularly interested in neurodegeneration. However, it is worth bearing in mind that since ageing is a risk factor for a wide range of age-related illnesses similar processes are likely to be occurring in non-neuronal tissues.

1.4.2 Neurodegeneration and Alzheimer's disease

The role of ageing in age-related diseases still remains unexplained. Ageing is the greatest risk factor for neurodegenerative diseases like AD and PD, the two most prevalent forms of neurodegeneration (Bishop et al., 2010; Cummings and Cole, 2002; Lees et al., 2009). Now we know that at least two cellular metabolic pathways regulate the ageing process, allowing us to modify them either genetically or pharmacologically to positively affect lifespan (Bishop et al., 2010; Fontana et al., 2010; Partridge, 2010). Moreover, recent evidence has shown that targeting age-related cellular signalling pathways (such as the IIS/mTOR network) increases survival rates in animal models of several neurodegenerative diseases, slowing or halting the pathogenic events and, overall, increasing their healthy lifespan (Caccamo et al., 2010; Cohen et al., 2009; Kerr et al., 2011; Killick et al., 2009; Malagelada et al., 2010; Spilman et al., 2010; Tain et al., 2009).

One of the central mechanisms of neurodegeneration in the brain is the collapse of proteostasis that leads to proteotoxicity (Cohen and Dillin, 2008; Dillin and Cohen, 2011). As with mitochondrial genes, those related to the UPS pathway are equally down-regulated during ageing (Bishop et al., 2010). This might be relevant to neurodegenerative diseases in which there is an accumulation of abnormally processed proteins such as in AD and PD (LaFerla et al., 2007; Lansbury and Lashuel, 2006; Lees et al., 2009; Querfurth and LaFerla, 2010; Wong and Cuervo, 2010). Recently it was experimentally shown that older flies are more susceptible to the toxic effects of the AD associated amyloid beta 1-42 peptide (Af31_42), as they die faster than younger flies when Af31_42 is expressed in their CNS (Rogers et al., 2012). AD is the most prevalent neurodegenerative disease. Its

aetiology is not fully understood, however its characteristic brain lesion of neurotic

plaques and the discovery that mutation in the amyloid precursor protein have made amyloid the centre of AD pathogenesis (Hardy, 2009; Hardy and Selkoe, 2002; Querfurth and LaFerla, 2010). $Af3_{1\_42}$ is the main pathological hallmark of AD (Hardy, 2009; LaFerla et al., 2007; Querfurth and LaFerla, 2010). The study by Rogers and colleagues suggested that the age-related decline in proteasomal function could be partly responsible for the susceptibility of older flies to Af31_42 (Rogers et al., 2012). Another natural cellular mechanism by which cells are able to clear damaged organelles or altered proteins is autophagy. The ageing process negatively affects the function of the AL system; hence it has been in part considered responsible for the accumulation of damaged organelles and proteins in ageing (Cuervo, 2008; Cuervo et al., 2010). As mentioned before, defects of autophagy in neurons lead to overt pathology in flies and mice (Hara et al., 2006; Juhdsz et al., 2007; Komatsu et al., 2006). Perhaps regulation of the degradative machinery could be an advantageous intervention to release neurons from the burden of damage accumulation and proteotoxicity.

The above clearly highlights the intricate relationship between the ageing process and neurodegeneration, and suggests, that anti-ageing interventions up-regulating degradation mechanisms could be of great relevance for the understanding and treatment of neurodegenerative diseases.

1.5 Treating ageing?

1.5.1 Healthy ageing: how to achieve it?

To better understand the ageing process and how to "treat" it, is fundamental to clearly define end points for research into ageing. This will include defining what physiological ageing is, when does it become pathological ageing and when overt pathology exists. For example, it has been a continuous concern for the field how to establish the differences between physiological memory decline associated with the ageing process, the establishment of mild cognitive impairment as a prodromal for dementia, and overt Alzheimer's disease. Complicating matters, the burden of disease in old age affects multiple tissues and organs at the same time, which translates into multiple diseases or co-morbidities. For example an obese patient with type 2 diabetes mellitus, hypertension, dyslipidemia (triglyceridemia, low HDL and high VLDL) is at increased risk for a coronary and atheroembolic embolic event, renal failure (and other complications inherent

of diabetes and the metabolic syndrome) and even at increased risk of developing a mood disorder like depression, memory loss, and dementia. Patients like this are becoming more and more prevalent particularly in developing economies. Healthcare for patients with multiple co-morbidities should include a multidisciplinary approach and targets for interventions should be carefully considered by the entire medical team. In such an approach a geriatrician (a specialist in diseases of old-age) should also be consulted. However, it is rather clear that a longevity phenotype should be free of disease, but then what does one need to measure when considering end points for ageing research. It cannot be expected for an 85 year old to maintain the cardiac and renal function of a healthy 18 year old. Or should we? Up to now we have not tested anti-ageing interventions in people (except for DR). A careful examination for function of multiple systems during life in different populations but particularly for each individual will help to determine when the decline starts, when and which compensating mechanisms the system take place and what would be the best approach for a particular patient. General practitioners and family doctors will be instrumental here. When a patient gets a consultation with a geriatrician it means that they are in overt pathology, as this is what currently happens around the world. However, a person attending regular check ups will only visit the general or family practitioner. This will entail regular and programmed visits to assess multisystem functionality. Appropriate electronic record keeping will be indispensable. As a person grows old functional decline creeps in, but this will certainly vary between individuals. Preventative ageing interventions will require a clear medical history. However, it is likely that by considering the general population, an average optimal time-to-intervene could be derived for the majority of the population. For example we know now that when there is increased risk of developing prostatic cancer, regular check ups should start earlier than in the general population. A similar list of risk factors could be drawn for interventions in ageing. Once functional decline for each system is set, check ups for that system will have to become regular, and if at higher risk than for the general population, these tests should occur earlier. Defining a longevity phenotype will be fundamental (Slagboom et al., 2000).

1.5.2 In pursuit of the DR mimetic

The widespread health benefits of DR have encouraged the search for drugs that can mimic the effects of DR, i.e., DR mimetics (Cabreiro and Gems, 2010; de Cabo et al., 2014; Ingram et al., 2004; Lane et al., 2004). The concept of a DR mimetic was put

forward by Lane, Ingram and Roth when trying to identify pharmacological agents capable of reproducing the beneficial lifespan-extending effects of DR, without reducing food intake (Lane et al., 1998). In pharmacology a drug mimetic (from the Greek for imitative) is an agent capable of eliciting or inhibiting a process without the need of its natural activator or inhibitor. In cardiovascular medicine, sympathomimetic drugs are those that elicit similar effects as the catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline) These drugs can act either by directly stimulating a- or I3-adrenergic receptors (an example of a direct (32-adrenergic receptor agonist would be salbutamol or albuterol used for asthma control), but also those that indirectly act by increasing the availability of endogenous catecholamines at the site of action, for example by inhibiting is transport (hence clearance) from the extracellular space back into the cell (examples would be amphetamines and cocaine). In the case of a DR mimetic, the molecular target would be less clear (given the pleiotropic effects of DR). However, this pharmacologic agent should be able to extend lifespan and resemble some of the beneficial health effects of food restriction. Though not usually considered, a DR mimetic could also be a drug that mimics the action of food restriction, this could probably be achieved with the use of anti-obesity drugs (Bray and Ryan, 2014; Rodgers et al., 2012). However, it should be noted that such agents have not been tested for lifespan extension in humans nor model organisms. This approach could be tested in model organisms like worms and flies where screens for drugs reducing food intake can be easily performed. Moreover, the use of these organisms can facilitate determination of whether such compounds reduce food intake by mechanisms localised in the gut, brain and/or other metabolic tissues (Gasque et al., 2013). However, the classical interpretation of a DR mimetic is the ability to confer all or some of the effects of DR (lifespan and healthspan) without reducing food intake. DR is already practised by people, for example Okinawans in Japan are thought to be a natural DR population (Gavrilova and Gavrilov, 2012; Willcox et al., 2009). Other groups, like the members of the Caloric Restriction Society also self-restrict their food consumption under the impression that reducing their food intake will protect them against the diseases of old age and slow the ageing process (Holloszy and Fontana, 2007). This is an unlikely behaviour to be adopted by the majority of the population even when the promise of a healthy increased lifespan is on offer. Dietary interventions have been at the core of the first line of treatment for many chronic degenerative diseases, yet though they have proven to be effective in reducing symptoms and improving quality of life, the adherence is rather short term in comparison to drug interventions (Delamater, 2006; Kwan et al., 2013).

Thus, identifying drugs with anti-ageing or pro-longevity properties has become critical for the ageing field.

Perhaps the most important property of a DR mimetic should be its inability to extend lifespan beyond conditions maximised for DR, they should be epistatic. In model organisms, multiple protocols to extend lifespan have been developed; this makes it ever more challenging to perform epistatic experiments between potential DR mimetics and DR *per se*. I consider that these experiments should be performed with the protocol capable of extending lifespan the most, as this DR intervention is likely to recapitulate the ceiling effect for lifespan extension under food restriction.

1.5.3 Drugs to improve ageing

1.5.3.1 Rapamycin and the rapalogs

The hopes for identifying drugs with anti-ageing properties was boosted in 2009 when a report showed that the drug rapamycin was able to extend the lifespan of mice even when fed late in life (Harrison et al., 2009). Ever since, others have replicated this result using different rapamycin protocols (Delamater, 2006; Kwan et al., 2013). Rapamycin is a drug approved for human consumption as an immunosuppressant and chemotherapeutic agent (Guertin and Sabatini, 2009). It has a specific pharmacological target; rapamycin inhibits mTORC1 (Wullschleger et al., 2006; Zoncu et al., 2011). As discussed previously, mTORC1 controls protein translation and autophagy. When rapamycin is administered to cells, or multicellular organisms, it reduces translation and increases autophagy (Bjedov and Partridge, 2011). Although these mechanisms were not shown to be responsible for the lifespan extension in mice per se, inhibition (by lower phosphorylation) of the downstream effector p70-S6K was shown as proof for mTORC1 inhibition (Harrison et al., 2009). Moreover, research in *Drosophila* showed that rapamycin treatment reduced translation and increased autophagy in vivo (Bjedov et al., 2010). These processes were shown to be required for the lifespan extending properties of rapamycin as over-expression of a constitutively active form of p70-S6K or preventing the increase of autophagy (by RNAi-mediated knockdown of Atg5), were sufficient to block the lifespan extending effects of rapamycin (Bjedov et al., 2010). Rapamycin has also been shown to extend lifespan of yeast and worms (Powers et al., 2006; Rallis et al., 2013; Robida-Stubbs et al., 2012). The fact that a drug approved for human consumption can extend the lifespan of evolutionary

distant organisms has sparked interest for the identification of other drugs, already labelled for disease treatment, having anti-ageing properties.

In addition to extending lifespan across evolutionary distant organisms, rapamycin treatment conferred resistance to the redox cycler paraquat and to starvation (Bjedov et al., 2010). Whether resistance to oxidative stress requires the up-regulation of autophagy and/or reduction of translation is still unclear. Resistance to starvation by rapamycin treatment did not seem to require 4E-BP, S6K inhibition or autophagy up-regulation, suggesting that the starvation phenotype is likely to be mTORC1 independent. Alternatively, other downstream targets of mTORC1 could be involved in the response to starvation (Bjedov et al., 2010). The effects of rapamycin have also been shown to modulate disease progression. For example, rapamycin treatment is protective in fly and mouse models of neurodegenerative diseases like Alzheimer's and Parkinson's disease (Majumder et al., 2011; Malagelada et al., 2010; Spilman et al., 2010; Tain et al., 2009). It also rescues the cardiac and skeletal muscle defects associated with lamin A/C deficiency in mice (Ramos et al., 2012), age-related macular degeneration in rats (Kolosova et al., 2012), some forms of cancer (Johnson et al., 2013), amongst other age-related (and not) diseases. However, in spite of extending lifespan and protecting against age-related pathologies, rapamycin associates with some complex side effects. For example, immunosupression, eodema, impaired wound healing, dermatologic alterations and metabolic changes like hypertriglyceridemia, glucose intolerance, and reduced insulin sensitivity (Lamming et al., 2013). Indeed, rapamycin also lead to increased triglyceride levels in *Drosophila* (Bjedov et al., 2010), which probably accounts for the starvation resistance effect associated with rapamycin (Bjedov et al., 2010; Emran et al., 2014). A recent study using mice showed that the metabolic side-effects of rapamycin are likely to be secondary to mTORC2 inhibition, particularly in liver (Lamming et al., 2012). In spite of the side effects, the success of rapamycin for promoting healthy ageing has generated interest in rapalogs, drugs with similar structure and/or function to rapamycin. Perhaps more selective inhibitors of mTORC1 could indeed have more beneficial effects to promote true healthy ageing (Blagosklonny, 2012a; Lamming et al., 2013).

When flies are fed with the mTOR inhibitor rapamycin and tested over a wide range of yeast concentrations, a similar scenario is observed. The degree of lifespan extension is attenuated under the lower DR-range of yeast concentrations, but increases as the yeast concentration is increased towards fully fed conditions (Bjedov et al., 2010). Interestingly, rapamycin is also more effective at extending lifespan in undernourished

conditions, which might be involved in the starvation-resistance effect induced by rapamycin (Bjedov et al., 2010; Emran et al., 2014). These experiments suggest that genetic down-regulation of mTOR or rapamycin in *Drosophila* share overlapping mechanisms, but might also have independent effects. In *C. elegans* where TOR-RNAi increases lifespan of wild type worms by over 25%, the same RNAi treatment to the eat2(ad1116) mutant worm did not further extend lifespan (Hansen et al., 2007), which suggests that TOR down-regulation and DR act in the same molecular pathway. Methionine restriction, a nutrient-specific form of DR, induces autophagy in yeast cells and is epistatic to mTOR inhibition for lifespan extension. Furthermore, deletion of several of the atg genes (5, 7 and 8) or reduction of the function of the vacuole (the yeast organelle closely related to the lysosome), block the lifespan benefits of methionine restriction (Ruckenstuhl et al., 2014).

1.5.3.2 Metformin and the microbiota

The antidiabetic drug metformin has shown to extend lifespan in *C. elegans* and mice, but not in *Drosophila* (Anisimov, 2013). Metformin supplementation late in life to mice increases median and maximum lifespan. The lifespan extension was associated with reduced cholesterol levels (total cholesterol and LDL), improved glucose tolerance and locomotor ability, increased antioxidant defence, and reduced markers of inflammation. Lifespan extension by metformin in mice was not associated with altered mitochondria' respiratory complex function, but with AMPK activation. Interestingly the transcriptional profile of animals treated with metformin showed similarities to the transcriptomic response of animals under DR (Martin-Montalvo et al., 2013).

The first study to show lifespan extension by metformin was performed in *C. elegans*. Metformin was shown to extend median lifespan by approximately 40%. The lifespan extension associated with prolonged locomotor healthspan. Lifespan extension by metformin required the presence of AMPK, the LKB 1 homolog PAR-4 and the transcriptional activator SKN-1 (Onken and Driscoll, 2010). A second independent group elegantly showed that AMPK and SKN-1 mediated protection against metformin- (or biguanides as phenformin, another biguanide, was also used) induced toxicity, rather than lifespan extension. The authors showed that for lifespan extension to occur, metformin altered folate metabolism in the *Escherichia coli* in which worms are co-cultured (Cabreiro et al., 2013). *E. coli is* to worms not only a nutritional source, but also its microbiota

(Cabreiro and Gems, 2013). This study has led to the interesting hypothesis that the regulation of the microbiome can potentially affect lifespan across evolution (Cabreiro and Gems, 2013; Heintz and Mair, 2014; Ottaviani et al., 2011).

It is increasingly recognised that cardiovascular diseases and metabolic dysfunction in diabetes and obesity lead to dysbiosis, i.e., microbial imbalance inside the body (Riley et al., 2013; Tilg and Moschen, 2014). As metformin is associated with gastrointestinal side effects (Fowler, 2007), it is not entirely surprising that metformin could alter the composition of the microbiome. There is some evidence to suggest that metformin alters the microbiome in rats and mice (Lee and Ko, 2014; Pyra et al., 2012), which suggests evolutionary conservation and relevance for human health, but in-depth characterisation is further required. The mechanisms by which the microbiome regulate healthspan are still poorly understood, but evidence suggests a complex interaction between the immune system and other cytoprotective mechanisms, and also metabolic changes (Clemente et al., 2012; Tilg and Moschen, 2014). Indeed, a recent study provided evidence of the complex metatranscriptomic response of the gut microbiome to a host of xenobiotic compounds (Maurice et al., 2013). As we are in constant interaction with the environment and its toxins the relevance of the microbiome as a first line of defence against them should be further explored. I have mentioned that xenobiotics and toxins have been reported to extend lifespan in invertebrates (Calabrese et al., 2011; Goldberg et al., 2010), however whether an interaction between these compounds and the microbiome influences longevity awaits exploration. A recent study in mice showed that DR positively alters the gut microbiota leading to reduced antigen load, which suggest that the changes in the microbiota induced by DR modify the gut microarchitecture protecting against infection (Zhang et al., 2013). The gut microbiota in humans changes with age (Biagi et al., 2010) and exploration into the interaction of these changes with healthspan and lifespan should bring rather exciting scientific advances.

Metformin was shown to extend lifespan independent of daft 6 and further extend the lifespan of the long-lived IIS mutant age-1. However, most importantly metformin was unable to extend the lifespan of the eat-2 mutants. As this genetic intervention is used as a DR protocol, this suggest that metformin is a true DR mimetic as it is unable to extend lifespan under conditions maximised for lifespan under diet restriction (Onken and Driscoll, 2010). Similarly, an independent group showed that metformin becomes detrimental for lifespan when supplemented without the presence of ***E. coli*** (Cabreiro et al., 2013). Removal of the bacteria is a common DR protocol employed in C. ***elegans***

research (Mair and Dillin, 2008). Whether this approach suggests that metformin is a true DR-mimetic, or only highlights the nature of the symbiotic interaction of the worms and the bacteria (Cabreiro and Gems, 2013) should be considered carefully.

1.6 Thesis Outline

In the following sections I will describe the rationale of the work presented in the following chapters. During my work researching for DR mimetics I had the privilege of testing the potential beneficial effects of several drugs for promoting longevity and healthspan (Figure 1.13). Most of them did not show robust enough effects, though some like the pro-diabetic alloxan, the anti-convulsive valproic acid and the antibiotic rifampicin were the ones that I would pursue after lithium. Interestingly valproic acid has already been shown to extend lifespan in *C. elegans* (Evason et al., 2008). However, the drug that I decided to focus on was lithium. I have had experience with this drugs during my medical training, not precisely in the clinic as anyone would assume, but for treating streptozotocin-induced diabetic rats. In previous work I showed that induction of diabetes in male rats increased immobility time in the Porsolt's forced-swimming test, a paradigm for learned helplessness used to screen for antidepressants. Diabetic patients are at increased risk of depression and by inducing insulin-dependent diabetes I was able to show increased learned helplessness (Castillo-Quan et al., 2010). Furthermore, the immobility time was restored to control levels after lithium was administered.

Lithium has been shown to extend lifespan in three independent *C. elegans* studies. Yet no molecular mechanism has been identified (McColl et al., 2008; Tam et al., 2014; Zarse et al., 2011). A study has also shown that low doses of lithium can reduce mortality in *Drosophila*, while high doses increase mortality (Matsagas et al., 2009). Moreover, a recent study correlating lithium concentrations in drinking water in Japanese counties reported that higher doses of lithium associated with reduced mortality for all causes (Zarse et al., 2011). I sought to further explore the role of lithium as a pro-longevity drug using the fruit fly *Drosophila melanogaster*.

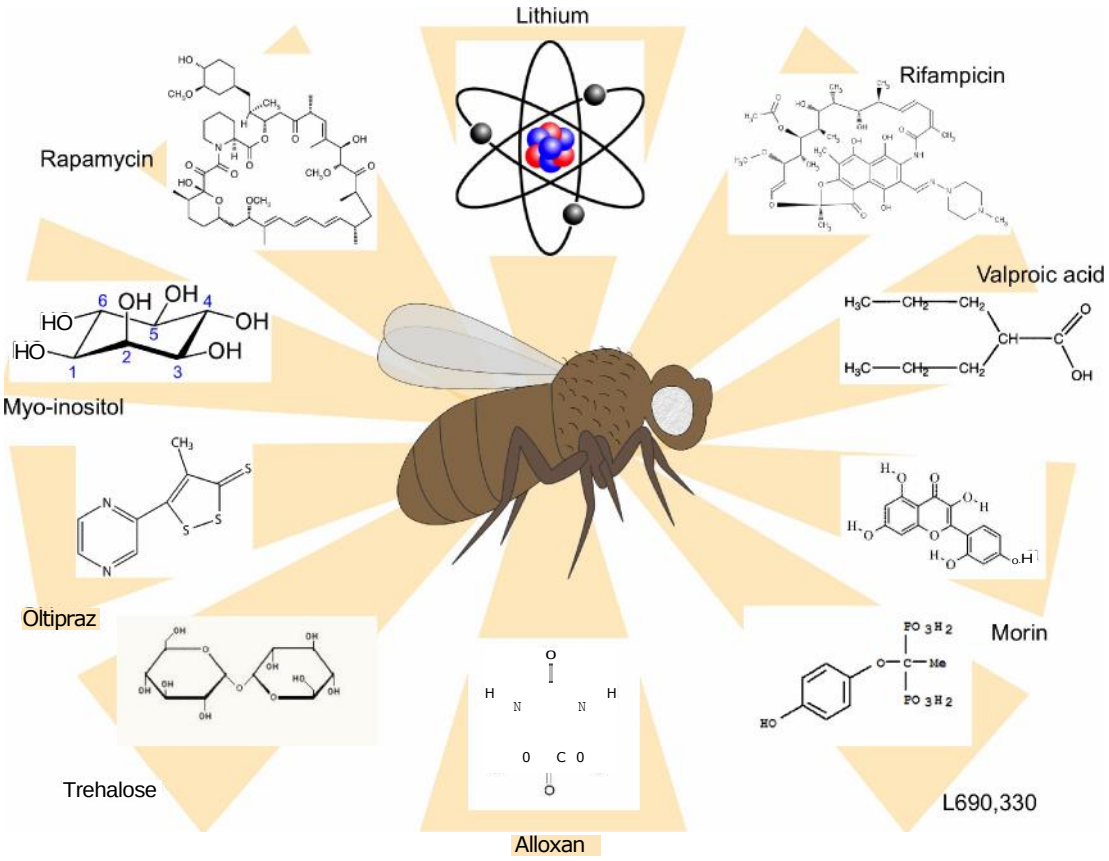


Figure 1.13 Compounds tested in *Drosophila* for lifespan and healthspan. Some of the drugs and compounds tested during the course of the work presented in this dissertation. Data obtained from the use of rapamycin, myo-inositol and morin will be presented during the course of this work. Of the drugs I tested and showed promising results to improve parameters of health (either in control flies or in an A(31_42 neurodegeneration model) were valproic acid, rifampicin and alloxan.

1.6.1 Is lithium a DR mimetic (Chapter 3)?

The use of compounds to extend lifespan and healthspan in *Drosophila* is very limited. Much more work has been done in *C. elegans* (Alavez and Lithgow, 2011, 2012). The amenable manipulation of the fly genome in correlation with the number of behavioural and phenotypical measurements that can be obtained make *Drosophila* an invaluable tool to explore not only lifespan, but also healthspan benefits. In Chapter 3 I sought to assess whether lithium was able to extend lifespan when supplemented in the food medium in flies of more than one genetic background. Previous work in the laboratory has shown that lithium improved the locomotor ability of a neurodegeneration model (Sofola et al., 2010). Additionally, Dr. Ivana Bjedov had shown that lifespan extension in control flies could be achieved at certain doses (unpublished data; see Figure 3.2). I corroborated these experiments and decided to analyse what was the correlation between the lifespan extension induced by lithium and DR. To explore the potential DR mimetic properties of

lithium several explorations should be performed. The first one is whether lithium reduces feeding behaviour by taste aversion, for example. If flies stop eating or reduce their food intake this would be a way of self-imposing DR. I therefore explored this possibility. Second, can lithium extend lifespan beyond that maximised by DR. To explore this possibility I conducted a 4-condition DR tent (previously described) to assess whether lithium shifted the DR tent or extend lifespan under most conditions tested. Lastly I analysed potential changes in the nutrient sensing-network IIS/mTOR.

Besides lifespan I explored the possibility that lithium could be extending lifespan by a trade-off mechanism by which lifespan is extended at the expense of reduced fecundity. Interventions down-regulating the nutrient-sensing network, including DR, reduce fecundity. These interventions also correlate with altered metabolism, therefore I explored potential changes in carbohydrate and lipid parameters.

Finally, I also assessed some pharmacological aspects of lithium that could have relevance for therapeutic approaches. For most of the experiments described I supplemented lithium chronically after two days post-eclosion (adulthood). However, I wondered whether lithium would modulate longevity when administered late in life. Additionally I assessed the "molecular memory" of lithium, i.e., whether effects in longevity and stress resistance are maintained after the drug is removed. I also evaluated the interaction lithium and rapamycin have on longevity, stress-resistance and metabolism.

1.6.2 What are the mechanisms of lithium to promote healthy ageing (Chapter 4)?

Lithium is considered a dirty drug. Although it is often used as a GSK-3 inhibitor, it can act on several signalling pathways. In Chapter 4 I used a genome-wide unbiased approach to interrogate the transcriptome and translatome in aids of understanding the molecular mechanisms behind the lifespan extension by lithium. For this I performed microarray transcriptional analyses and polysome-profile microarrays. I will describe several approaches taken to analyse the changes observed. Additionally, I was also able to analyse how the transcriptional response of lithium correlates with other interventions that promote lifespan extension in *Drosophila*.

1.6.3 Is GSK-3 involved in lithium's ability to extend lifespan (Chapter 5)?

The most widely recognised target of lithium is GSK-3. Mammals express two GSK-3 isoforms, GSK-3a and GSK-313. *Drosophila* only possess one homolog called shaggy *(sgg)*. In chapter 5 I explore the role of *sgg* in modulating lifespan and healthspan. No previous study has carried out such investigations in any organism. Additionally I explored the role of *sgg* as a downstream target of lithium's action Finally given the tools available I explored the possibility that different tissues might respond differently to *sgg* modulation. Using the arsenal of tissue-specific drivers I was able to determine that not all tissues respond in the same way, perhaps revealing different basal levels required for maintaining cellular function.

1.6.4 What drives neurodegeneration in *Drosophila* neurons expressing $AI3_{1—42}$ (Chapter 6)?

In the final results chapter (6) I explore non-traditional phenotypes in an Af31_42 model of Alzheimer's disease. So far it has been characterised that flies expressing A131_42 in adult neurons show reduced lifespan, acceleration of age-related locomotor decline and electrophysiological dysfunction. Therefore, I decided to analyse whether other parameters could also be deregulated in these flies. I sought to analyse the response to several forms of stress including oxidative, xenobiotic and starvation. During the course of these experiments I found that these phenotypes were altered by what seemed to be the earliest sign of neurodegeneration reported so far. This phenotype had a great impact on lifespan and metabolism, which revealed that expression of Af31_42 in neurons affect the general physiology of the flies.

Chapter 2

General methodology

2.1 *Drosophila melanogaster:* strains and genetics

One of the major advantages of working with the fruit fly *Drosophila melanogaster* is the vast amount of laboratory strains. The Partridge laboratory has a generous inventory of genetic stocks allowing appropriate use and generation of different crosses. All stocks were kept at 18°C, while experimental crosses and transgene flies were kept at 25°C and 65% humidity on a 12:12 light : dark cycle using standard sugar/yeast (SY) medium (see 2.2.1). Flies were reared at standard larval density (-300 flies per 200mL bottles) and eclosing adults were collected over a 2 hour period after which they were mated for 48 hours before collecting females for experimental treatments (Bass et al., 2007).

2.1.1 White Dahome^{y} (WDah)

The control white *Dahomey* (w^{pah}) stock has been maintained in large population cages with overlapping generations since 1970. The w^{pah} stock was derived by incorporation of the ^{w1118} mutation into the outbred *Dahomey* background by backcrossing (Bass et al., 2007). The w^{pah} strain is naturally infected by the intracytoplasmic bacterium *Wolbachia,* which modulates lifespan effects of the IIS pathway (Griinke et al., 2010; Ikeya et al., 2009; Negri, 2012).

2.1.2 White 1118 (w1118)

The ^{w1118} *Drosophila* stock is an inbred and isogenic strain vastly used for neurodegeneration studies. This strain is often used as a control in ageing studies to demonstrate that pro-longevity effects found in the w^{pah} strain are independent of genetic background (Bjedov et al., 2010; Slack et al., 2010).

2.1.3 Backcrossing

Backcrossing transgenic lines is necessary to ensure that any differences seen between mutant and control flies are due to the expression of the transgene alone, and not due to confounding mutations or insertional mutagenic effects (Partridge and Gems, 2007). Some studies have shown that insufficient standardization of the genetic background and inappropriate backcrossing or use of inappropriate controls can produce artificial lifespan extension (Burnett et al., 2011; Toivonen et al., 2007). For this reason, before the transgenic stocks could be used for experimentation, they had to be prepared so that their genetic backgrounds were uniform, differing only at the locus where the transgene was integrated. This was achieved by a series of backcrossings. In the first cross, transgenic males were mated with wild type females. In this way, the cytoplasmic content (including mitochondrial genome) is passed to the offspring, which is selected either by visible phenotype (eye colour for example) or by PCR following specific mutation. After this initial cross virgin females were collected and crossed to wild type males, and this cross was repeated at least another five times until six backcrossing events were completed. Transgenic lines were then made homozygous or balanced and kept as a stock for experimentation.

2.1.4 GAL4-UAS system

The GAL4-UAS system is considered one of the most powerful biochemical tools in the fly world (Brand and Perrimon, 1993). Its major advantage is that it can restrict the expression of genes of interest in a tissue or cellular specific manner using native gene promoters. The GALA gene encodes for the yeast *Saccaromyces cerivisiae* Ga14, a transcriptional activator protein. For example, the elav-GAL4 *Drosophila* line will expresses the Ga14 protein under the elav promoter. Elav encodes for embryonic lethal abnormal vision, a pan-neuronal protein, therefore elav-GAL4 is a neuronal driver. On the other hand, the upstream activation sequence (UAS) is an enhancer to which Ga14 binds to activate gene transcription. UAS controls the expression of the gene of interest only allowing transcription in cells expressing Ga14. In the case where the green fluorescent protein (GFP) is under UAS control, i.e., UAS-GFP, and this line is crossed with an elav-GAL4 line, the final genotype elavGAL4/+ > UAS-GFP/+ will express GFP only in neurons (Osterwalder et al., 2001).

2.1.5 Gene-switch system

The gene-switch (GS) system uses the GAL4/UAS system with the restriction that the transcriptional activity depends on the presence of steroid hormones or chemically related compounds (Ford et al., 2007; Osterwalder et al., 2001; Roman et al., 2001a). In the GAL4/UAS system the binding of the transcription factor GAL4 to an upstream activating sequence (UAS), results in the transcription of the UAS-linked transgene (Brand and Perrimon, 1993). In the GS system GAL4 is fused with the regulatory domain of the human progesterone receptor (Ford et al., 2007; Osterwalder et al., 2001; Poirier et al., 2008; Roman et al., 2001b). In the absence of the activator mifepristone (RU486), the Ga14 protein and UAS transgene can coexist in the fly without expression of the transgene under the control of UAS. When flies expressing the GS driver and the UAS line are fed RU486, the Ga14-gene-switch is able to bind to the UAS and therefore the transgene is expressed (Figure 2.1) (Osterwalder et al., 2001; Roman et al., 2001b).

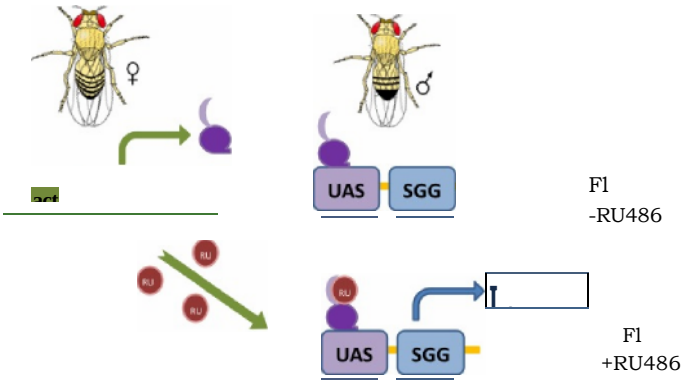


Figure 2.1 GeneSwitch expression system. This diagram illustrates the cross between a gene-switch driver (act-GeneSwitch, actGS) and a UAS line (UAS-sgg) to obtain a line carrying both the driver and UAS line, which results in the following line: actGS/+;UAS-*sgg*/+. Transcription only occurs when flies ingest medium supplemented with RU486 (RU). Flies with the same genotype but not given RU are used as perfect genetic controls.

2.2 *Drosophila* food medium

2.2.1 Sugar-yeast medium (SY)

Our standard laboratory medium (SY) was used for the development and maintenance of all flies, unless otherwise stated. SY medium was made in the following way: 15 g of agar were dissolved in 700 mL distilled water by heating until boiling. After this 100 g of autolysed yeast powder and 50 g of sugar were added until boiling. 170 mL of distilled water were added and left to cool down. When temperature was between 50-60 $^{\circ}$C, 30 mL of nipagin (100 g/L) and 3 mL propionic acid (both anti-fungal preservatives) were added.

Food was then dispensed (4 mL per vial) into plastic vials. Food was allowed to set at room temperature before storing at 4°C (Bass et al., 2007).

2.2.2 Grape juice medium

Grape plates were used as a surface for egg laying and collection. Collected eggs were subsequently dispensed into bottles containing SY medium. Grape plates were made as follows: 25 g agar were dissolved in 500 mL distilled water and brought to the boil. To this 300 mL of red grape juice were added and brought to the boil again. After adding an additional 50 mL of distilled water the medium was allowed to cool to below 60°C. As with the SY medium 21 mL of nipagin (100 g/L) were added. The medium was dispensed into plastic petri dishes and allowed to set at room temperature before storing at 4°C.

2.2.3 Starvation medium

Starvation medium was prepared by adding 15 g of agar to 700 mL of distilled water. This was brought to boil and cooled down before dispensing into vials. No additional ingredients (including anti-fungals) were added to avoid any potential source of nutrients.

2.2.4 Dietary restriction (DR) regime

For DR experiments a yeast dilution protocol was established using as a reference the SY medium previously described. For details of the precise recipe see Appendix 7.

2.3 Fly husbandry and culturing

2.3.1 Male and female separation

Some anti-ageing interventions that extend lifespan are not able to do so in male flies (Clancy et al., 2001), or even in mice (Selman et al., 2009); in other cases the response shows some dimorphism, where the response is attenuated in males (Bjedov et al., 2010; Miller et al., 2013). Therefore, it is a common practice in ageing studies to test males and females separately (Partridge and Gems, 2007). Additionally, continuous mating can impact several physiological parameters including survival (Barnes et al., 2008; Chapman and Partridge, 1996). Female and male flies can be distinguished very easily under a

microscope whilst flies are anesthetised with CO2 (Greenspan, 2004). For most experiments only female flies were used, though some phenotypes were also corroborated in males to ensure sex-independent effects. Female and male flies were sorted using a fine paintbrush while anesthetised. Flies were usually separated at day 2 post-eclosion after a 48 hours mating period.

2.3.2 Virgin collection

To obtain certain desired genotypes, it was often necessary to cross male and female flies of different genotypes. This ensured at least one copy of the chosen transgene into their progeny. This was achieved by collecting unmated female flies (or virgins). Virgin collection was carried out for backcrossing (see section 2.1.3) and to obtain desired genotypes for experiments. Female *Drosophila* flies do not respond to courting males during the first 8 hours after eclosion at 25°C (Greenspan, 2004). Therefore newly emerged female flies were collected within 6 hours after clearing rearing bottles.

2.4 *Drosophila* handling and survival

2.4.1 Lifespan assay

An important determinant for ageing analysis is the age at which flies initiate the assay. For this, it was necessary to ensure that the parents of the experimental flies were the same age at egg laying and reared under the same conditions. In this way, I could control for the effects of parental age on lifespan (Priest et al., 2002). 'Egg squirt' protocols were undertaken to ensure that all flies in the experiment were raised at similar larval densities (-300 eggs per bottle containing 70 mL of food), thereby avoiding the possibility that any differences in lifespan could be accredited to differences in larval density within which the flies were reared (Priest et al., 2002). Flies were allowed to lay eggs over less than 24 hours on grape medium plates, with live yeast paste to encourage mating. The eggs were collected from the plate by washing with phosphate buffered saline (PBS) solution and collected into falcon tubes. The eggs were allowed to settle to the bottom of the tube. Using a 100 ilL Gilson pipette —15-18 lit of egg suspension was dispensed into 200 mL glass bottles containing 70 mL SY medium. This equates to —300 eggs per bottle.

After 10 days of development flies emerge from their pupae and were then

transferred into fresh bottles. The flies were then allowed to mate in these new bottles for 48 hours. After this flies were sorted using a paintbrush under CO_2 anaesthesia, and divided onto vials with 15-20 flies per vial.

Flies were transferred to fresh vials of food three times a week throughout life. The number of dead flies found during each transfer was recorded. Accidental deaths and escapees were distinguished from deaths and were censored from the experiment. From these data, a survivorship graph was generated, making it possible to compare survival curves over time between different genotypes or experimental conditions. Lifespan curves were analysed with log-rank test.

2.4.2 Stress assays

To determine the role of genetic, environmental and/or pharmacological interventions, survival curves obtained in a similar manner than for lifespan experiments (previous section) were carried out using different chemicals to induce cellular stress (see sections 2.6.2 and 2.6.3).

2.5 Behavioural investigations

2.5.1 Feeding assay

To explore the feeding behaviour of flies of different genetic backgrounds, environmental and/or pharmacological interventions, flies were reared and grouped as previously described. Five vials per group were included with five flies each. The feeding behaviour assay relies on the fly proboscis extension (Wong et al., 2009). To analyse their behaviour on the food and ensure that the proboscis extension was observed continuously for two hours and recorded on data sheets, later plotted and statistically analysed. To maximize the reliability of the observed effects the observer was blinded to the conditions/genotypes tested.

2.5.2 Fecundity assay

Fecundity has historically been heavily linked to longevity (Partridge et al., 2005). Although this association has been uncoupled (Grandison et al., 2009a), interventions to

prolong lifespan without affecting fecundity have been limited. Such intervention would in reality be a healthy lifespan treatment. To assess fecundity female flies were let lay eggs for a period of —24 hours in vials containing standard medium. Vials containing eggs were frozen and kept at -20°C for a short time until eggs were counted. Total of number of eggs were divided between number of flies per vial and t-test was used to determine statistical value.

2.5.3 Climbing assay (negative geotaxis)

Locomotor age-related decline is an insightful screen of the functional capacity of flies that is also sensitive to some anti-ageing interventions (Gargano et al., 2005; Jones et al., 2009; Kerr et al., 2011). The paradigm called negative geotaxis consisted of a climbing assay in which 10-15 flies were placed in a 35 cm column (1.5 cm diameter) with a conic bottom end. Flies were tapped down and observed during 45 seconds. The column was separated into three areas: top, middle and bottom by two lines; one was 10 cm from top and the other was 3 cm from bottom). After 45 seconds flies located in each of these three sections were recorded into scoring sheets. Each column was evaluated three times to minimize trial error and a minimum of three vials per genotype/condition was evaluated in order to be able to perform statistical analyses. The recorded scoring showed the mean number of flies in total (ntot), at top (ntop), and at bottom (nbottom), which allowed obtaining a performance index. The performance index (PI) was calculated as $^1/2$ (ntot + ntop — nbottom / ntot). The performance index was plotted against time per each genotype and condition. Results were analysed with two-way ANOVA and Tukey post-hoc using JMP 7.0.

2.6 Drugs and other chemicals

2.6.1 Mifepristone (RU486)

As explained earlier the GeneSwitch system uses mifepristone (RU486) as in inducer of gene expression where GeneSwitch drivers are being used (Osterwalder et al., 2001; Poirier et al., 2008). Our laboratory has characterised that the dose used in our experiments does not impair longevity, fecundity or metabolism. RU486 (Sigma, M8046) was dissolved in 100 % ethanol and supplemented to standard SY medium before dispensing into vials. Food supplemented with RU486 was stored at 4°C until use.

2.6.2 Paraquat

Flies were kept in pre-determined conditions similar to those in lifespan assays for a fixed period of time after they were transferred in to food containing paraquat. For paraquat preparation 1M N,AP-dimethy1-4,4'-bipyridinium dichloride (paraquat, Sigma 856177) was dissolved in ddH2O after which was added to 0 SY medium (20mM final concentration; see Appendix 7). All stress assays were tested without yeast in the medium to avoid interaction between the yeast and the stressor. At least one experiment using yeast was carried out to ensure that the effect observed was not confounded by a yeast-deprivation response. The medium used in all figures shown throughout this work had all the other components of the standard medium including sucrose. Flies were typically assayed with 15 per vial. Deaths were scored every 2 hours after the detection of the initial wave of death. Data was plotted as a survival curve and analysed with log rank test.

2.6.3 Hydrogen Peroxide (11202)

For 11202 preparation 30% 11202 from Sigma was added to 0 SY at a final concentration of 5%. Flies were typically assayed with 15 per vial. Deaths were scored at least 3 times per day, plotted as a survival curve and analysed by long rank test.

2.6.4 Chloroquine

Chloroquine diphosphate salt (Sigma 6628) was prepared by directly adding the salt to 0 SY medium to obtain a final concentration of 6%.

2.6.5 Phenobarbital

Phenobarbital (Sigma P1636) was prepared by directly adding the salt to 0 SY medium to obtain a final concentration of 6%.

2.6.6 DDT

Dichlorodiphenyltrichloroethane was prepared by diluting in 100% ethanol and adding to 1 SY medium to obtain a final concentration of 0.03%.

2.7 Biochemistry and molecular biology methods

2.7.1 Triglyceride assay (TAG)

To quantify triaclyglyceride levels a colorimetric assay kit by Thermo Scientific was used (Broughton et al., 2005; Slack et al., 2010; Bjedov et al., 2010); 6 replicas of 5 female flies were homogenised in 0.05% Tween 20 and incubated for 5 minutes at 70°C. Samples were then centrifuged for 5 minutes at 7000 rpm. The supernatant was transferred to fresh eppendorfs and centrifuged for 10 minutes at maximum speed. For each sample, 175u1 was transferred to a fresh eppendorf. 10u1 of each sample was dispensed into a well on a 96-well plate, with each sample in triplicate. To each well, 200u1 of Thermo Infmity Triglycerides solution was added and the plate was left to incubate at 37°C for 10mins, after which time I measured absorbance in each well at 574nm. The lipid standards were treated in the same way as the samples all throughout and were prepared as 7 serial dilutions in 0.05% Tween 20. These were 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 μg/μl triglyceride.

Samples were normalised to protein levels using the Thermo Scientific bicinchoninic acid protein (BCA) assay (see section 2.7.7).

2.7.2 DNA extraction and Single-Fly Polymerase Chain Reaction (PCR)

To analyse single flies for specific mutations one fly was placed in a 1 5 mL eppendorf and squashed with a blue pestle after which 50 ilL of squish buffer (990 ilL of squish buffer (10 mM Tris-HC1 pH 8.0, 1 mM EDTA, 25 mM NaC1) + 10 ilL of proteinase K) were added. The liquid was transferred to a PCR tube and incubated for 1 hr at 37°C and then at 95°C for 10 mM. The sample was spin after which it was either stored at 4°C or prepared for PCR.

The PCR reaction consisted of (per fly) 1 ilL of extracted DNA, 2.5 ilL 10X PCR buffer (15 mM $MgC1_2$), 0.5 ilL dNTPs (10 mM), 0.5 ilL of each primer, 0.25 ilL TAQ polymerase and 19.75 ilL of ddH2O. All reagents were obtained from Qiagen, UK. The PCR cycles were run using a thermal cycler (Eppendorf UK limited), with the following protocol: 1 cycle of 94°C for 15 mM (initial melting step to denature the hot-start TAQ polymerase), 30 to 35 cycles of 95°C for 30 sec (for denaturing DNA), 50-60°C for 30 sec (to anneal the primers), and 72°C for 2 mM (elongation step) Finally, 1 cycle of 72°C for

7 to 10 min as a final elongation phase. Samples were then either stored at -20°C or ran on an agarose gel. Find PCR primers used for single fly PCR in Appendix 5.

2.7.3 Gel electrophoresis

1 to 2 gr of agarose (Sigma, UK) were dissolved in 98 or 99 mL of TAE (Tris base, acetic acid, EDTA) buffer and heated in a microwave, until close to boi, after which is was slowly cooled down by placing under running tap water. Once cooled 3 piL of ethidium bromide were added to the agar solution. The mixture was placed in a gel tray and a comb was added to create wells for the samples. The gel was allowed to set for 1 hr. A marker ladder and samples were added in conjunction with 6x loading dye (40% glycerol, 6x TBE buffer and 0.25% bromo blue) at a final volume of 20-25 'IL. Electrophoresis was performed between 70-100 V until samples had ran enough, and just before running out of the gel. The samples were then visualised under UV light using a UV transiluminator.

2.7.4 Quantitative Real Time PCR (qRT-PCR)

Total RNA was extracted from 15 flies using Trizol (GIBCO) according to the manufacturer's instructions. The concentration of total RNA purified for each sample was measured using an *Eppendorf biophotometer*. 1 tg of total RNA was then subjected to DNA digestion using DNAse I (Ambion), immediately followed by reverse transcription using the Superscript II system (Invitrogen) with oligo(dT) primers. Quantitative PCR was performed using the PRISM 7000 sequence-detection system (Applied Biosystems), SYBR Green (Molecular Probes), ROX Reference Dye (Invitrogen), and Hot Star Taq (Qiagen, Valencia, CA) by following manufacturer's instructions. Each sample was analysed in triplicate with both target gene and two reference genes (RP49 and Act5c) primers in parallel. Find qPCR primers used in Appendix 6.

2.7.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All SDS-PAGE for western blots was performed using the Criterion (Bio-Rad) protocol. 12% resolving gel was prepared by placing 5.03 mL ddH2O in a 50 mL falcon tube to which 3.75 mL of 1.5 M Tris-HC1 (pH 8.8), 150 piL of 10% SDS, 4.5 mL of 40% acrylamide/bis (37.5:1) were added. This mixture was vortex. To this same mixture 7.5 'IL

of TEMED and 10% of APS (freshly prepared) were added. The mixture was quickly vortex before dispensing in the Criterion cassettes and propanol added to eliminate bubbles. After 45 to 60 min, the propanol was removed and the stacking gel was added. 4% stacking gel was prepared as follow 9 5 mL of ddH2O in a 50 mL falcon tube and the following were added: 3.78 mL of 0.5 M Tris-HC1 (pH 6.8), 150 ilL 10% SDS and 1.485 mL of 40% acrylamide/bis (37.5:1). The sample was vortex before 15 ilL TEMED and 75 'IL of 10% APS were added and mixed again. The mix was added on top of the resolving gel up to the top of the cassette. A comb with the appropriate number of lanes was placed at the top of the cassette and allowed to set for at least 1 hr before immediately used or stored at 4°C for 12 hrs max before use.

2.7.6 Western blotting

For whole fly experiments 10 female flies were homogenised in 200pL 2x Laemmli loading sample buffer (100mM Tris-HC1 pH6.8, 20% glycerol, 4% SDS) containing 5% 0-mercaptoethanol. For head sample preparation, heads were split and were homogenised in the loading buffer at 21.11, per head. Samples were heated at 95°C for 5 minutes after which extracts were cleared by centrifugation. 10 'IL or approximately 4Optg of protein extract were loaded per lane on a polyacrylamide gel. After separation, proteins were transferred to a nitrocellulose membrane where they were incubated overnight with a selected primary antibody and thereafter an appropriate secondary antibody.

2.7.7 Protein Quantification

Samples were dispensed into a microplate well (all samples in triplicate). BSA albumin standards were used with the same diluent as the samples (for example 0.05% Tween 20 for triglyceride assay). The following standard concentrations were prepared: 2000, 1500, 1000, 750, 500, 250, 125, 25, 0 μg/mL To each well 200 ilL of the 'working reagent' supplied in the kit was added, and the plate was left to incubate at 37 °C for 30 minutes. The absorbance was then measured at 562 nm

Chapter 3

Pharmacology of Lithium for Ageing: a DR mimetic?

"I see no limit to how long human life can be extended if scientists learn how to turn on antiaging genes in the young or how to prepare cocktails of drugs that serve the same purpose as genetic engineering."
Michael R. Rose

3.1 Abstract

The pharmacology of ageing is even more recent than the genetics of ageing. Though drugs capable of extending lifespan have been reported for over a decade, it was not until rapamycin was shown to extend the lifespan of mice that this new avenue of research into ageing really took off. It has become clear now that synthesising new drugs capable of mimicking the already proven genetic interventions will not be the only way to prolong healthspan for longer. Alternatively testing the potential anti-ageing properties of known and licensed drugs could accelerate the establishment of anti-ageing medicine. In this chapter I aimed to show that the drug lithium chloride, used as a medication in the treatment of bipolar disorder, has anti-ageing properties. I found that indeed lithium is a pro-longevity drug that extended lifespan independent of sex and genetic background. Unlike IIS mutants and rapamycin treatment, lithium supplementation did not affect fecundity at doses that extended lifespan. Lithium was able to extend lifespan at all food concentrations tested, making it unlikely to be a DR mimetic. I also explored the possibility of interactions with IIS and mTOR and showed that lithium did not seem to be acting through these pathways. Therefore lithium is a novel pro-longevity drug that can act independent of the traditional nutrient-sensing pathways offering an alternative to DR mimetics for promoting healthy ageing.

3.2 Introduction

At the present time one of the most important objectives of research into ageing is to identify drugs that can enhance healthy lifespan. As discussed in Chapter 1, genetic modifications have been extremely successful in extending the lifespan of evolutionarily distant organisms. However, for these interventions to have real applicability to humans,

they need to be mimicked by drugs. Therefore, a significant part of the biogerontology community is focusing on identifying drugs that can activate or inactivate pathways or processes recognised to increase healthy lifespan. Through the genetic manipulations described earlier, protein targets have been identified, therefore a natural approach would be the generation of compounds to inactivate or boost the proteins that have so far been shown to play an important role in the ageing process. This approach is likely to be successful given the strong genetic evidence. However, drug synthesis is a lengthy process that can take over a decade to have medically relevant use. Another approach is to test drugs already available and approved for human consumption for their use in extending healthspan, lifespan or both. Given that their use in humans is approved, their applicability and relevance becomes evident faster.

3.2.1 The use of drugs in the ageing-field

The hopes for identifying drugs with anti-ageing properties was boosted in 2009 when a report showed that the drug rapamycin was able to extend the lifespan of mice even when fed late in life (Harrison et al., 2009). Rapamycin is a drug approved for human consumption as an immunosuppressant and chemotherapeutic agent.

Table 1 Landmarks papers in the Biology of Ageing by number of citations

Year	Reference	Finding	Citations§
1935	(McCay et al., 1935)	Dietary restriction extends lifespan in rats.	117
1993	(Kenyon et al., 1993)	Characterization of the *C. elegans daf2/daf16* pathway to extend lifespan.	1412
1996	(Brown-Borg et al., 1996)	Altered somatotrophic axis extends lifespan in mice.	539
2001	(Clancy et al., 2001)	Loss of *chico* extends lifespan in *Drosophila.*	737
2001	(Tatar et al., 2001)	*dInR* hypomorph flies live longer.	799
2003	(Holzenberger et al., 2003)	Heterozygous loss of IGF-1R extends lifespan in female mice.	1034
2009	(Colman et al., 2009)	DR retards the onset of age-related pathologies in monkeys.	741
2009	(Harrison et al., 2009)	Rapamycin fed late in life to mice extends lifespan.	959

§ According to Scopus (only since 1996). Last visited 13th of September 2014.

Rapamycin as a pro-longevity drug has become a landmark finding in the biogerontology community (Table 1). Rapamycin is not a perfect drug, its use associates

with the establishment of altered metabolism, wound healing, immunosuppression among others. As discussed in Chapter 1, the metabolic alterations secondary to rapamycin treatment in mice have been attributed to mTORC2 inhibition. Therefore, the current effort is focused in identifying more specific mTORC 1 inhibitors, or even inhibitors for specific downstream targets of mTORC1 like S6K.

Our group has also shown that another drug approved for human use, and considered a DR mimetic, seems not to have an effect on lifespan. Metformin, a drug used in the treatment of type 2 diabetes, was unable to extend lifespan of male and female *Drosophila* (Slack et al., 2012). It is unclear why metformin is incapable of extending the lifespan of fruit flies, particularly when reports have shown that metformin can extend lifespan of *C. elegans* and mice (Cabreiro et al., 2013; Martin-Montalvo et al., 2013; Onken and Driscoll, 2010). However, Slack and colleagues only tested the effect of the drug in one genetic background and on one food concentration. Given that metformin is considered to be a DR mimetic, it is rather surprising that the effect of the drug was not evaluated on a full DR tent. If the medium used to test the drug was close to the peak of the DR tent (see Chapter 1) then no effect is to be expected of a DR mimetic at low concentrations (as observed), and the drug should become detrimental once the concentration is increased (as observed). Perhaps testing the drug in more than one genetic background and more than one food condition will be informative.

3.2.2 Lithium as a therapeutic agent

Lithium was first introduced to medical practice as a treatment for bipolar disorder (BPD). Over 60 years ago the Australian psychiatrist John Cade reported improvement of manic disorder when lithium salts were administered (Johnson, 1998; Soares and Gershon, 1998). Cade first observed that lithium supplementation to guinea pigs led to calming and lethargic effects. This discovery prompted Cade to evaluate the potential therapeutic properties in various psychiatric illnesses. Cade determined that the positive effects of lithium were specific for BPD and this drove him to propose that mania could be the result of lithium deficiency (Johnson, 1998; Lenox and Watson, 1994). Cade's hypothesis of lithium deficiency was proven false, however the therapeutic benefits of lithium in BPD are undeniable. In spite of this, the mechanisms by which this drug achieves its benefits are not yet fully understood (Lenox and Wang, 2003; Williams and Harwood, 2000).

Two proteins have taken centre stage as the effectors by which lithium may exert its therapeutic effects. Glycogen synthase kinase-3 (GSK-3) is one of them and is discussed in detail in Chapter 5 (Klein and Melton, 1996; Stambolic et al., 1996). The other enzyme inhibited by lithium is the inositol monophosphatase (IMPase) (Berridge et al., 1989; Harwood, 2005; Jope and Williams, 1994). IMPase inhibition leads to reduction of inositol levels, which are required for the maintenance of inositol lipids. The second messengers inositol 1,4,5-triphosphate and diacylglycerol require inositol for their synthesis and their reduction compromises signal transduction (Berridge et al., 1989; Harwood, 2005). I will discuss the effects of inositol in Chapter 5.

3.2.3 Pharmacology of lithium

BPD is a chronic and debilitating illness characterized by periods of depression and mania (Malhi and Tanious, 2011; Malhi et al., 2012). It has been considered to be the sixth leading cause of disability in the developed world (D'Souza et al., 2011). Lithium is the first choice and the gold standard medication for the treatment of BDP, but it also has therapeutic value in the treatment of alcoholism, schizoaffective disorders and cluster headaches (Malhi and Tanious, 2011; Timmer and Sands, 1999).

Lithium is a univalent cation and must be administered with an anion. Several preparations exist, but the most widely used are lithium carbonate (capsule) and lithium citrate. Lithium chloride is not used in the clinic because of its high toxicity, but this is the preferred formulation in research given its solubility, which allows low dose administration (Timmer and Sands, 1999).

Lithium is completely absorbed in the upper gastrointestinal (GI) tract, achieving peak serum levels within a couple of hours of oral ingestion. It does not bind to proteins and is distributed in total body water (Malhi and Tanious, 2011; Timmer and Sands, 1999). However, it preferentially accumulates in certain tissues like kidneys, thyroid gland and bone, over muscle and liver for example (Timmer and Sands, 1999). Peak levels in the brain are delayed by approximately 24 hours in comparison with plasma due to lower permeability of the blood-brain barrier, and the concentration in cerebrospinal fluid (CSF) is only 40% of serum due to transport out of CSF by the endothelium and arachnoid membranes. It is excreted almost entirely by the kidney without any hepatic metabolism, and its half-life will depend on several factors like age, treatment duration and renal function (Malhi and Tanious, 2011; Timmer and Sands, 1999). After a single dose the half-

life of lithium will be between 12 and 27 hours, but it can increase to over double (up to 58 hours) in the elderly (Timmer and Sands, 1999).

The therapeutic window for lithium lies between 0.4 mM/L and 1 mM/L as measured in serum (Figure 3.1A). Concentrations over 1 mM/L increase the risk of adverse side effects and above 1.5 mM/L side effects like slurred speech, tremor, muscle weakness, seizure and irreversible renal damage are imminent. Even when serum concentrations are kept within the therapeutic window side effects can still occur (Malhi and Tanious, 2011; Paton et al., 2010).

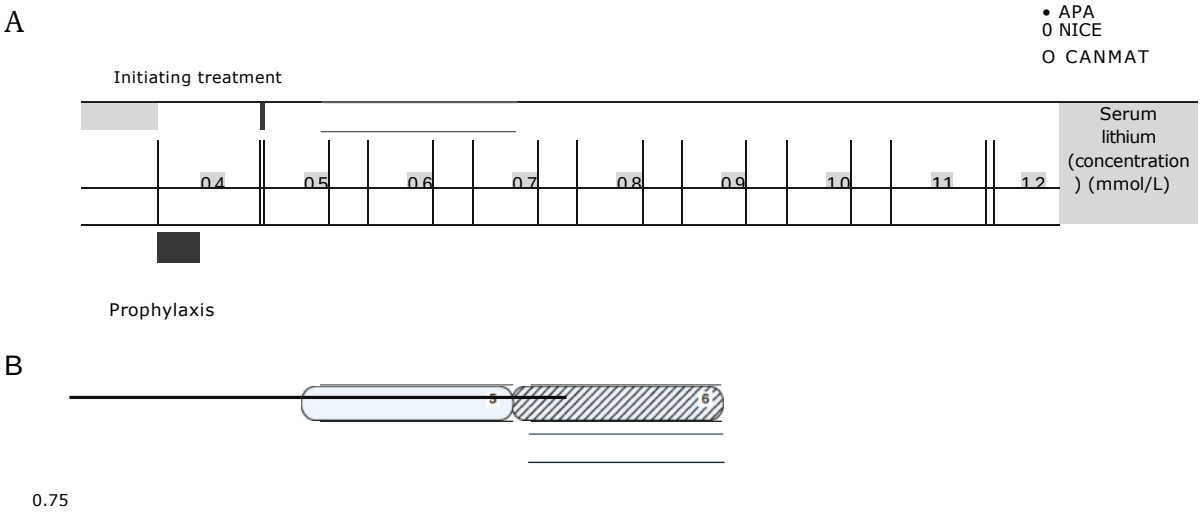
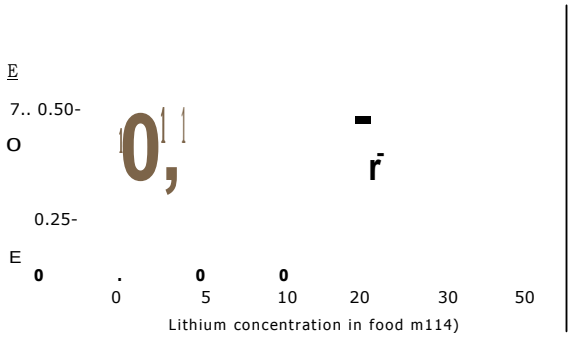


Figure 3.1 Concentration of lithium in humans and *Drosophila.* (A) Recommended lithium concentrations in serum as suggested by medical associations and guidelines: American Psychiatrist Association (APA), National Institute for Health and Clinical Excellence, UK (NICE), and Canadian Network for Mood and Anxiety Treatments. The numbers indicate the recommended plasma concentrations for patient treatment. 1) represents the concentration at which it should be started (0.5 mM/L) and slowly increased up to (1.2 mM/L, the maximal recommended concentration); 2) represents the maximal concentration required for prophylaxis; 3) is the plasma concentration often used by psychiatrist; 4) plasma concentration more effective for controlling mania symptoms; 5) concentration for stabilising patients; and 6) represents the recommended plasma concentration for patients previously using lithium who have relapsed. Taken from (Malhi and Tanious, 2011). (B) Detected concentrations of lithium in flies plotted against the concentration in the medium. Taken from (Dokucu et al., 2005).



In patients over the age of 60 the average and highest dose has been documented to be reduced by almost half of that prescribed for younger adults (from 1000 mg/d to 689 mg/d and from 2400 mg/d to 1350 mg/d) (D' Souza et al., 2011). Older patients seem to be more susceptible to the toxic effect of the drug.

Lithium has also been administered to *Drosophila* for the study of its role in many behavioral traits. Interestingly these studies have highlighted that the concentrations supplied in the fly medium (Figure 3.1B) yields concentrations at least ten times lower inside the fly (Dokucu et al., 2005).

A

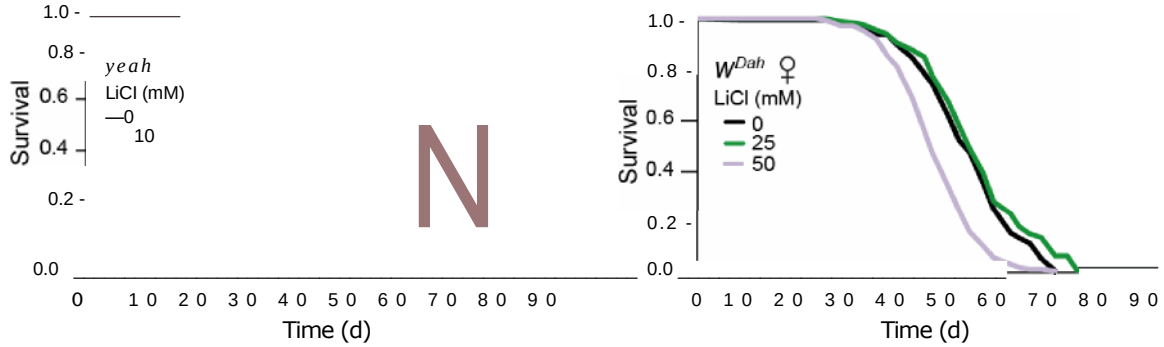


Figure 3.2 Lithium increases lifespan of $w^{p^{11}}$ female flies. 150 females per condition were fed either vehicle (ddH_2O) or lithium chloride (LiC1) supplemented in the SY medium and their survivals were analysed. (A), flies fed 10 mM LiC1 showed a significant lifespan extension ($p < 0.01$, log-rank test). **(B),** flies fed 25 mM LiC1 did not show a significant difference in lifespan compared with flies fed vehicle (0 mM LiC1) ($p > 0.05$, log-rank test). Flies fed food supplemented with 50 mM LiC1 showed a significant lifespan reduction ($p < 0.01$, log-rank test). N = 150 female flies per dose condition. Lifespan experiments were performed by Dr Ivana Bjedov.

3.2.4 Is lithium a potential drug for anti-ageing interventions?

The first documented evidence that lithium could extend lifespan came from the Lithgow laboratory. McColl and colleagues showed that lithium extends lifespan of *C. elegans* at various doses before becoming toxic. Moreover, they showed that lithium was able to further extend lifespan of the long-lived *daf-2* insulin receptor mutant and that this lifespan extension was independent of the transcription factors daf-16/FOXO, since lithium was able to extend lifespan of *daf-16* mutant worms. This suggests that lithium does not act through the IIS pathway to extend lifespan (McColl et al., 2008). The lifespan-extending properties of lithium have been replicated independently in *C. elegans,* by two independent groups (Tam et al., 2014; Zarse et al., 2011). Furthermore, the Rose laboratory reported that low doses of lithium reduced the mortality rates of *Drosophila* (Matsagas et al., 2009). These findings seem promising, especially in light of the epidemiological observation that

drinking water supplemented with lithium is associated with reduced mortality in humans (Zarse et al., 2011).

Unpublished work from our laboratory has shown that lithium can indeed extend lifespan in *Drosophila* (Figure 3.2). In light of this I sought to determine whether this lifespan extension was reproducible and independent of sex and genetic background. I also investigated how lithium induced lifespan extension fits within the nutrient-sensing pathways. In the present chapter I assessed the ability of LiC1 to extend lifespan independent of DR and the nutrient sensing network. In the following chapter (Chapter 4) I explored the mechanisms by which lithium could be operating to extend healthy lifespan by means of an —omics approach. Moreover, taking advantage of the fact that the fruit fly is very amenable to genetic manipulations I attempted to verify the validity of the candidate mechanisms through epistatic interactions. In Chapter 5 I study the role of glycogen synthase kinase-3 (GSK-3) in mediating the effects of lithium. I also provide evidence that supplementing lithium to a neurodegeneration fly model improves pathological features.

3.3 Methodology and experimental design

3.3.1 *Drosophila* strains

Drosophila strains ^{w1118} and w^{poh} were used in the experiments described in this chapter as well as in the following chapters (see Chapter 2). The ubiquitous driver daughterless-GeneSwitch driver (daGS) was used to drive the expression of the UAS-InR^{DN} to down-regulate the IIS pathway. Flies lacking the transcription factor *dfoxo* were also used (Slack et al., 2011).

3.3.2 Dietary restriction

The specific preparation of the DR media has been described in Chapter 2. For this chapter I used the following yeast dilutions: 0.2, 0.5, 1.0 and 2.0. Lithium was supplemented as described in the following section.

3.3.3 Lithium preparation and delivery

Lithium chloride (LiCl; Sigma L0505) was dissolved in ddH2O at a concentration of 5 molar and then supplemented to 1SY medium (yeast medium, see chapter 2 section 2.2.1), unless otherwise stated. Food was then dispensed into plastic vials and stored at 4°C until used. Cold refrigerated vials were placed at 25°C for a minimum of 2 hours before flies were transferred into them.

3.3.4 Rapamycin preparation and delivery

Rapamycin (LC Laboratories #R-5000) was dissolved in 100% ethanol at a concentration of 50 mM and then added to the fly medium at the desired concentration.

3.3.5 Immunoblot analyses

Protein extraction and western blot techniques were described in Chapter 2. Primary antibodies used in this chapter were: pSer505-AKT (Cell Signaling Technologies #4054, 1:1000), total-AKT (Cell Signaling Technologies #9272, 1:1000), pThr398-S6K (Cell Signaling Technologies #9209, 1:1000), custom made total-S6K (provided by Dr. Cathy Slack, 1:1000) and I3-actin (Abcam #ab4801, 1:5000). Appropriate HRP-conjugates secondary antibodies (Abcam) were used (1:12000).

3.4 Results

3.4.1 Lithium modulated lifespan in a dose-dependent manner

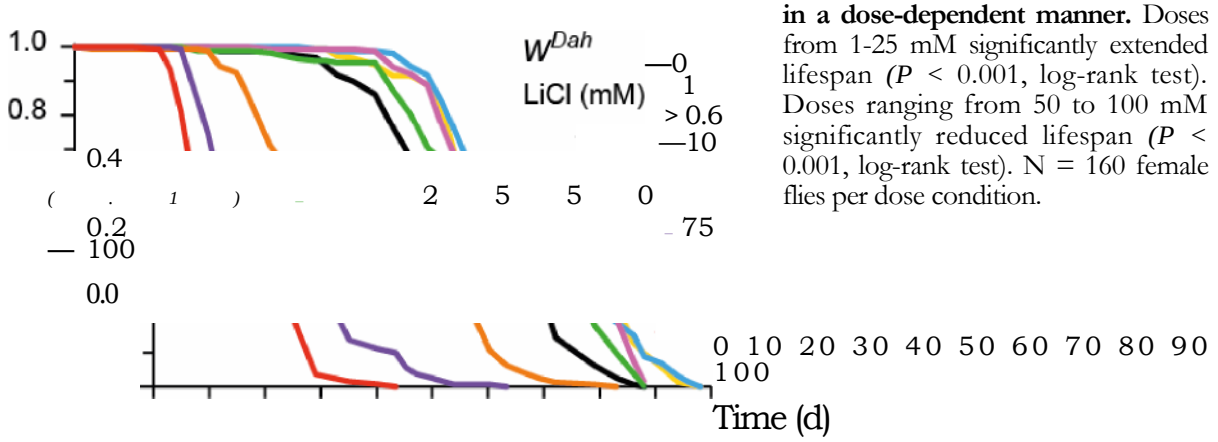


Figure 3.3. Lithium affected lifespan in a dose-dependent manner. Doses from 1-25 mM significantly extended lifespan (*P* < 0.001, log-rank test). Doses ranging from 50 to 100 mM significantly reduced lifespan (*P* < 0.001, log-rank test). N = 160 female flies per dose condition.

As earlier described, lithium can extend the lifespan of *C. elegans* (McColl et al., 2008; Zarse et al., 2011), and can reduce mortality of *Drosophila* (Matsagas et al., 2009). Therefore, I sought to explore the longevity effects of lithium in *Drosophila*. Dr Ivana Bjedov conducted the first set of experiments in the w^{pah} strain. As shown in **Figure** 3.2, once mated w^{pah} females fed food supplemented with lithium at 10 mM showed increased median and maximum lifespan. On the contrary, 25 mM lithium was unable to extend lifespan and 50 mM lithium proved to be toxic. I decided to further characterise the lifespan effects of lithium by increasing the concentration range from 1 to 100 mM **(Figure** 3.3). I again used w^{pah} female flies and observed that concentrations ranging from 1 to 25 mM could extend lifespan. However, the lifespan extension at 25 mM was trial-dependent, sometimes showing a positive effect, others without difference from controls or producing a slight lifespan shortening effect. Doses from 1 to 10 mM extended median lifespan by 16% (-10 days) and maximal lifespan up to 18% (-14 days; *P* < 0.001, log rank test compared to the control group). In contrast, concentrations from 50 to 100 mM significantly reduced lifespan in a dose-dependent manner (Figure 3.3; *P* < 0.001, log rank test).

3.4.2 Lithium extended lifespan independent of sex, genetic background and fecundity

The degree of lifespan-extension by a mutant (or any other intervention) can vary with the strain genetic background (Grandison et al., 2009b; Kaeberlein et al., 2004). To further confirm the pro-longevity effects of lithium and to test whether the effects are independent of genetic background, a second (Figure 3.4) and third (Figure 3.5) set of experiments were independently set up with varying concentrations of lithium using the inbred isogenic *white1118 (w1118)* genetic background. Female flies were supplemented with lithium concentrations ranging from 1-10 mM (Figure 3.4A; $P < 0.05$, log-rank test). 1 mM lithium extended median lifespan by 2 days, while 5 and 10 mM extended median lifespan by 4 and 7 days respectively.

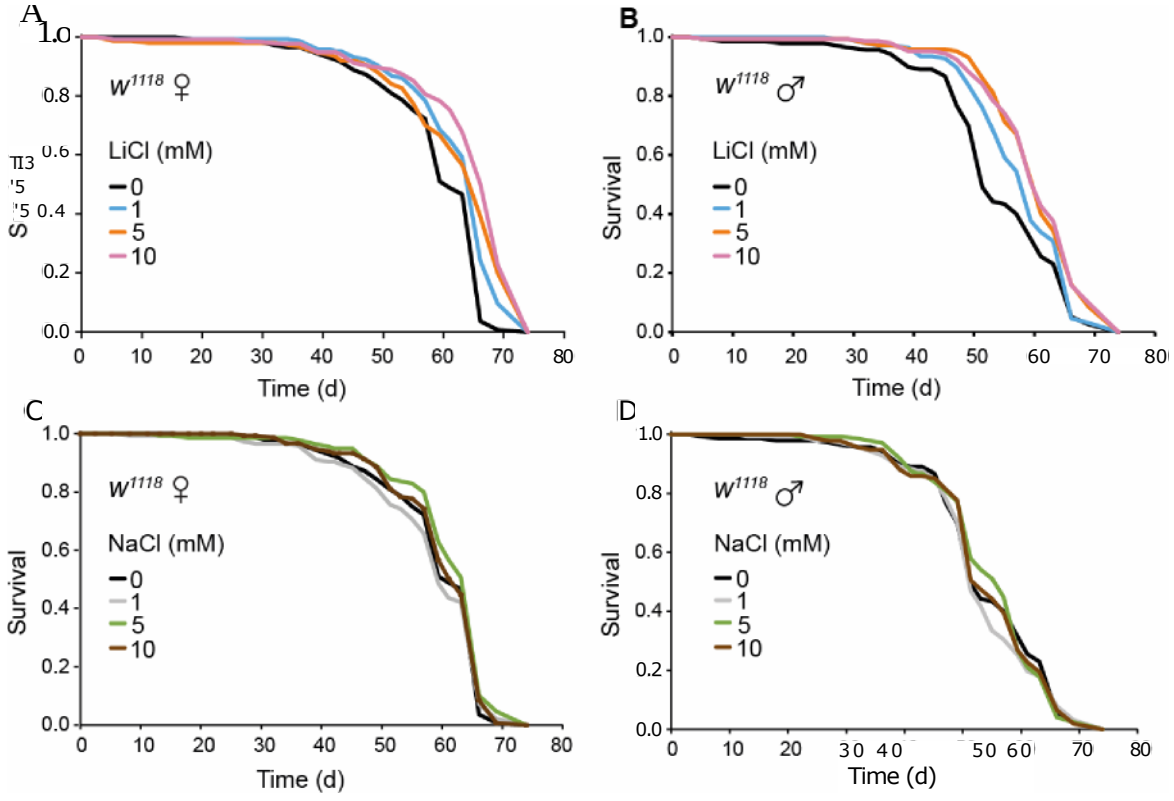


Figure 3.4 Lithium and sodium chloride are tested for longevity in the *w1118* genetic background. (A) and (B) show lifespan effects of LiC1 in female and male flies, respectively. (C) and **(D)** show lifespan properties of NaC1 supplemented at similar concentrations as LiC1 in female and male flies, respectively. N = 150 flies per dose/gender condition. These survival experiments were performed in collaboration with Ms. Li Li.

A number of interventions that extend lifespan are to some extent sex-specific (Maklakov and Lummaa, 2013). I therefore tested whether lithium could also extend lifespan of *w1118* male flies, using the same doses of LiC1 previously described for *w1118*

females (Figure 3.4B). Male flies fed medium with 1 mM lithium showed a significant *(P* < 0.05, log-rank test) median lifespan extension (5 days), with no maximal lifespan effect. 5 and 10 mM similarly extended median lifespan by 18% (11 days), and maximum by 4 days.

Addition of LiC1 to the fly medium could increase its osmotic properties and hence affect lifespan (Ja et al., 2009; Lee et al., 2009; Piper et al., 2010). To control for this potentially cofounding effect, I performed experiments using equivalent molar concentrations of NaCl. None of the concentrations tested significantly affected lifespan of female (Figure 3.4C) or male (Figure 3.4D) flies.

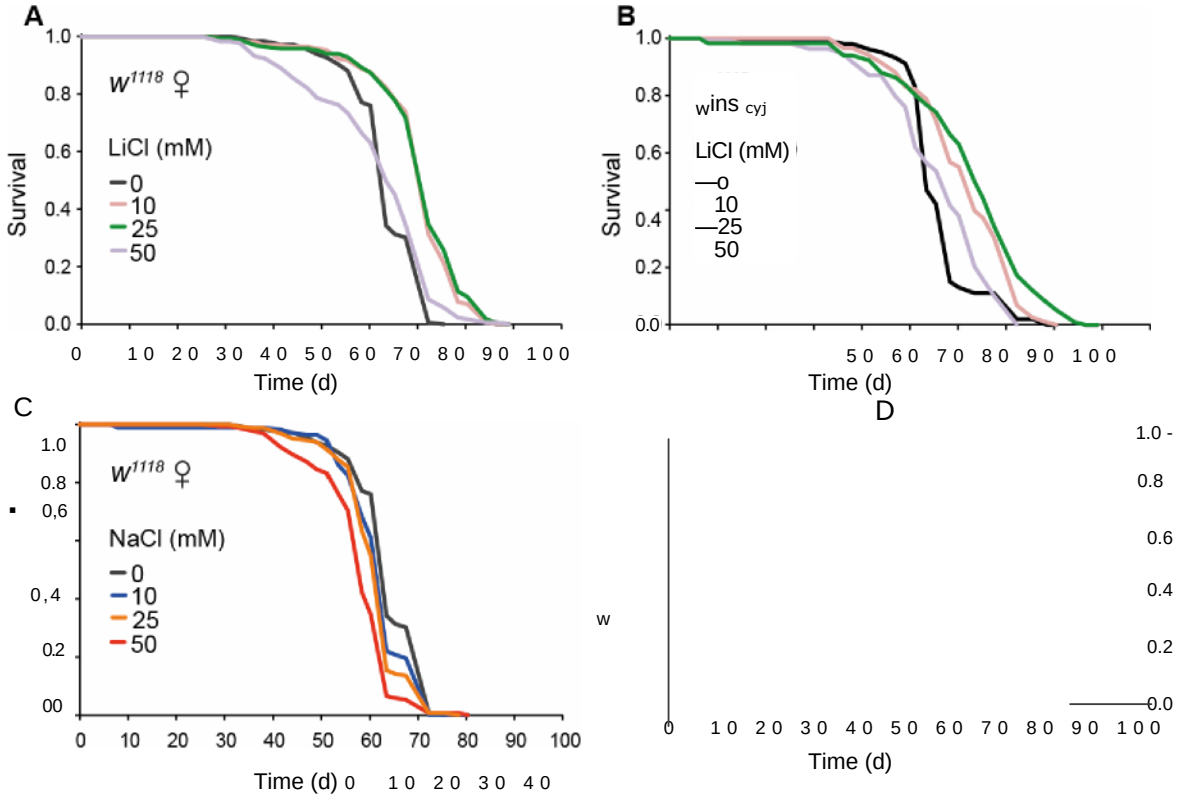
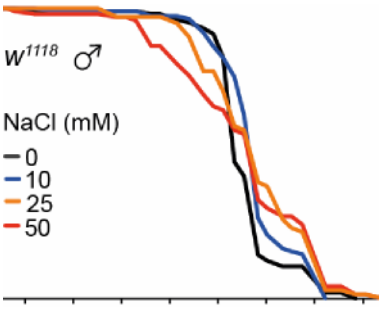


Figure 3.5 Lifespan response to higher doses of LiC1 and NaC1 using *w1118* female and male flies. (A) Female flies expose to 10, 25 and 50 mM lithium. (B) Similar concentration supplemented to male flies. (C) **(D)** show the lifespan response to equimolar concentrations of NaC1 in female and male flies respectively. N = 150 flies per dose.

and 

A second set of experiments with *w1118* flies used 10, 25 mM and 50 mM lithium. 10 and 25 mM increased median and maximum lifespan (Figure 3.5A; *P* < 0.001 log-rank test). 10 mM lithium resulted in a 13% (8 days) median lifespan extension and a maximum of 10% (7 days). When lithium was supplemented at 25 mM, female flies showed a similar median

and maximum lifespan extension as when fed 10 mM lithium. I tested similar

concentrations in male flies (Figure 3.5B). Supplementing the fly medium with 10 mM lithium extended median lifespan of male ^{w1118} flies by 18.5% (-10 days) and maximum lifespan by -4% (2.5 days) (*P* < 0.001, log rank test) while lithium treatment using 25 mM extended median lifespan by 23% (12 days) and maximum by 13.5% (-10 days) (*P* < 0.001, log rank test) and 50 mM lithium did not significantly extend lifespan (*P* = 0.4849, log rank test). I also tested the effect of supplementing NaC1 at similar concentrations. At all doses tested, NaC1 did not replicate the lifespan extension effects of LiC1, in either male (Figure 3.5D) or females (Figure 3.5C) of the ^{w1118} background. Lithium could thus increase lifespan in two genetic backgrounds and in both sexes, suggesting that its effect is strain and sex independent.

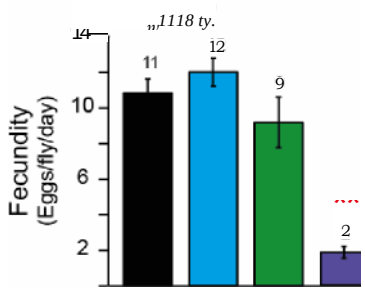


Figure 3.6 Effect of lithium of female fecundity. Egg laying was tested after 15 days of lithium supplementation. Data is presented as average eggs laid per female in 24 hrs. Error bar represent SEM. N = 150 females. ** *P* < 0.001 (ANOVA post hoc Tukey-Kramer).

Several genetic and pharmacological interventions that extend lifespan reduce fecundity (Partridge et al., 2005; Regan and Partridge, 2013). To determine whether extension of lifespan by lithium was associated with reduced fecundity, I measured egg-production. Lithium did not reduce egg laying at doses that extended lifespan. However, at the higher doses at which lithium reduced lifespan, it also reduced fecundity (Figure 3.6). My results, therefore, did not support the idea that lithium extended lifespan by reducing fecundity.

3.4.3 Lithium ameliorated age-related locomotor decline

Mutations in the IIS pathway that extend lifespan have been linked to improvement in several functions of health including cardiac function and locomotor ability in flies (Gargano et al., 2005; Wessells et al., 2004). I therefore sought to investigate whether lithium treatment could improve locomotor ability in female flies of the ^{w1118} genetic background (Figure 3.7A). Female flies were treated and assessed across time with either 10 or 25 mM lithium. Female flies fed 10 mM lithium were partially protected against age-

related locomotor decline (*P* < 0.01; two-way ANOVA post hoc Tukey Kramer). However, flies treated with 25 mM lithium performed similarly to controls (*P* > 0.05; two-way ANOVA). I also determined whether lithium could improve age-related locomotor dysfunction in the $^{-}14^{Dah}$ background over a wider range of concentrations (1-75 mM; Figure 3.7B). Surprisingly, lithium was able to partially protect against the decline at 75 mM (*P* < 0.05; two-way ANOVA post hoc Tukey Kramer), but not at doses that extended lifespan (*P* > 0.05; two-way ANOVA for doses from 1-50 mM lithium)

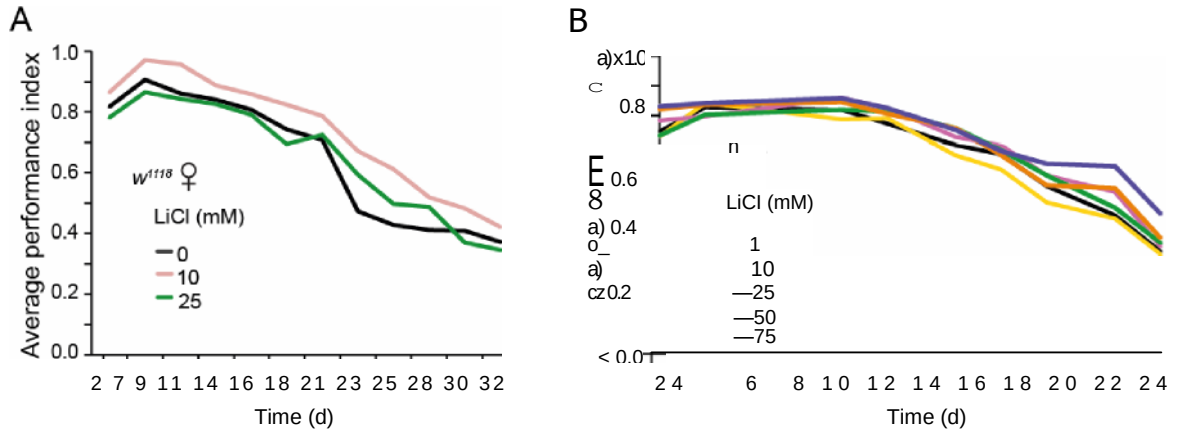


Figure 3.7 Lithium protected against age-related locomotor decline. (A) Negative geotaxis analyses of *w"8* female flies fed either 10 or 25 mM lithium. Experiment performed in collaboration with Ms. Li Li **(B)** Negative geotaxis analyses of w^{pah} female flies fed lithium concentrations ranging from 1-75 mM. Experiment performed in collaboration with Mr. Michael Schannack.

These results did not conclusively support a role for lithium in protecting neuromuscular function during ageing. Only 10 mM lithium showed partial protection in the ^{w1118} genetic background, but I was unable to observe a similar effect in the le^{ah} background. Given these inconclusive results it is hard to determine whether lithium protects against age-related locomotor decline. Additionally, other tests to assess locomotor function (flight test, speed, overall daily activity or electrophysiology recording in the giant fibre system) should be performed to more conclusively determine the healthspan benefits of lithium on the function of the nervous and muscular systems (Augustin and Partridge, 2009).

3.4.4 Lithium reduced triglyceride levels and sensitised against starvation

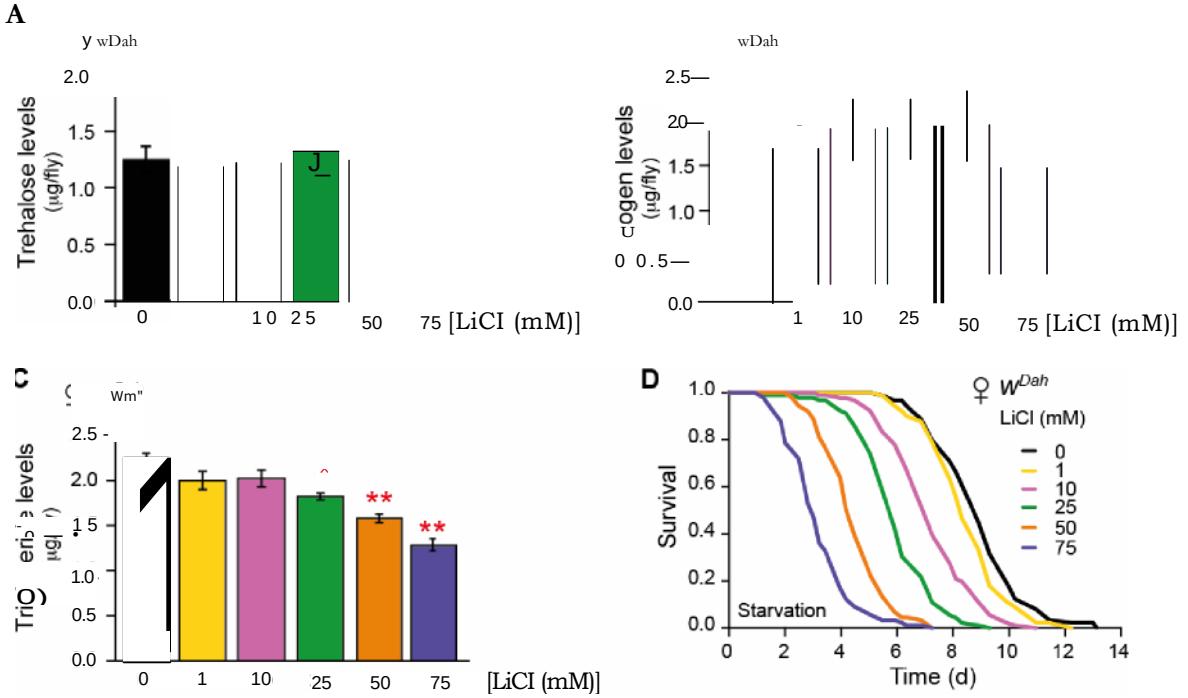


Figure 3.8 Metabolic effects of lithium in carbohydrate and lipid stores, and the response to starvation. Lithium did not modify (A) trehalose levels nor **(B)** glycogen levels, but dose-dependently reduced (C) triglyceride levels. For these experiments (A) and **(B)** 5 biological replicates of 2 flies were measured after 15 days of lithium treatment. No statistical significance was found ($P > 0.05$; ANOVA post hoc Tukey-Kramer). For experiment in (C), 5 flies per 5 biological replicates were measure after 15 days of lithium treatment. Data in (A-C) are presented as median ± SEM. **(D)** Starvation was measure by transferring flies pre-treated for lithium for 15 days.

Genetic manipulations that extend lifespan by reducing signalling through the nutrient-sensing, insulin/IGF-1 signalling (IIS) and mechanistic target of rapamycin (mTOR) network, often associate with abnormalities in carbohydrate and lipid metabolism (Baker and Thummel, 2007; Tatar et al., 2014; Wang et al., 2014). Ubiquitous reduction (or knock-out) of specific components of the IIS pathway extends lifespan in mice, but can make them insulin-resistant at younger, but not older ages (Foukas et al., 2013; Selman et al., 2008a). Long-lived *Drosophila* with IIS down-regulation show increased levels of triglycerides and carbohydrate metabolites (Baker and Thummel, 2007; Broughton et al., 2005). Similarly, rapamycin treatment, which inhibits mTOR and extends fly and mouse lifespan, increases triglyceride levels in flies and in mammals compromises glucose and lipid metabolism (Bjedov et al., 2010; Harrison et al., 2009; Houde et al., 2010; Lamming et al., 2012). To assess whether the lifespan-extending effect of lithium was associated with altered carbohydrate metabolism, I measured the levels of trehalose and glycogen,

two storage carbohydrates in *Drosophila*, after 15 days of lithium treatment and, over a wide range of lithium concentrations, I was unable to detect a significant change in the levels of either compound (Figure 3.8A and B). I also measured the levels of triglycerides, the main lipid storage in flies (Ballard et al., 2008; Kanlein, 2012) and, after 15 days of lithium treatment, observed a dose dependent reduction in w^{ah} female flies (Figure 3.8C). Lithium thus did not affect the storage of carbohydrates, but reduced triglycerides in a dose-dependent manner

In *Drosophila*, triglyceride levels are associated with starvation resistance (Ballard et al., 2008). I aimed to analyse the response of lithium pre-treated flies to nutrient deprivation. To test starvation-resistance of lithium-treated flies, I pre-fed them with lithium for 15 days and then transferred them to vials containing only water/agar. Lithium dose-dependently reduced lifespan under starvation conditions (Figure 3.8D; $P < 0.001$, log rank test).

Given these results I also analysed the triglyceride levels in ^{w1118} lithium treated flies and observed a dose-dependent reduction (Figure 3.9A) which matched with starvation sensitivity (Figure 3.9B). Thus the effects on triglycerides and starvation are conserved in two genetic backgrounds.

A

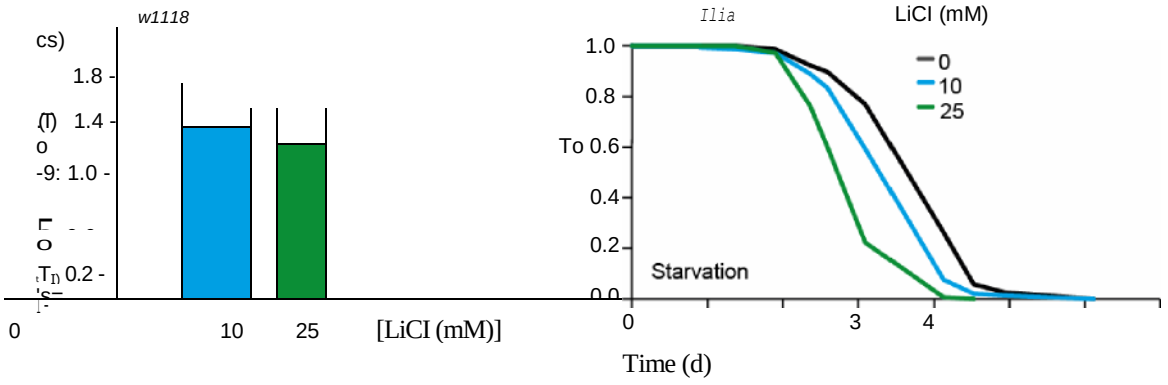


Figure 3.9 Effect of lithium on triglyceride levels and starvation in w1118 flies. (A) Triglyceride levels were analysed in flies pre-treated for 15 days. Data are presented as average of 5 biological replicates of 5 flies per condition ± SEM. * $P < 0.01$ (ANOVA, post hoc Tukey-Kramer). **(B)** Survival curve of flies pre-treated with lithium for 15 days and the subjected to starvation.

3.4.5 Lithium extended lifespan beyond dietary restriction

Dietary restriction (DR), a reduction in food intake short of malnourishment (Fontana et al., 2010; Levine et al., 2014; Solon-Biet et al., 2014), extends healthy lifespan in diverse

species. Lithium and DR could extend lifespan by overlapping or independent mechanisms. I therefore sought to determine whether lithium and DR extend lifespan by distinct or overlapping mechanisms. I assessed whether lithium could extend lifespan beyond the maximum achievable by DR. I varied the yeast concentration in the food in the presence of a constant concentration of sucrose as my DR protocol (Bass et al., 2007). When the yeast component of the food was reduced a typical tent-shaped response was observed, with peak lifespan at 1.0X yeast concentration **(Figure 3.10** and **Appendix 8** for full survival curves). If lithium acts through similar molecular mechanisms to DR, then lithium would not be able to further extend lifespan when it is maximised by DR, and DR would not be able to extend lifespan when it is maximised by lithium (Clancy et al., 2002). I used 4 yeast conditions (0.2X, 0.5X, 1.0X and 2.0X). The optimal concentration for lifespan was observed a 1.0X with a median lifespan of 70.5 days **(Figure 3.10A)** and maximal of 86.1 days **(Figure 3.10B).** Yeast dilutions of 0.5X and 0.2X were shorter lived in comparison, with median lifespans of 67.5 and 22 days respectively. On the contrary, doubling the yeast concentration (2.0X) also shortened lifespan, with a median of 62.1 days. A similar effect was observed for maximum lifespan **(Figure 3.10B).** In addition I supplemented lithium to all of these food conditions. The concentrations used were (in mM): 1, 2.5, 5 and 10. Interestingly, lithium was able to significantly extend median and maximum lifespan under all yeast conditions tested *(P* < 0.001, log rank test; **Figure 3.10A and B; Appendix 8),** including very low yeast content (0.2x yeast). This was surprising given that flies pre-treated with lithium were sensitive to starvation **(Figure 3.9D).** The optimal median lifespan extension under malnourished conditions (0.2x yeast) was obtained with 2.5 mM lithium which increased it by —30% **(Appendix 8A).** Doses from 1-5 mM of lithium were optimal for extending lifespan on 0.5x yeast **(Appendix 8B),** while all doses tested (1-10 mM LiC1) on the optimal yeast dilution extend lifespan **(Appendix 8C).** Interestingly the longest median lifespan (absolute value) achieved with lithium under full feeding conditions was the same as the longest median lifespan extension (absolute value) observed under maximised DR **(Figure 3.10A** and **Appendix 8E).** Under full feeding conditions median lifespan was extended by —18% *(P* < 0.001, log rank test).

These results suggest that the optimal concentration of lithium to extend lifespan might depend on the nutritional status of flies, or its dietary intake, but lifespan extension could be achieved in all yeast dilutions given the appropriate dose. Surprisingly median lifespan was extended more under malnourished or fully fed conditions than in the

intermediary DR regimes, perhaps suggesting some overlapping mechanisms between lithium and DR.

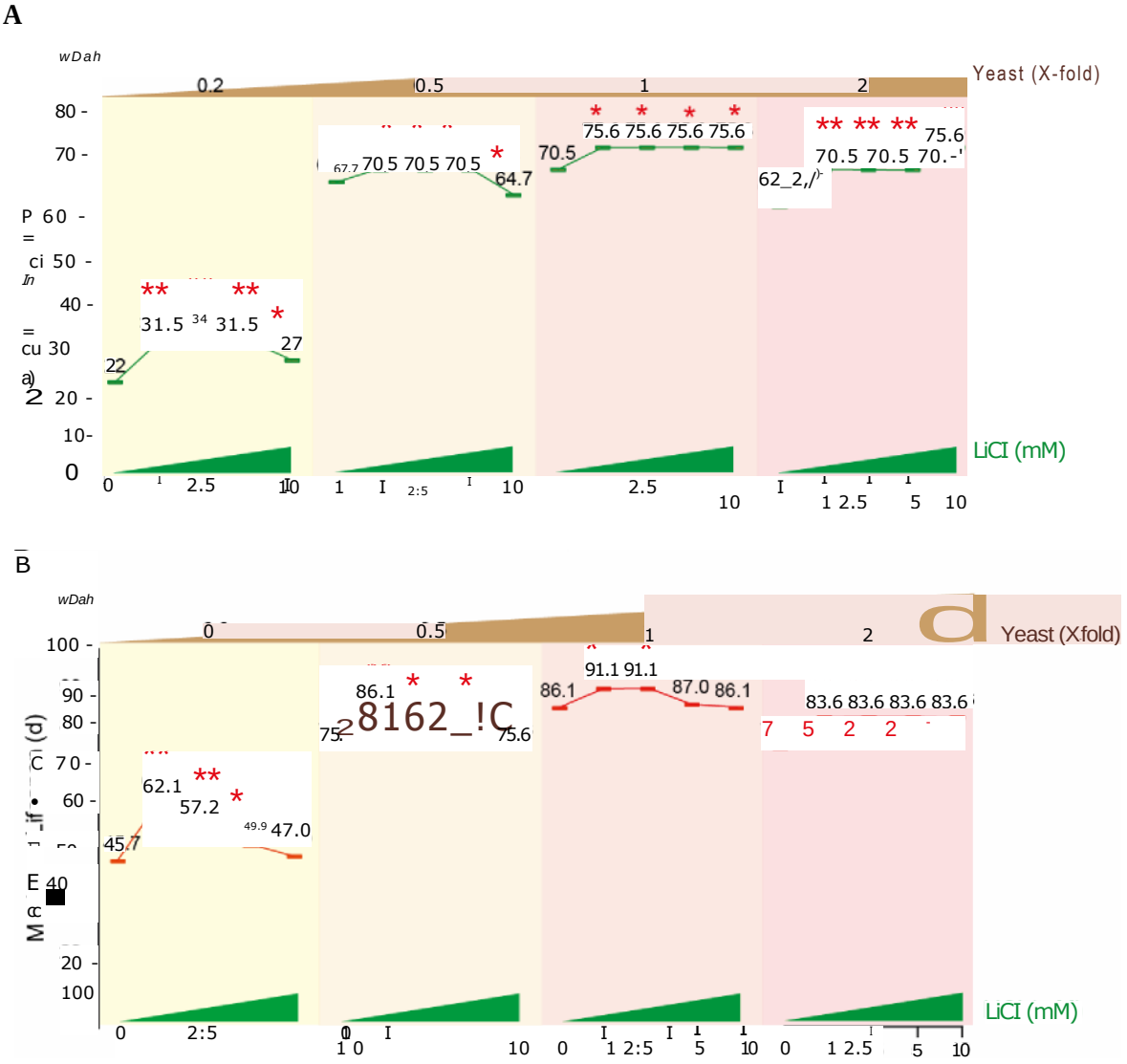


Figure 3.10 Median and maximal lifespan effect of lithium tested on four different yeast concentrations. The effect of different lithium concentrations to extend lifespan was tested against increasing concentrations of yeast in the fly medium. (A) represents median lifespan of female flies, while **(B)** shows maximum lifespans. N = 160 flies per condition. * $P < 0.01$; ** $P < 0.001$ (log-rank test). For full survivals see **Appendix 8.**

3.4.6 Lithium did not alter mTOR activity

I therefore considered the possibility that lithium could be modulating the nutrient-sensing network. One mechanism by which lithium could be exerting lifespan benefits is through the inhibition of the nutrient-sensing mTOR pathway. Down-regulation of mTOR activity by genetic manipulation or pharmacologic inhibition is a robust intervention to extend lifespan across taxa (Bjedov and Partridge, 2011; Johnson et al., 2013). mTOR exists in

two complexes, mTORC1 and mTORC2, with different downstream effectors (Guertin and Sabatini, 2009; Wullschleger et al., 2006). The lifespan extending effects of rapamycin have been mainly attributed to down-regulation of mTORC1 (Bjedov et al., 2010; Harrison et al., 2009), though it has been shown that long-term rapamycin treatment also inhibits signalling through mTORC2 in certain cell types (Sarbassov et al., 2006). To determine whether lithium could influence activity through the mTOR pathway, I probed for phosphorylation levels of S6K, downstream of mTORC1, and phosphorylation of Akt, downstream of mTORC2. Both kinases are phosphorylated and activated when mTOR is active (Wullschleger et al., 2006). Lithium was unable to change the phosphorylation levels of S6K at any dose tested (Figure **3.11A).** I also evaluated the ability of lithium to modulate Akt phosphorylation, without observing significant evidence of change (Figure **3.11B).** Therefore it is unlikely that lithium modulates lifespan through the mTOR pathway.

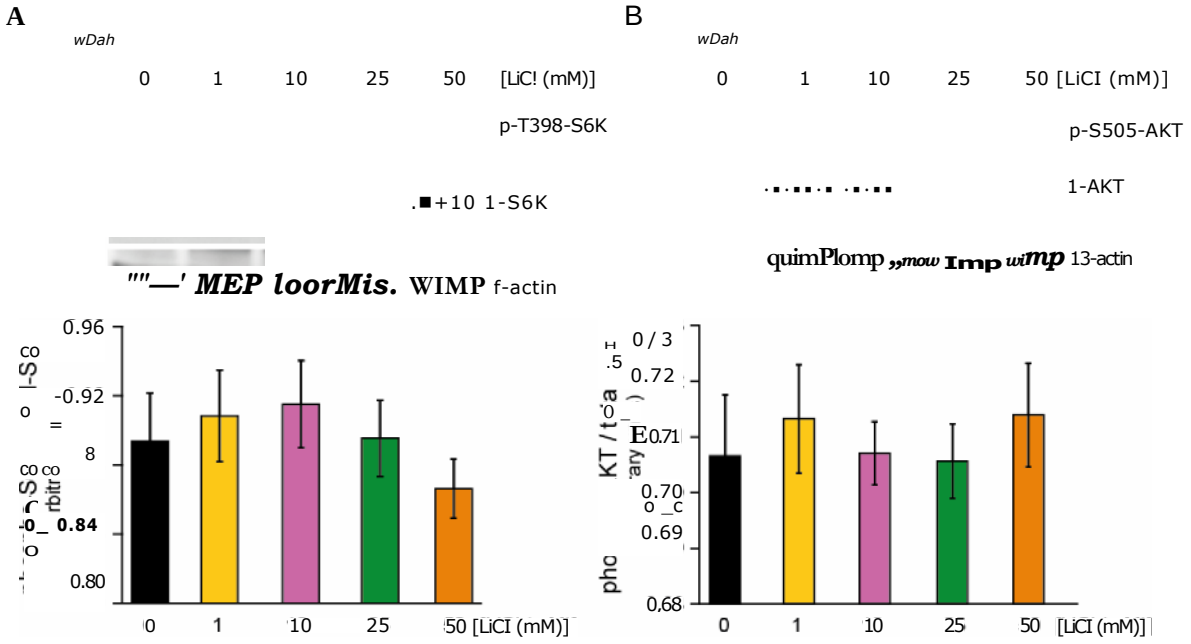


Figure 3.11 Lithium modulation of mTOR activity. (A) Activity of mTORC1 was measured by analysing the phosphorylation levels of S6K against total S6K. (B) Activity of mTORC2 was analysed by substracting the phsosphorylation levels of pAkt (S505) to total AKT. Data are presented as median ± SEM.

3.4.7 Lithium did not require the transcription factor dFOXO to extend lifespan

Genetic manipulations that down-regulate signalling through the IIS pathway extend lifespan from *C. elegans* to mice. Moreover, human longevity has been associated with

genetic variants in the transcription factor FOXO (in humans specifically FOXO3A), required for lifespan extension by reduced IIS in worms and flies (Kenyon et al., 1993; Slack et al., 2011; Willcox et al., 2008). To determine whether dFOXO is required for lithium to extend lifespan, I treated *dfoxo-null* flies with two lithium doses previously shown to extend lifespan in wild-type flies (1 and 10 mM). *dfoxo-null* flies were significantly shorter lived than the controls (Figure 3.12; $P < 0.001$, log rank test). Lithium supplemented in the fly medium at 1 mM extended median lifespan of flies lacking *dFOXO* by 8% (4.7 days), but did not change their maximum lifespan. The survival curve was significantly different from the un-treated *dfoxo-null flies* ($P < 0.02$, log rank test). A similar situation was also observed with 10 mM lithium, the same median lifespan extension, without change in maximum lifespan. I also used a lower dose of lithium, 0.5 mM LiC1, observing also an 8% median lifespan extension, but also a maximum lifespan extension of 8% (-5.5 days; $P < 0.02$, log rank test). These results suggest that dFOXO is not required for lithium to extend lifespan.

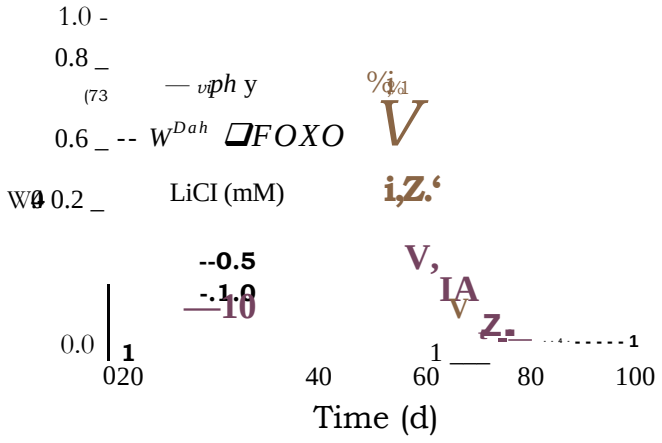


Figure 3.12 Lifespan effect on a *dFOXO* null background. The lifespan effect of lithium was assessed in flies lacking the transcription factor dFOXO. N = 150 flies per condition

3.4.8 Lithium further extended lifespan of an insulin mutant and has additive effects with rapamycin

Given that dFOXO is not required for lithium to confer longevity benefits, I investigated whether lithium could extend the lifespan of already long-lived flies with IIS down-regulation. To test this hypothesis, I over-expressed a dominant negative version of the *Drosophila* insulin receptor (InR^{DN}). This intervention was previously reported to extend lifespan when the InR^{DN} *(UAS- InR^{DN})* was over-expressed throughout development or when expressed only during adulthood (Slack et al., 2011), by using a modified version of the Ga14/UAS system, the GeneSwitch System (Osterwalder et al., 2001). Over-expression

of the InR^{DN} only during adulthood significantly extended lifespan (Figure 3.13A; ***P*** < 0.001, log rank test). Median lifespan was increased by 18% (12 days) and maximum lifespan by —13.5% (10 days). When these flies were treated with lithium, median lifespan was further extended by 7% (5 days), but maximum lifespan only marginally changed by 1 day. However, the survival curves were significantly different from the lithium-untreated flies over-expressing the InR^{DN} ***(P*** < 0.05, log rank test). Overall, the combination of IIS down-regulation and lithium treatment extended median lifespan by 26.6% (18 days) and maximum lifespan by 15% (12.3 days). These results suggest that lithium and IIS down-regulation could be used in combination to achieve maximal benefits for longevity.

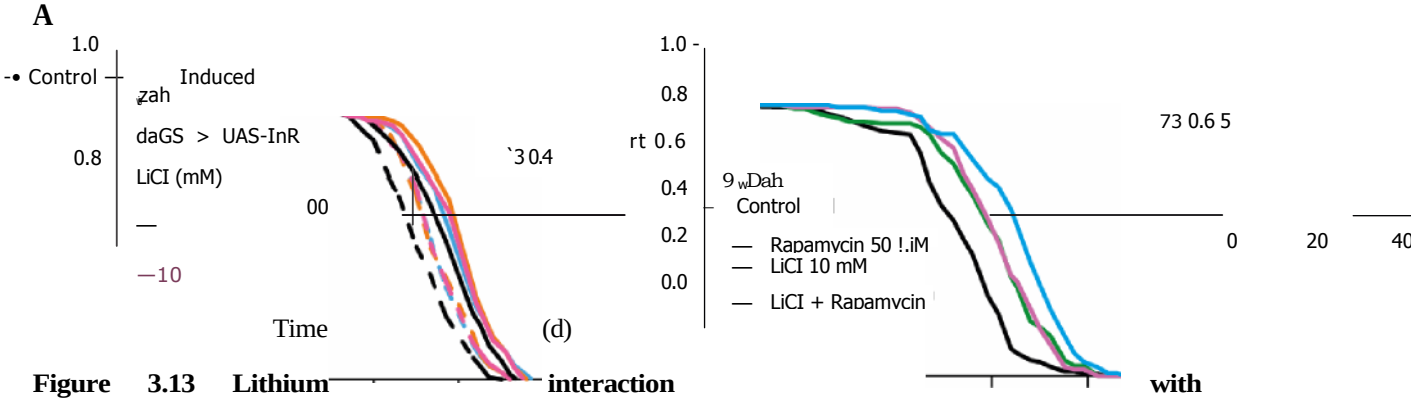


Figure 3.13 Lithium interaction with IIS and mTOR down-regulation for lifespan. (A) The effect of lithium treatment on a long-lived insulin mutant was done by over-expressing a dominant negative InR in the whole fly and then supplementing these flies with lithium. **(B)** The effect of combining lithium and rapamycin was carried out using control flies. Experiment in (B) was performed by Dr. Ivana Bjedov.

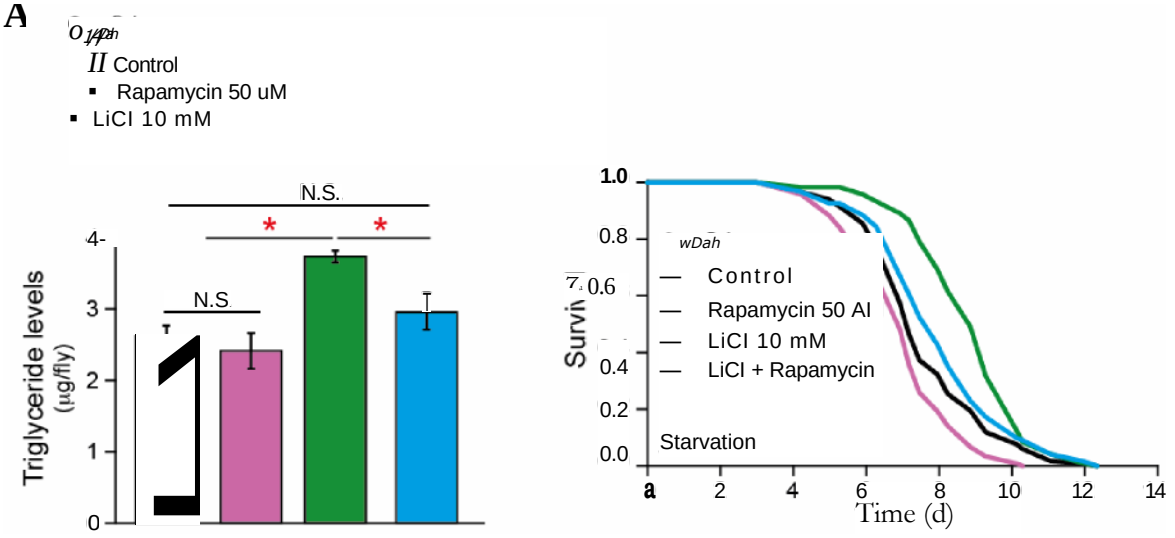
I then decided to analyse whether combining lithium and rapamycin could have beneficial effects for lifespan. I used similar doses to the ones described an evaluated the survival profile of these flies. Both lithium and rapamycin were able to significantly extend lifespan ***(P*** < 0.001 for both treatments, log rank test). The lifespan effect were comparable, 10 mM lithium extended median lifespan by 9.5%, while 50 11M rapamycin extended lifespan by 13%. They both extended maximum lifespan by 5%. A statistical comparison of the survival curves did not show significance ***(P*** = 0.9892, log rank test). When they were supplemented in combination, flies lived significantly longer than either treatment supplemented as monotherapy. The overall median lifespan extension when both lithium and rapamycin were administered was —21%, with maximum lifespan extension of 11% (Figure 3.13B). Overall, these results suggest that the combination of lithium plus rapamycin could be an advantageous poly-pharmacological approach for ageing.

Taken together my results suggest that lithium extends lifespan independently of

the nutrient-sensing network, including the mTOR and the IIS pathways, and that lithium plus lower IIS or rapamycin could be additive for lifespan.

3.4.9 Lithium blocked the effect of rapamycin to increase triglycerides

Figure 3.14 Lithium and rapamycin interaction to modulate triglyceride levels and starvation response. (A) Flies pre-treated for 15 days were analysed for the effect of triglyceride levels. Each bar represents 5 biological replicates of 4 flies. Data are presented as average ± SEM. * $P < 0.01$ (ANOVA; post hoc Tukey-Kramer). (B) Survival curves depicting the response to starvation in flies pre-treated with lithium, rapamycin or the combination of both drugs.



As previously mentioned, one of the undesirable side-effects of rapamycin treatment is altered metabolism resembling the metabolic syndrome (Chang et al., 2009; Fraenkel et al., 2008; Houde et al., 2010). In *Drosophila*, rapamycin causes elevation of triglycerides (Bjedov et al., 2010). I have shown here that lithium has the opposite effect of rapamycin since it dose-dependently lowers triglycerides (Figure 3.8C and **Figure 3.9A). I** therefore hypothesised that lithium might be able to block the dyslipidemia caused by rapamycin. I used a concentration of lithium that did not significantly change triglycerides (10 mM) and compared it to 50 mM rapamycin, previously shown to extend lifespan (Bjedov et al., 2010). Lithium did not change the triglyceride levels, unlike rapamycin, which significantly increased it ($P < 0.01$ ANOVA, post hoc Tukey-Kramer; **Figure** 3.14A). When both treatments were administered simultaneously, lithium was able to block the increase in triglyceride levels by rapamycin ($P < 0.05$ ANOVA, post hoc Tukey-Kramer for comparison between rapamycin vs. rapamycin plus lithium), making the combination

treatment similar to controls ($P = 0.09$ ANOVA, post hoc Tukey-Krammer for comparison

between control vs. rapamycin plus lithium).

To confirm that the modulation in lipid levels was metabolically relevant, I challenged flies pre-treated for 15 days under a similar protocol to starvation. Rapamycin treatment makes flies resistant to starvation (Bjedov et al., 2010), while I showed here that lithium sensitises against starvation. Given that triglyceride levels correlate with the response to nutrient deprivation, I speculated that the effect of lithium plus rapamycin would cancel each other. I pre-treated the flies with lithium, rapamycin or a combination of both for 15 days after which I analysed their survival under starvation. Lithium significantly reduced lifespan under nutrient deprivation ($P < 0.01$, log rank test), while rapamycin conferred resistance ($P < 0.001$, log rank test; **Figure** 3.14B). When lithium and rapamycin were administered in concert the starvation response as a monotherapy for lithium or rapamycin was blocked, as the response was not statically different from controls ($P = 0.069$, log rank test). These results suggest that the addition of lithium to rapamycin treatment could be advantageous to block the dyslipidemia induced by rapamycin treatment in *Drosophila*.

3.4.10 Lithium extended lifespan when supplemented late in life

Finally, I decided to investigate the effect of supplementing lithium at different periods of the adult *Drosophila* life. I first assessed whether supplementation at older ages would still show the same dose-response effect with a similar therapeutic window. I kept flies in control fly medium (0 mM LiC1) and switched them to food containing a range of doses between 1 and 75 mM. I carried out the switch after 30 days post-eclosion and analysed their survival (Figure 3.15A). 1 mM lithium extended median lifespan by 5% (4 days) and maximum by 13% (8 days; $P < 0.05$, log rank test). 10 and 25 mM extended lifespan by 9% (6 days), but while 10 mM extended maximum lifespan by 4.5% (3.5 days), 25 mM extended maximum lifespan by 8% or 6 days ($P < 0.01$, log rank test). 50 and 75 mM significantly reduced lifespan ($P < 0.001$, log rank test). 50 mM reduced median lifespan by 5% and maximum by 10.5%. Supplementation with 75 mM lithium after 30 days post-eclosion reduced median lifespan by 36.5% and maximum by 25.5%. Thus, when lithium is started after 30 days post-eclosion lifespan extension can be achieved with doses from 1-25 mM.

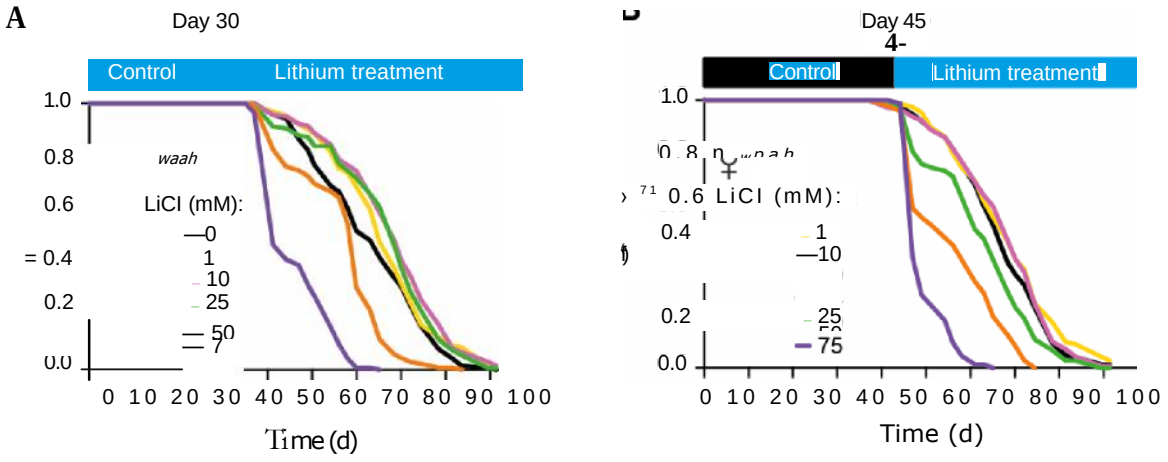


Figure 3.15 Late-onset lithium treatment (A) Lithium treatment at different doses was started 30 days post-eclosion. **(B)** Lithium treatment was initiated after 45 days of separating into females. N = 150 per condition.

I then decided to investigate whether the supplementation of lithium could be started even later, after 45 days post-eclosion. At this point all the group of flies had a few deaths. Flies were randomly allocated to the different concentrations of lithium. Interestingly none of the doses that extended lifespan after 30 days post-eclosion were able to significantly extend lifespan (Figure 3.15B). 1 and 10 mM did not change lifespan and statistical analyses of the survival curves revealed no significant difference ($P > 0.05$ log rank test). Food supplemented with 25 mM lithium and started after 45 days post-eclosion significantly shortened median lifespan by 4 days ($P < 0.02$, log rank test), while maximum remained similar to the control group. 50 and 75 mM lithium also significantly reduced lifespan ($P < 0.001$, log rank test). Interestingly, the detrimental response to high doses of lithium 50 and 75 mM was very quick as median lifespan was achieved after 2 days in media supplemented with 75 mM lithium and 7 days when supplemented with 50 mM. By comparing the shape of the curves of flies supplemented 50 or 75 mM lithium after 30 or 45 days post-eclosion it is evident that some degree of age-related toxicity exists. Thus, though lifespan extension can be achieved when lithium is supplemented late in life, this will require some time to promote longevity. Also it seems that lithium toxicity is exacerbated at older ages.

3.4.11 Lithium extended lifespan when fed for a brief period

I then wondered whether short treatment periods could have long lasting effects for lifespan. Therefore, I only supplemented lithium for a brief period of time after which the

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flies were switched off from lithium to control medium. I first supplemented a range of lithium concentrations from day 2 post-eclosion for 5 days before switching to medium not supplemented with lithium (Figure 3.16A). Interestingly none of the concentrations significantly changed lifespan (*P* > 0.05 log rank test). This was particularly surprising for the higher concentrations, which are quite toxic when supplemented continuously (Figure 3.3).

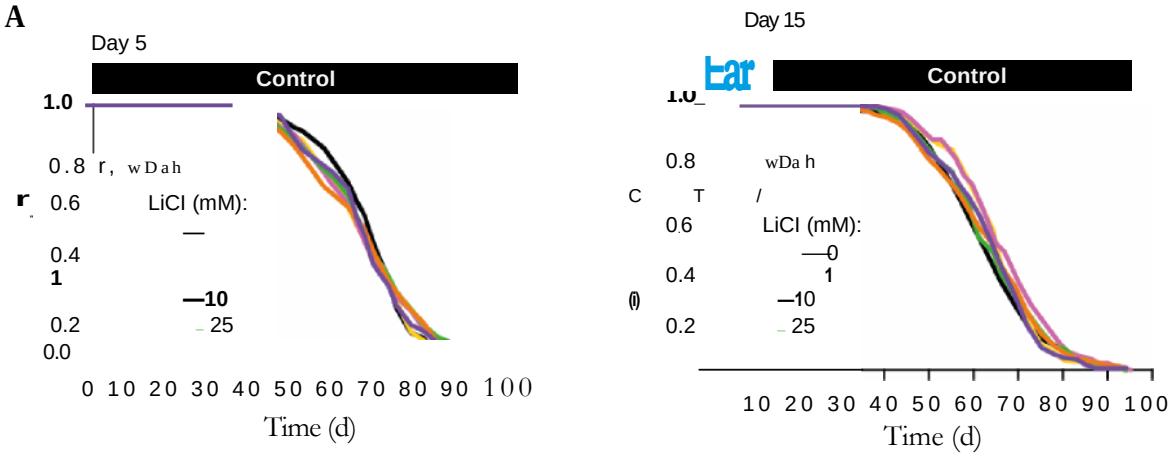


Figure 3.16. Switch off lithium experiments. Lithium was supplemented only for 5 days (A) or 15 days (B) after which all groups received fly medium not containing lithium. N = 150 per condition.

I decided to explore the possibility that lithium required to be supplemented for longer periods to promote long lasting effects. I followed a similar protocol and fed flies different concentration of lithium for 15 days after which they were switched off the drug to control medium (Figure 3.16B). Both 1 and 10 mM lithium extended median lifespan by 8% (5 days), while only extended maximum lifespan by 1 day (*P* < 0.05 log rank test). Once more none of the concentrations between 25 and 75 mM changed lifespan significantly (*P* > 0.05 log rank test). Thus, lifespan extension can be achieved when lithium is supplemented only during a brief period early in adulthood, yet the drug has to be supplemented for more than five days before switch off.

3.5 Discussion

3.5.1 Lithium is a pro-longevity drug

I showed here that lithium extended lifespan of male and female flies independent of genetic background. Lithium modulated lifespan in a dose-dependent manner It extended lifespan at low concentrations (1-25 mM) while it reduced lifespan at higher doses (50-100 mM). McColl et al., showed that lithium was able to extend lifespan of *C. elegans* at doses ranging from 5-50 mM, the optimal concentration being 10 mM which extended lifespan on average 36% (McColl et al., 2008). An independent study by Tam et al., showed that 10 mM lithium only mildly extended median lifespan of *C. elegans* without changing maximum lifespan, but they showed that lithium improved locomotor function (Tam et al., 2014). Moreover, Zarse et al., only reported lifespan extension in *C. elegans* after administration of 10 11M lithium (Zarse et al., 2011). These dose differences are not entirely surprising as I observed a considerable variation between experiments using the same genetic background over three years of experimentation. Moreover, as I will discuss later, the optimal concentration for lifespan extension varied according to the yeast concentration in the fly medium.

Several interventions are modulated by genetic background. For example, in yeast cells some genetic manipulations that mimic DR and extend lifespan are strain-dependent (Kaeberlein et al., 2004). In *Drosophila* triple knockout of dILPs 2,3 and 5 increased median lifespan by 29% in the w^{pah} background, but failed to regulate longevity in the w^{1118} background (Griinke et al., 2010). I was able to show that lithium extended lifespan in two genetic backgrounds, making the intervention likely to be strain-independent. Lithium treatment of female flies of the w^{pah} background resulted in median lifespan extension by as much as 18%, and 13% in ^{w1118} females. Furthermore, I was able to show that the lifespan effect is sex-independent as lithium also extended the median and maximum lifespan of male ^{w1118} flies.

Interventions that promote longevity in *C. elegans* and *Drosophila* often do so at the cost of reproductive fitness. For example, DR in female flies leads to a significant reduction in egg-laying (Barnes et al., 2008; Bass et al., 2007; Metaxakis and Partridge, 2013). Similarly, mutations in the IIS pathway or rapamycin treatment also reduce number of eggs laid (Bjedov et al., 2010; Clancy et al., 2001; Griinke et al., 2010; Slack et al., 2010). The effect on reproductive fitness in long-lived organisms is explained by the disposable soma theory of ageing that assumes that given an increase in damage

accumulation with age the organism has to decide to invest energy either in reproduction or somatic maintenance. When somatic maintenance is favoured, lifespan extension is ensured at the cost of low or no reproductive capacity (Kirkwood, 2005; Kirkwood and Austad, 2000). I was unable to observe an effect on female egg-laying at doses that extended lifespan. However, as lithium became toxic female fecundity was significantly reduced. This result is contrasting with the observation in *C. elegans* in which the optimal dose for lifespan extension was associated with an acceleration of the age-related loss of fecundity (McColl et al., 2008). It would be interesting to know whether lithium can regulate lifespan of *C. elegans* independent of the germ-line. Perhaps the processes by which lithium modulates ageing in *C. elegans* and *Drosophila* are considerably overlapping, yet differ in other traits like reproduction. This is not the only discrepancy detected between our results and those obtained in *C. elegans*. As previously mentioned Tam and colleagues observed that longevity in *C. elegans* is associated with improvements in locomotor ability (Tam et al., 2014). I tested the effect of lithium treatment on age-related locomotor decline in two genetic backgrounds and only observed that lithium retarded the locomotor dysfunction at doses that shortened lifespan in the $^{-}w^{pah}$ background, but at a relevant dose for longevity in w^{1118} flies. Our group has previously shown that low doses of lithium can improve locomotor function in an AP1_42 *Drosophila* model of Alzheimer's disease (Sofola-Adesakin et al., 2014; Sofola et al., 2010). It is unclear why doses that promote longevity in *Drosophila* would not protect against age-related locomotor decline, while doses that shorten lifespan would. I consider that this should be further explored as it might reveal divergent mechanisms to regulate longevity and neuromuscular function during ageing.

In conclusion, lithium can extend lifespan in yeast, worms and flies suggesting that lithium is an evolutionary conserved pro-longevity drug. Moreover, lithium concentrations in drinking water have been associated with reduced mortality for all causes in a Japanese population raising the possibility that lithium could modulate ageing in mammals.

3.5.2 Lithium extends lifespan when administered late in life or briefly in early adulthood

As anti-ageing therapies become widely used there will be a debate as to when should they be initiated to promote longevity. Though long-term use (even if started early in adulthood) of a drug that slows down ageing does not seem too much to ask when the

broad-spectrum benefits are considered, it will certainly require changes in behaviour. In practice, a drug with potential anti-ageing properties should be able to extend lifespan when administered late in life. This is in part for practical considerations. If this drug was impacting in reproductive fitness, treatment initiation should only be considered in the post-reproductive stage, particularly in females. Additionally, long-term exposure to drugs can lead to unforeseen side effects. Rapamycin, for example, has been shown to extend lifespan when administered late in life making it, from this point of view, an ideal anti-ageing medication (Harrison et al., 2009). In *C. elegans* metformin was shown to modestly increase mean lifespan when administered from middle age, though the optimal dose was lower than when administered earlier on (Cabreiro et al., 2013).

To analyse whether lithium would be able to increase lifespan when administered late in life I considered two ages for treatment initiation. First I analysed the pro-longevity effects of lithium after 32 days post eclosion (48 hours of mating after eclosion were not considered in the lifespan curves). Interestingly, all doses (1, 10 and 25 mM) that extended lifespan when treatment was initiated early on in adulthood were still able to increase median lifespan if started 30 days later (Figure 3.15A). This differs from the effect of metformin previously mentioned (Cabreiro et al., 2013). However, when lithium treatment started after 45 days (when the first few flies had started dying) it was unable to increase lifespan, and indeed showed accelerated lithium toxicity at older ages (Figure 3.15B). While flies under 75 mM lithium initiated at 30 days took approximately 7 days to reach median lifespan, this same concentration started after 45 days achieved median lifespan in just 2 days.

Alternatively I also analysed the effect of "switching off' lithium treatment, i.e., stopping the treatment after a brief period of supplementation. For these experiments I analysed the effect of feeding lithium to flies for two periods, either 5 or 15 days after they were switched off. After 5 days of supplementation of doses ranging from 1-75 mM no effect on lifespan was observed. Though the lack of effect for lower doses was not completely unexpected I found very surprising that the higher concentrations did not reduce lifespan. On the other hand, when lithium was supplemented for 15 days median lifespan was significantly extended at doses of 1 and 10 mM (thought the effect on maximum lifespan was minimal) and again no lifespan effect was observed at doses from 25-75 mM (Figure 3.16B). To the best of my knowledge these types of experiments have never been reported before and can provide novel insights into the pharmacology and therapeutics of lithium treatment.

These experiments reveal several important features of lithium. First, lithium is able to extend *Drosophila* lifespan when administered late in life as rapamycin in mice and metformin in *C. elegans* and mice (Cabreiro et al., 2013; Harrison et al., 2009; Martin-Montalvo et al., 2013). Second, there is a threshold period or therapeutic time frame during the ageing process at which lithium is able to successfully exert its longevity effects. After this point low doses of lithium will be unable to change the course of ageing and lifespan (also meaning they will not be toxic), but as the concentration increases lifespan will be shortened even quicker than at younger ages. Lithium toxicity during human ageing has been well documented (Malhi et al., 2012; Timmer and Sands, 1999). Even though targeted therapeutic concentrations are considered to be narrow (0.5-0.8 mM/L), at older ages the recommended concentrations are suggested to be at the lower end (0.5-0.6 mM/L) of the therapeutic window (Wijeratne and Draper, 2011). Similar serum concentrations are achieved in patients over 60 years old with lower drug doses than in younger patients. Furthermore, lithium is less prescribed for the treatment of BPD in older patients potentially due to increased risk of toxicity (Paton et al., 2010). Several physiological changes in kidney function, total water and extracellular volume, comorbidities, and drug-drug interactions due to polypharmacy in the elderly are considered to contribute to the increased age-related toxicity (D'Souza et al., 2011).

My results showed that lithium might be unable to extend lifespan of flies when administered very late in life (45 days). At least two points should be considered here, first at this age flies barely eat (Wong et al., 2009), hence circulating concentrations of the drug inside the fly obtained (when supplemented at the lower end of the range of concentrations tested) might be suboptimal. Secondly, as with humans, it might be tricky to find the optimal range to extend lifespan in flies as going beyond the narrow range might increase toxicity. To test this, it will be necessary to develop a standardized protocol, allowing measuring the concentration of the drug inside the flies and to be able to monitor it *in vivo* across their lifespan. Additionally, the drug might require some time to induce or repress the molecular targets required for lifespan extension. Thus, if the treatment is initiated too late, the organism might be unable to mount a strong enough molecular response that would allow longer survivals. In humans and mammalian models lithium has been proposed to act by two possibly unrelated mechanisms for the treatment of mania and depression. For example, it has been documented that anti-manic properties can take up to 6-10 days to develop, while its anti-depressive effects take up 6-8 weeks to come into effect (Malhi and Tanious, 2011). It is possible that the mechanisms for anti-manic and

anti-depressive effects are different, and the one(s) regulating longevity in flies might be more similar to the anti-depressive ones in humans, hence they might require more time to be elicited.

Third, the "switch off' experiments reveal that lithium has "molecular memory" as the pro-longevity effect is kept even after the drug is removed for a long period of time. Fourth, the toxic effects of lithium on lifespan require continuous treatment or long-term exposure (longer than 15 days) when initiated early on in adulthood. The latter was made evident by the fact that neither 5 nor 15 days of lithium treatment started after 2 days post-eclosion were sufficient to shorten lifespan at the highest dose tested (75 mM) which would dramatically shorten median lifespan to approximately 30 days when administered continuously. The fact that long-term treatment was not necessary to achieve lifespan extension in flies suggests that short-term treatments could be as effective as long-term treatments. In humans, long-term treatment with lithium is associated with progressive and permanent renal damage, which is the most dreaded toxic side-effect of lithium (Malhi and Tanious, 2011). Dosing for the treatment of BPD is often broken down in several doses per day. However, single doses and alternate day regimes have also been considered to be efficient in maintaining brain concentration levels within the therapeutic window (Malhi and Tanious, 2011; Wijeratne and Draper, 2011). When testing lithium as a pro-longevity drug in mammals alternate day dosing or just brief periods of time should be considered to reduce undesirable side effects and maximise the potential health benefits.

Taken together my results suggest that several dose regimes can be used to extend lifespan in *Drosophila.* As these treatment protocols have never been tested before in any other organism they should be used as a starting point for the analyses of lithium in ageing, particularly in mammals. My results suggest that either late-life treatment initiation or brief treatment protocols are sufficient to promote longevity.

3.5.3 Lithium regulates metabolism and the response to starvation

Metabolic pathways are at the heart of the ageing process (Barzilai et al., 2012). Changes in major pathways and metabolites can contribute to the healthy ageing phenotype or even accelerate ageing through metabolic dysfunction and disease (Bartke and Westbrook, 2012; Houtkooper et al., 2010; Perrone et al., 2012; Soltow et al., 2010; Tomds-Loba et al., 2013). I explored potential metabolic shifts associated with lithium in carbohydrate and lipid metabolism without detecting changes in trehalose and glycogen levels. However,

lithium dose-dependently reduced triglyceride levels. Alterations in metabolism often associate with lifespan extension in *Drosophila*. Unsurprisingly, reduced IIS leads to overt gluco-metabolic alterations. Long-lived flies show increased levels of trehalose and glycogen, and elevated circulating glucose (Alic et al., 2011a; Broughton et al., 2005; Griinke et al., 2010; Slack et al., 2010). Furthermore long-lived flies with reduced IIS or rapamycin treatment, or flies subjected to DR show increased levels of triglycerides (Bjedov et al., 2010; Burger et al., 2007; Emran et al., 2014; Slack et al., 2010). Though the relevance of these alterations for longevity is not clear, they have become longevity-associated traits in flies given their constant reports in ageing studies in *Drosophila*. This is not the case for mammals in which DR, IIS mutants, S6K1 deletion or metformin treatment are associated with improved metabolic profiles during ageing, i.e., increased insulin sensitivity, lower lipids and glucose levels (Bliiher et al., 2003; Colman et al., 2009; Selman et al., 2008a, 2009). In a way lithium seems to regulate *Drosophila* metabolism in a different way to the classic anti-ageing interventions in the nutrient sensing network. An exception to this statement would be metformin treatment, which also reduces triglyceride levels in flies, but without a longevity phenotype (Slack et al., 2012). In mammals lithium produces nephrogenic diabetes insipidus, several electrolyte alterations and weight gain, though these phenotypes are probably secondary to the effect of lithium on kidneys, parathyroid gland and bone, and thyroid gland, respectively (Jefferson, 2010; Timmer and Sands, 1999). In a study where lithium was supplemented to rats, increasing concentrations of lithium associated in a dose-dependent manner with reductions in total lipids, cholesterol, triglycerides and nonesterified fatty acids (Fleischman et al., 1974). I did not measure the effects on such specific lipid metabolites, but the effect on triglycerides seems to be conserved in evolution from flies to rats.

I observed that concomitant with altered lipid metabolism, lithium treatment led to a dose-dependent sensitivity to starvation. Most insulin mutant flies are resistant to starvation. However, they also have high triglyceride levels (Broughton et al., 2005; Slack et al., 2010; Griinke et al., 2010; Alic et al., 2011). Rapamycin treatment in flies also leads to starvation resistance, but is associated with increased triglyceride levels as well (Bjedov et al., 2010; Emran et al., 2014). Triglycerides are the main fat storage of flies and have been shown to predict the response to starvation (Ballard et al., 2008). Though starvation resistance has been viewed as a desired phenotype in long-lived organisms, I will show in Chapter 7 that short-lived *Drosophila* with increased triglyceride levels are resistant to starvation, suggesting that these traits can be uncoupled. Taken together, these

observations suggest that starvation resistance and longevity are independent and that resistance to nutrient depletion in flies is indeed the result of accumulated energy stores which help to cope when no food in supplied.

In conclusion lithium spares carbohydrate energy stores in *Drosophila* but dose-dependently reduces triglycerides levels making flies sensitive to starvation.

3.5.4 Lithium is unlikely to be a DR mimetic

I carried out a set of experiments to determine whether lithium promoted longevity through DR or the nutrient sensing network. In *Drosophila,* the ingredient in the medium with the biggest effect on lifespan is the yeast (Bass et al., 2007; Mair et al., 2005; Piper et al., 2010). Varying the concentration of yeast leads to a typical DR tent where the optimal concentration for lifespan extension is somewhere in the middle of the yeast dilution. Further dilution leads to lifespan shortening due to malnourishment, while increases in the yeast concentration also shorten lifespan, probably due to the deleterious effect of over feeding (Bass et al., 2007; Piper and Partridge, 2007).

I supplemented a range of lithium concentrations (1, 2.5, 5 and 10 mM) in the fly medium containing different concentrations of yeast (0.2, 0.5, 1 and 2X yeast; X = 100 g/L) and assessed survival under these conditions. Lifespan peaked at 1X when lithium was not present in the medium. The lifespan obtained with this concentration of yeast was therefore the optimal one for lifespan under DR. Lithium supplemented in all yeast conditions was able to extend median lifespan. In 0.2X the best concentration to extend lifespan was 2.5 mM, while the best in 2X was 10 mM lithium. Interestingly at 0.5X 10 mM lithium significantly reduced lifespan. Several conclusions can be drawn from these experiments. First, lithium could extend lifespan in all yeast concentrations tested. Given that it further extended lifespan under conditions maximised for diet it is unlikely that lithium acts as a DR mimetic. A DR mimetic would have led to a right-shifted tent, where lithium would have not changed (or reduced) lifespan at the optimal yeast concentration for lifespan, and reduced lifespan at all concentrations below that. The lifespan extension would have only been observable under full feeding or over nourished conditions (Clancy et al., 2002; Mair and Dillin, 2008). Second, I was able to document that the optimal concentration of lithium to extend lifespan changed under different yeast dilutions. This suggests some interaction between DR and lithium, i.e., they share some overlapping mechanisms. I therefore decided to test whether lithium affected the IIS and mTOR

nutrient sensing network. I did not detect changes in mTOR activity as evaluated by immunoblot analyses. Lithium was able to extend lifespan of flies lacking *dFOXO* and further extend lifespan of long-lived flies with IIS down-regulation. Taken together these results did not support a role for the nutrient sensing network in the lifespan extension provided by lithium.

McColl and colleagues also investigated the interaction of lithium with DR and the IIS pathway. Using the *eat2* mutant, a genetic model of DR in *C. elegans,* they were able to show a further lifespan extension when lithium was supplemented. They also observed lifespan extension in a *daf16* mutant and further lifespan extension in a long-lived *daf2* mutant (McColl et al., 2008). My results in *Drosophila* are in accordance to the published data in *C. elegans* suggesting that lithium can indeed extend lifespan beyond DR and the IIS pathway. Unfortunately, the analyses of McColl and colleagues (also Zarse et al., and Tam et al.,) did not include epistasis with mTOR mutants or rapamycin treatment limiting a comparative analyses of the involvement of the mTOR pathway in lithium's longevity phenotype between *C. elegans* and *Drosophila.*

3.5.5 Lithium and Rapamycin: a polypill?

As previously mentioned lithium can interact with many drugs increasing its toxicity. Drugs that alter renal function can either increase or decrease lithium concentrations. For example, while thiazides (a group of diuretics used in hypertension and other cardiovascular diseases that increase diuresis through inhibiting the reabsorption of sodium and chloride ion in the distal convoluted tubules in the kidney) increase it, loop diuretics (which act on the Na^+-K^+2C1^- symporter in the loop of Henle decreasing the hypertonic renal medulla hence reducing the reabsorption capacity and promoting diuresis) decrease it. Angiotensin converting enzyme inhibitors used for the treatment of hypertension and heart failure and the non-steroidal anti-inflammatory ibuprofen increase lithium concentrations (Timmer and Sands, 1999). As the older population is likely to be taking multiple medications (polypharmacy) it will be of great interest to analyze the interaction of the pro-longevity effect of lithium in the context of other drugs.

Additionally, it has been proposed that given that ageing affects multiple pathways, affecting these pathways simultaneously could be advantageous for healthy ageing (Le Couteur et al., 2012; Ingram et al., 2006). I decided to explore the possibility of combining lithium and rapamycin for three reasons. First, lithium and rapamycin seem to act on

different pathways in *Drosophila*. Second, while lithium reduces triglyceride levels, rapamycin increases it. Third, a combination of lithium and rapamycin has been shown to be more beneficial than either treatment on its own for ameliorating neurodegeneration (Sarkar et al., 2008). I explored the possibility that lithium could block the increase in triglycerides promoted by rapamycin treatment. For this I used a concentration of lithium that was unable to significantly reduce triglycerides, but that extended lifespan. I chose to use 10 mM lithium. When lithium and rapamycin were simultaneously fed to flies lithium was able to block the hypertriglyceridemia produced by rapamycin, in spite of having no detectable effect when supplemented as monotherapy. I documented that rapamycin and lithium cancelled each other's response to starvation, which validated the finding in lipid levels.

Amongst the undesirable side effects of rapamycin treatment the metabolic alterations have generated particular interest (Lamming et al., 2012, 2013). A drug that promotes healthy ageing should not increase the prevalence of insulin resistance and diabetes. I have shown here that simultaneous administration of lithium and rapamycin can limit this undesirable effect of rapamycin on metabolism in flies.

Blocking the effect of rapamycin on lipids might not be enough therapeutic reason for combination treatment in ageing. Although the combination provided some metabolic benefits, this did not neccesarily translates in improved lifespan. The combination of both drugs could have led to cancelling each other effect on longevity or even becoming toxic hence shortening lifespan. I therefore analyzed the effect of combining the drugs on survival. Flies that received rapamycin and lithium in combination outlived those that only received either treatment as monotherapy. This result shows that the combination of both drugs can be advantageous for lifespan extension.

Combining drugs in a polypill has been proposed as a future direction in biogerontology (Le Couteur et al., 2012; Gems, 2014). The concept of the polypill was proposed by Wald and Law for the prevention of ischaemic heart disease (IHD) and stroke. Wald and Law proposed that the combination of a cholesterol lowering drug (statin), three drugs for the treatment of blood pressure (each at half-standard dose), folic acid and aspirin, which they called the polypill, would reduce IHD events by 88% and stroke by 80%. People taking this formulation would benefit from an average of 11 years free of these conditions (Wald and Law, 2003). Several clinical trials are on their way to document the benefits of long-term treatment with fixed-dose combination (FDC) strategies (Sanz and Fuster, 2013). In light of the findings of these trials and my findings

with lithium and rapamycin combination, I propose that a polypill approach could be advantageous in ageing for the following reasons:

1. Polypharmacy has become synonymous of drug treatments in the elderly. Increased prevalence of multiple pathologies (comorbidity) will more than often require the use of multiple medications to control symptomatology (Capobianco and Lio', 2013; Sitar, 2012).
2. As the number of drugs required for disease treatment rise, the complexity of the therapeutic intervention follows. Undesired drug-drug interactions might occur. This is not considering that the elderly population has been documented to have the highest rate of self-medication, which could complicate the polypharmacy approach when not reported to the team of physicians (Sitar, 2012).
3. Adherence to a large number of drugs decreases over time. This has been one of the major advantages of FDC strategies over multiple single medications found in clinical trials.
4. The appropriate combination of drugs can be therapeutic but also preventative. In the case of FDC strategies, the drugs being combined can be of great benefit in patients over the age of 55 years if provided at very low concentrations. Reducing the risk of coronary events and strokes could prove to be a major public health intervention.
5. Combining drugs that act on independent pathways to promote health can be beneficial, especially if they are able to block feedback loops and adaption mechanisms that lead to undesirable side effects.

It is too early to predict whether lithium and rapamycin could ever become a viable polypill treatment for ageing. I consider that perhaps adding aspirin and metformin could provide further enhancements. The road to the polypill in ageing is still long and narrow, but my findings are interesting enough to start speculating about it.

Chapter 4

~~Genome-wide -OMICS of lithium for ageing: identifying~~ a molecular mechanism

"Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less."
Marie Curie

4.1 Abstract

In spite of its widespread use for the treatment of BPD, lithium's mechanisms of action in this disease are unknown. Several proteins and signalling pathways have been proposed, but evidence is conflicting. In this chapter I aimed to characterise the transcriptional response of Drosophila (heads and thoraces) to lithium chloride. Transcriptional microarrays and also polysome profile arrays were conducted to detect changes in the transcriptome and translatome. We found transcriptomic data to support that lithium and HS down-regulation act by non-overlapping mechanisms as their transcriptional signatures were not shared. This is in accordance with the data presented in the previous chapter showing that lithium does not require the transcription factor dFOXO and can extend the lifespan of already long-lived insulin receptor mutant flies. However, the transcriptional response of lithium treated flies significantly overlapped with that of flies over-expressing the transcription factor cap 'n' collar C (CncC) and of flies treated with the xenobiotic phenobarbital. Indeed, the genes up-regulated by lithium mapped to the three phases of detoxification. I confirmed that lithium indeed protects against toxins as it protected against several chemical stressors. Moreover, I also found that lithium down-regulated components of mitochondrial complex 1 both at the transcriptional and translational level. These correlated with reduced respiratory control ratio by mitochondrial respiratory complex I from isolated mitochondria of lithium treated flies. My genome-wide OMICS approach has uncovered two potential mechanisms by which lithium could be conferring its beneficial effects.

4.2 Introduction: pharmacogenetics and pharmacogenomics

Over the last 15 years and with the advent of technology capable of surveying the entire genome, ageing research has adopted tools in aid of understanding the molecular processes behind interventions that promote longevity. In the beginning most of these observations were purely correlative. With the development of appropriate bioinformatics tools, processes, networks and interaction maps have been described. In invertebrates, in particular, these observations have been taken a step forward by directly manipulating the genes found by these genome-wide approaches. For example, when mRNA expression changes of one or more genes were found to be up-regulated, RNAi technology was used to determine whether that increased expression was correlative, necessary or indispensable for the longevity phenotype. Such methods had been taken before genome-wide assessment was possible, but with candidate genes rather than with an unbiased approach. The latter when associated with a drug has been called pharmacogenetics. On the other hand, pharmacogenomics refers to the use of genetic epistasis experiments coupled with genome-wide technology in response to drugs (Alavez and Lithgow, 2011, 2012).

4.2.1 The molecular targets of lithium: the use of microarrays

Lithium's track record in the clinic is quite peculiar. It is the most prescribed drug for the treatment of BPD, yet we do not understand how it acts to control the disease. It is true that we do not know the molecular targets for all drugs used in Medicine. However, given the simple molecular architecture of lithium it is quite surprising that a therapeutic target has not been identified. In part we are still unsure what to look for in mood disorders. The aethiopathogenesis of BDP is still unclear. Therefore, identifying a specific protein or enzyme, or even a pathway has been challenging.

To understand both the molecular processes driving BPD and the effect of lithium several lines of study have been taken and should be regarded as complementary.

1. Not every patient or group of patients respond to lithium treatment. There is evidence that good/poor response to lithium has a genetic component as it runs in families. Therefore, understanding the genetic makeup of BPD patients has therapeutic value. This allows tailoring of treatment, but aids at characterising genetic subsets of the disease (Serretti and Drago, 2010; Severino et al., 2013).
2. Understanding gene expression changes in brain or periphery of patients with BPD and the response of these same genes to lithium could narrow the molecular targets.

For example, Sun et al., characterised the transcriptional response of post-mortem prefrontal cortex of patients with BPD. They identified changes in mitochondria' respiratory chain complexes, phosphatidylinositol signalling, and glycolysis and gluconeogenesis. Among the changes in the respiratory complexes they found that when patients where stratified by use or not of lithium, those not using lithium (but other mood stabilisers) showed down-regulation of subunits of complexes I and IV particularly, while patients treated with lithium showed up-regulated levels of these same genes (Sun et al., 2006).

3. Genome-wide responses to lithium in cellular and animal models. Seelan and colleagues treated human neuronal cells with lithium for 33 days before analysing the transcriptional response. They found that TRB3 which encodes for tribbles homolog 3, a pro-apoptotic protein was the most down-regulated transcript, while PRDX2, encoding peroxiredoxin 2, an antioxidant enzyme, was the most up-regulated mRNA. Given that several genes showed differential expression they built a protein interactome that revealed that the MAPK pathway was the most regulated by lithium (Seelan et al., 2008). Chetcuti treated male mice with lithium for 7 days and analysed the transcriptional response in whole brain. They only found a small subset of genes differentially expressed over four-fold. Of these genes they were only able to corroborate (by qPCR) the changes in a handful of genes including (but not limited to) transcription elongation factor B (GO: regulation of transcription), metallothioenin 3 (GO: metal ion homeostasis), and proteasome subunit, 13 type 5 (GO: ubiquitin dependent protein) (Chetcuti et al., 2008).

4. Genome-wide responses to lithium in healthy subjects. Watanabe and colleagues treated healthy subjects with therapeutic doses of lithium for 2 weeks and assessed transcriptomic changes in leukocytes after 1 and 2 weeks of lithium treatment and also 2 weeks after treatment had stopped. The top five GO categories for up-regulated genes were: response to stimulus, cellular response to stimulus, response to stress, immune system process, and cell surface receptor signalling pathway. The top five GO categories for down-regulated genes were response to bacterium, modification of morphology or physiology of other organisms involved in symbiotic interactions, cell killing, response to fungus, and killing of cell of other organisms. This study has provided evidence for stress response and the regulation

of immune function as potential mediator of lithium therapeutic action (Watanabe et al., 2014).

All of these approaches have potential value for identifying the pathogenic drivers of BPD, but also the molecular targets of lithium. However, the genomic data for these studies has been poorly analysed. This is the particular case of transcriptomic analyses in cell models and rodents. Though the genome-wide approach is unbiased, researchers tend to make arbitrary cut-off points and focus just on a subset of genes, rather than analysing the entire genome response. Taking only the most up-regulated and down-regulated genes severely narrows the unbiased potential approach.

4.2.2 The use of transcriptomics in ageing

Research into ageing has also benefited from the incorporation of microarray and other OMICS methods for the complementation of lifespan analyses and phenotyping. In mice studies the analyses has been more correlative. For example, Dhahbi and colleagues proposed that transcriptional signatures obtained by microarrays could be used as predictive biomarkers of DR mimetics. They identified that of five treatments tested in mice, metformin was the one that most closely resembled the transcriptional response of chronic DR (Dhahbi et al., 2005). Another exciting application has been the comparison of transcriptional responses in different species. For example, McElwee, Schuster, Blanc and colleagues compared the transcriptional profile of four long-lived organisms across a significant evolutionary distance. They analysed *C. elegans daf-2* mutants *(daf-2 vs daf-16;daf2), Drosophila* CHICO heterozygous mutants *(chico$^{/}$FF vs +I+),* Ames dwarf mice (Prop-1 ^{dm} *vs +I+)* and Little mutant mice (Ghrhr^{lielit} *vs +I+)* (McElwee et al., 2007). They found that although there was little conservation at the level of individual genes, functional categories did reveal shared processes. The three species showed up-regulation of categories involved in cellular detoxification, carbohydrate metabolism and oxidoreductase activity. They also shared down-regulation of macromolecular biosynthesis and protein biosynthesis (McElwee et al., 2007). They identified these processes as evolutionary conserved mechanisms that could potentially mediate lifespan extension and healthy ageing.

In *Drosophila* one of the first attempts to elucidate changes during the ageing process and their relationship with DR showed that transcriptional changes during ageing

are slowed down by DR (Pletcher et al., 2002). Microarrays have also been used in *Drosophila* to test whether transcriptional changes during ageing and oxidative stress share common transcriptional signatures. As mentioned before, the ageing community was for a long time driven by the mistaken hypothesis that ROS caused ageing (Blagosklonny, 2008; Gems and Partridge, 2013). Hence, though the two experimental examples I will mention here were perhaps misconceived, they do offer insights into the dynamic and complex nature of the transcriptional changes during ageing. Landis *et al.*, compared the RNA abundance of young (10 days) vs. old (61 days representing approximately median lifespan) flies, and also 3-day old flies subjected to 100% oxygen as a surrogate for oxidative stress. They found that 38% of gene expression changes that occurred in the aged flies overlapped with the hyperoxia treatment. Amongst the genes that shared a transcriptional response were those involved in the response to heat shock (hsp genes), antioxidants and immune-response genes all being up-regulated. Similarly, ageing and hyperoxia shared the down-regulation of preoteases, alkaline phosphatases and triglyceride lipases (Landis et al., 2004). Zou *et al.*, also compared the genome-wide transcriptional changes during ageing and the response to the redox cycler paraquat in *Drosophila* and found 33% correlation between changes induced by the oxidative stressor and the ageing process. In contrast to the study by Lund et al., Zou and colleagues observed that while some genes involved in cytoprotective mechanisms were up-regulated during ageing like Hsp26, others were down-regulated like glutathione S-transferase D1 (Zou et al., 2000). The complicated nature of the transcriptional changes during the ageing process is magnified by the fact that some of these studies evaluate whole flies, while we know now that different tissues will age at different rates, and their requirements for specific transcript are very different.

Transcriptional response to interventions that extend lifespan in *C. elegans* have also been pioneering (Golden et al., 2006; Pincus and Slack, 2008). DAF-16 is known to be required for the lifespan extension by IIS down-regulation. Thus, effort in identifying the downstream effectors has been a priority in biogerontology (Murphy, 2006; Tullet, 2014). Murphy and colleagues used microarray analysis to identify gene expression changes in several IIS worm mutants, allowing them to obtain a common transcriptional signature. By contrasting these changes with the transcriptional response elicited when *daf-16* is blocked (by RNAi for example) uncovered two sets of genes, one with the potential to increase lifespan (were up-regulated in IIS long-lived worms, but repressed by lack of daf-16), and a second set with potential lifespan shortening effects (opposite

direction) (Murphy et al., 2003). Amongst the up-regulated genes by IIS down-regulation were several involved in stress response including genes with cytochrome P450 activity, Hsp's, metallothionenin-1, catalase, SODs. They were able to show that knocking down several of these genes by RNAi shortened the lifespan of the *daf-2* long-lived worms, but none to the extent of *daf-16* RNAi. These results suggested that there is no sole effector of lifespan extension downstream of DAF16, but rather is the concerted transcriptional response which makes the worms live longer and be healthier (Murphy et al., 2003). Moreover, DAF16 is unlikely to act on its own. Using DNA adenine methyltransferase identification Shuster et al., assessed genes bound by DAF-16 and correlated them with expression change in *daf-2 vs daf-16;daf-2* mutant worms. They identified that some of the genes regulated by *daf-2* are not direct targets of *daf-16,* but that *daf-16* regulates their expression by activating other transcription factors. This was particularly true for genes involved in repair and detoxification (Schuster et al., 2010). In *Drosophila* a similar scenario seems to be true, Alic and colleagues assessed dFOXO targets using chromatin immunoprecipitation (ChIP) coupled with microarrays (ChIP-chip) and found that only a fraction of the transcriptional response of IIS is directly mediated by dFOXO. They pointed towards additional factors like GATA, other forkheads and dHR96 as potential regulators downstream of dFOXO (Alic et al., 2011b).

4.2.3 Transcriptional response to lithium in yeast, *C. elegans* and *Drosophila*

To the best of our knowledge only three studies (one per organism) reporting genome-wide approaches in these organisms have been performed. I will described them here as they might be of relevance for the analyses of our own data.

By using an integrated transcriptomic, proteomic and metabolomic approach Bro and colleagues studied the effect of lithium on galactose-grown yeast cells. Among the most prominent changes at the transcriptional level was the down-regulation of categories related to transcription, translation (including ribosomal proteins and proteins involved in ribosomal biogenesis) and nucleotide metabolism, while up-regulated categories involved energy reserve and monosaccharide metabolism and stress response genes (Bro et al., 2003). Lithium increased glycogen and trehalose content and this was reflected at the transcriptional level. Interestingly genes with at least two stress-related elements in their promoter regions accounted for almost a third of the transcriptional response elicited by

lithium. Several proteins with predicted molecular heat shock function were identified by proteomics (Bro et al., 2003).

In *C. elegans*, the only study so far reported analysing the transcriptional response to lithium was carried out by McColl and colleagues. They identified 5 gene ontology (GO) categories enriched in worms treated with 10 mM lithium (the optimal dose for lifespan extension they identified) after only 48 hours of treatment. These categories included nucleosome, nucleosome assembly, acyltransferase activity, endoplasmic reticulum and chromosome organization and biogenesis. Unfortunately they only focused in one gene, a histone demethylase for the remainder of their analyses and did not perform epistasis experiments. They however showed that RNAi against this histone demethylase extended lifespan in *C. elegans* (McColl et al., 2008).

Kasuya and colleagues interrogated the genome of *Drosophila* heads by using microarrays in the hope of identifying genes and processes regulated by lithium in the nervous system with potential therapeutic applications. Flies were treated with 50 mM LiC1 for 24 hrs before analysed for mRNA abundance. Interestingly they identified and corroborated (by RT-PCR) the up-regulation of three genes within the detoxification pathway: Gst-D2, Cyp309a1 and CG5999 (encoding a UDP-glucoronosyltransferase). Analyses of GO revealed several categories involved in stress-response and oxidoreductase activity (Kasuya et al., 2009).

Table 2. Functional categories enriched in lithium treated *Drosophila* heads according to DAVID.
Taken from (Kasuya et al., 2009)

	Term	p Value[a]	Fold enrichment[b]
1	Branched chain family amino acid metabolic process (GO:0009081)	7.57E-04	69.1
2	Stress response (SP PIR keywords)	923E-05	43.8
3	Valine, leucine and isoleucine degradation (KEGG pathway: dme00280)	1.69E-05	15.9
4	Organic acid metabolic process' (GO:0006082)	820E-06	5.99
5	Carboxylic acid metabolic process' (GO:0019752)	820E-06	5.99
6	Oxidoreductase (SP PIR keywords)	3.18E-05	5.83
7	Mitochondrion (GO:0005739)	9.09E-05	4.29
8	Amino acid metabolic process (G0(0006520)	8.05E-04	6.11
9	Oxidoreductase activity (GO:0016491)	9.69E-04	3.16
10	Hydrolase (SP PIR keywords)	4.03E-04	3.15
11	Cytoplasmic part (GO:0044444)	1.66E-04	2.25
12	Cytoplasm (GO:0005737)	8.09E-04	1.92
13	Catalytic activity (GO:0003824)	3.48E-05	1.71

[a] Modified fisher exact p-value (EASE score).
[b] Enrichment factor for the lithium-responsive genes (told change > 1.2; FDR < 0.05).
The identical genes arc assigned to these terms for *Drosophila* genome.

Overall the transcriptional response to lithium in these three organisms suggests that functional categories involved in metabolic pathways, translation, oxidoreductase and mitochondria could be relevant for lithium's effect.

4.2.4 Stress and ageing: longevity and resilience

The ability of organisms to respond to stress is amongst the most primal and evolutionary conserved mechanisms ensuring survival. Cells are equipped with genes and proteins that orchestrate adaptive responses that modify their internal milieu to match their new environmental conditions (Leopold and Perrimon, 2007; McEwen, 1998). These responses have aided organisms in the wild to survive periods of famine, drought, etc. during their evolutionary history. Such adaptations not only control cellular and organismal survival, but also maintenance and repair (Gems and Partridge, 2008; Lithgow, 2006; Shore and Ruvkun, 2013). Therefore, it is not surprising that stress response mechanisms have become central to the regulation of longevity and the retardation of the ageing process (Gems and Partridge, 2013; Shore and Ruvkun, 2013).

Genetic manipulations that increase lifespan in organisms ranging from the roundworm *C. elegans* to mammals are more than often associated with a myriad of biochemical and physiological adaptations that confer resistance to several forms of stress (Clancy et al., 2001; Holzenberger et al., 2003; Migliaccio et al., 1999; Tullet et al., 2008). Amongst the stressful situations that pro-longevity interventions are often associated with, are the ability to cope with oxidative stress and the detoxification of foreign chemical substances (xenobiotics) (Gems and Partridge, 2008, 2013).

Genes involved in defence and detoxification are necessary and therefore up-regulated in response to stress, while others that are dispensable and energetically costly to maintain are down-regulated. Thus, it has become increasingly relevant to understand the transcriptional signatures of ageing and stress response (Alic et al., 2011b; Bai et al., 2013; Melo and Ruvkun, 2012; Murphy, 2006; Shore et al., 2012). Successful attempts have shown that indeed gene expression changes between long-lived *C. elegans, Drosophila* and mice share up-regulated genes involved in xenobiotic metabolism (McElwee et al., 2007). Moreover, up-regulation of these genes is a shared transcriptional signature between dietary restricted mice and long-lived mice with mutations in the somatotrophic axis (Steinbaugh et al., 2012). Interestingly, rapamycin, the drug that has opened the field of pharmacologic modulation of ageing and longevity (Bjedov and Partridge, 2011; Harrison et al., 2009), does not seem to regulate the expression of xenobiotic metabolising enzymes (Steinbaugh et al., 2012).

The expression of these proteins is known to be under the control of the transcription factor SKN-1/NRF-2, which also modulates lifespan (Sykiotis and Bohmann, 2008; Tullet et al., 2008). Another factor involved in xenobiotic metabolism is the

hormone nuclear receptor dHR96, which is presumed to act downstream of dFOXO and is a possible regulator of the transcriptional response to IIS down-regulation (Alic et al., 2011b; King-Jones et al., 2006). In *Drosophila* dHR96 regulates the transcriptional response to xenobiotics. Flies lacking dHR96 are sensitive to the barbiturate phenobarbital and the pesticide DDT (King-Jones et al., 2006). In *C. elegans,* the nuclear receptor NHR8 is required for resistance to colchicine and chloroquine, both xenobiotics (Lindblom et al., 2001).

Given that direct manipulation of the transcription factors regulating stress response seems to be an evolutionary conserved intervention to promote healthy ageing, chemical inducers of such pathways have the potential to promote longevity and retard the ageing process. Such compounds have been called `hormetins' for their ability to induce a hormetic response (Gems and Partridge, 2008; Lithgow, 2006; Rattan, 2008).

4.2.5 Hormesis: from toxicology to longevity

Originally from the field of toxicology hormesis refers to a dose-response phenomenon characterised by a low dose stimulation and a high dose inhibition, usually represented as an inverted U or J shaped curve (Calabrese and Baldwin, 2003; Calabrese et al., 2007). In biogerontology, hormesis refers to the lifespan extension observed with low grade stress, while higher doses of the same compound (or stronger stimulation of the biochemical pathways that modulate the stress response) would limit longevity (Calabrese et al., 2011; Gems and Partridge, 2008; Rattan, 2001). Hormesis also consists of a priming event. *C. elegans* subjected to a mild increase in temperature are subsequently protected against heat stress. This suggests that activation of the defence mechanisms involved in heat response allows primed worms to better cope with a heat challenge (Lithgow, 2001). A similar scenario has been proposed with the effects of oxidative stress. External chemicals can also induce hormesis. Recent reports of chemical inducers (including known poisons) of a hormesis-like response have shown that lifespan extension can be achieved at lower doses even when lifespan is compromised with higher doses (Frankowski et al., 2013; Schmeisser et al., 2013a, 2013b). The dose response for the promotion of longevity also fits an inverted U or J-shaped curve with a hormetic zone, i.e. the zone spanning the drug concentrations that increase lifespan (Calabrese, 2013; Gems and Partridge, 2008).

Interestingly lithium has already been included in a catalogue of hormetins (Calabrese, 2005a; Calabrese and Baldwin, 2000) for its capacity to induce chemical

hormesis for growth in plants (Allender et al., 1997), cellular survival (Suganthi et al., 2012) and its effects on cell morphology and signal transduction (Gao et al., 1993).

4.3 Methodology and experimental design

4.3.1 Gene-expression microarrays

Once-mated *w1118* female flies were pre-treated for 10 days with 10 mM lithium and snap frozen in liquid nitrogen, after which heads and thoraces were separated and analyzed for differential gene expression by Affymetrix microarrays. The experimental set up was performed by Dr. Luke TaM.

4.3.2 Polysome-profile microarrays

w1118 female flies were treated with 10 mM LiC1 or vehicle (ddH2O) for 10 days. After being snap frozen in liquid nitrogen, heads and thoraces were separated from the abdomen/ovaries to remove confounding differences associated with fecundity. Polysome profiles were generated as previously described with minor modifications (Dinkova et al., 2005). Heads and thoraces were homogenized on ice in 300 mL polysome extraction buffer (300 mM NaC1, 50 mM Tris-HCL (pH 8.0), 10 mM $MgC1_2$, 1 mM EGTA, 200 mg heparin/mL, 400 U RNAsin/mL, 1 mM phenylmethylsulfonyl fluoride, 0.2 mg cycloheximide/mL, 1% Triton X-100, 0.1% sodium deoxycholate). 800 mL additional polysome extraction buffer was added and mixed gently and placed on ice for 10 mM. Debris was the removed by spinning at 20,000g (4°C) for 10 mM and the supernatant was layered onto a 10-50% sucrose gradient in high salt resolving buffer (140 mM NaC1, 25 mM Tris-HCL (pH 8.0), 10 mM $MgC1_2$). Using a Beckman SW41Ti rotor (38,000 rpm at 90 mM, 4°C) polysomes and ribosomal subunits were separated before the gradients were fractionated. Fractions were collected using a Teledyne density gradient fractionator with continuous monitoring absorbance (252 nm), which was also digitally recorded. Fractions containing 1-3 and > 4, ribosomes per transcript were collected as low and high fractions respectively, directly into ice cold EtOH and precipitated overnight at -20°C along with GlycoBlue. RNA was then pelleted, washed and resuspended in RNase free H2O, to then be isolated using Trizol-LS as per manufacturer's instructions. To estimate the level of translation, ratios of intensities were calculated by dividing high fraction intensities by low

fraction intensities to give a translation ratio value for each transcript. Experiments were carried out in 3 independent replicates (80 heads/thoraces per replicate). Dr. Luke Tain carried out the polysome profiles and microarrays.

4.3.3 Gene-Ontology (Catmap analysis)

For functional analysis using all expressed genes, we used the Wilcoxon rank sum test implemented in Catmap (Breslin et al., 2004). Ranks of genes were based on the Bayes t-statistic for differential expression and, for a given functional category. The significance of the rank sum for all genes in the category was calculated analytically based on a random gene-rank distribution. Catmap analysis was performed by Dr. Dobril Ivanov.

4.3.4 Mitochondrial Isolation

Drosophila mitochondria were isolated as previously described (Miwa et al., 2003). Briefly, adult flies (n = 400 for each time point) were chilled on ice and gently pressed using a pre-chilled pestle and mortar. The pestle was moved in a vertical motion (with no horizontal motion) until the shape of the flies was no longer visible. The flies were then washed in STE + BSA buffer (250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.4 (4°C), 1 % BSA). The flies were then pressed further in 5 mL of STE + BSA buffer. The squashed flies were then passed through double-layered muslin cloth. The collected pulp was then spun for 3 mins at 4°C at 1,500 rpm to remove debris. The supernatant was then passed through a single layer of muslin into a clean centrifuge tube and spun for 10 mins at 4°C and 10,000 rpm to collect the mitochondria. The mitochondrial pellet was then suspended in 250 ilL STE + BSA buffer and stored on ice. A BCA assay (Sigma) was used to determine the protein concentration of the mitochondrial preparations. The isolated mitochondria were used immediately after preparation for experiments.

4.3.5 Mitochondrial Physiology Measurements

Oxygen consumption was measured using a Clark-type oxygen electrode thermostatically maintained at 25°C. Glutamate (5 mM) and malate (5 mM) or 5 mM pyruvate were added to measure Complex I-linked respiration, succinate (5 mM) with rotenone (5 1.1M) were added to measure Complex II-linked respiration. All data were obtained using an Oxygraph Plus system with Chart recording software.

4.3.6 Immunoblotting

To detect the Gst-D-eGFP reported antiGFP (2955 Cell Signaling Technologies, 1:1000) was used. The rest of the protocol was as described in Chapter 2.

4.4 Results

4.4.1 Lithium and IIS/FOXO do not share a transcriptional response

Lithium is known to be a complex drug that interacts with many cellular pathways and many candidate proteins have been put forward as its mechanism of action (Lenox and Wang, 2003; Phiel and Klein, 2001). In order to determine the molecular mechanism(s) associated with the longevity effects of lithium, we undertook a genome-wide approach by analysing the transcription profile of lithium treated flies. The bioinformatics analyses was performed in collaboration with Dr. Dobril Ivanov. Flies treated with 10 mM lithium for 10 days were subjected to microarray analysis and compared to un-treated control flies. Lithium up-regulated several genes involved in the detoxification pathway, namely enzymes with cytochrome P450 or glutathione transferase activity (Kasuya et al., 2009). These enzymes are at the core of the detoxification pathway, important for xenobiotic metabolism (Mattson, 2008b; Motohashi and Yamamoto, 2004). Up-regulation of similar enzymes has been reported to be a common transcriptional response of IIS down-regulation in worms and flies (McElwee et al., 2007). We first decided to investigate whether the transcriptional response differentially expressed by lithium was similar to that observed when IIS is genetically down-regulated. We used a recently published microarray analysis where the InR^{DN} was over-expressed as a means of reducing IIS (Alic et al., 2011b). We compared the overlap of genes elicited by both interventions without detecting statistical significance over the Bonferroni corrected P-value threshold of 0.0001 for up-regulated (P = 1.0; Fisher's exact test) or down-regulated (P = 0.00657; Fisher's exact test) genes (McElwee et al., 2007). The only significant overlap (P = 1.48e-08) was between genes down-regulated by lower IIS and those up-regulated by lithium treatment, which suggested that the transcriptional responses changed in opposite directions.

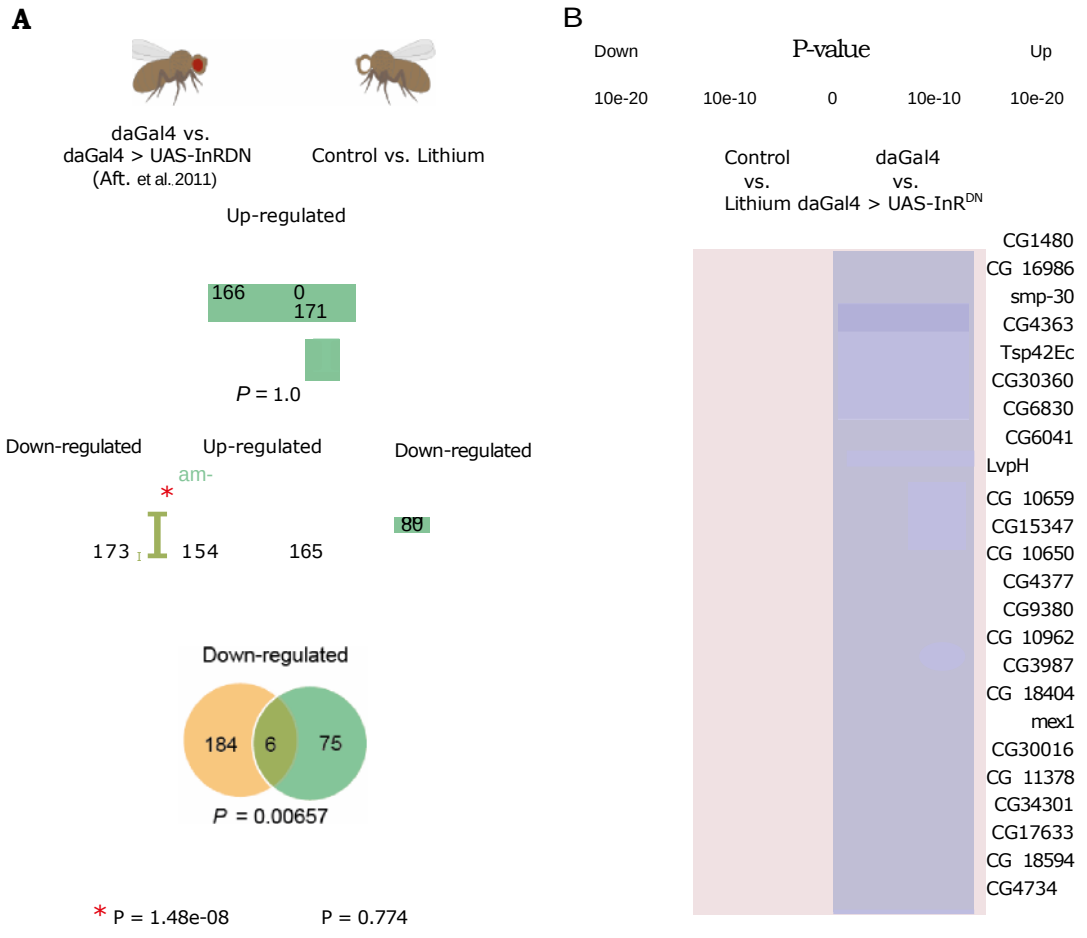


Figure 4.1 Comparative analyses of the transcriptional response of IIS down-regulation and lithium treatment. (A) Venn diagrams showing overlaps between genes regulated by IIS down-regulation by the over-expression of a dominant negative version of InR and the transcriptional response to lithium. Significance $P < 0.001$. (B) Heat map that shows genes that overlapped between the two treatments. Bioinformatics analyses performed by Dr. Dobril Ivanov.

Some of the phenotypes observed in flies with IIS down-regulation do not require the transcription factor dFOXO, including resistance to oxidative stress (Slack et al., 2011). We have also detected that the longevity effect of lithium is dFOXO-independent. To dissect whether the differentially expressed genes after lithium treatment were similar to that of IIS that do not require dFOXO, we compared them with those where the InR^{DN} was over-expressed in a *dfoxo* null background (Alic et al., 2011b). We were unable to detect a significant overlap in all possible comparisons ($P > 0.0001$, Fisher's exact test; Figure 4.2). Taken together our results suggest that the transcriptional signature of lithium does not resemble that of IIS down-regulation induced or not by dFOXO. Interestingly, the transcriptional data corroborated that IIS down-regulation and lithium were molecularly different, consistent with these treatments being additive (Figure 3.13).

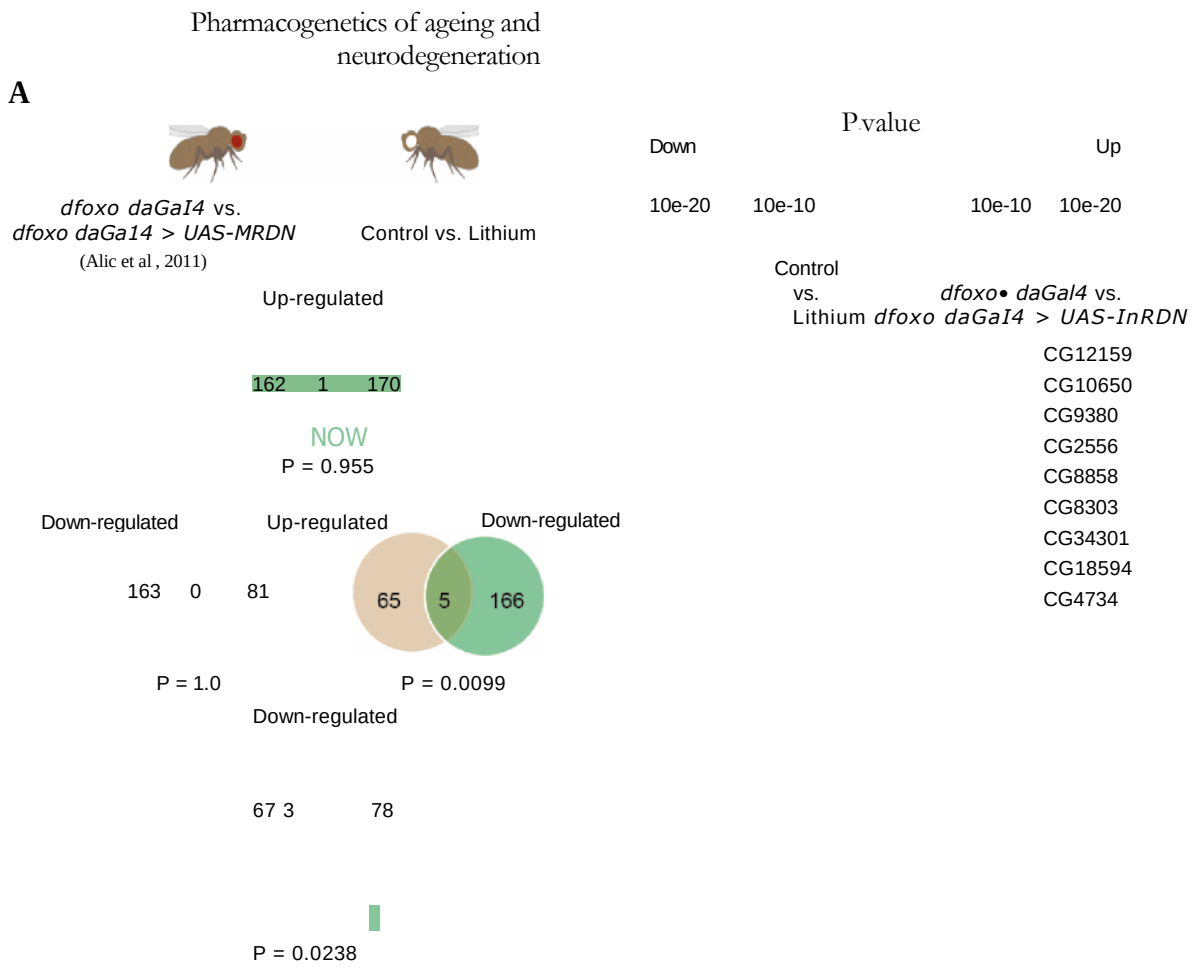


Figure 4.2 Comparative analyses of genes regulated by low IIS in a *dfoxo* null background and lithium. (A) Venn diagrams showing overlaps between genes regulated by IIS down-regulation in a dfoxo null background and the transcriptional response to lithium. Significance ***P* < 0.001.** (B) Heat map that shows genes that overlapped between the two treatments. Bioinformatics analyses performed by Dr. Dobril Ivanov.

4.4.2 Transcriptional response from lithium did not overlap with the transcriptional response of flies overexpressing HR96

The nuclear hormone receptor HR96, the single *Drosophila* orthologue of the mammalian pregnane X receptor and constitutive androstane receptor, has been implicated in the protection against oxidative stress and xenobiotics, which require the transcriptional induction of antioxidants and detoxification enzymes (King-Jones et al., 2006). Moreover, HR96 has also been implicated in the regulation of lipid metabolism, especially triglycerides (Sieber and Thummel, 2009). We decided to compare the transcriptional response elicited by over-expression of HR96 from a previously published microarray study (King-Jones et al., 2006) with our lithium data set (Figure 4.3). Genes differentially expressed by the two interventions significantly overlapped (*P* = 2.62e-11, Fisher's exact test). However, the transcriptional signature did not share the same directionality as the overlaps did not pass the Bonferroni P-value threshold for significance (*P* > 0.0001) when only genes up-regulated or down-regulated by both interventions were analysed (Figure

4.3). Moreover, a significant overlap going in opposite directions was detected (*P* = 2.34e10, Fisher's exact test) for genes down-regulated by the over-expression of HR96, but up-regulated in response to lithium. Therefore, our results do not support that HR96 over-expression and lithium treatment share a similar transcriptional signature, suggesting that lithium does not activate HR96.

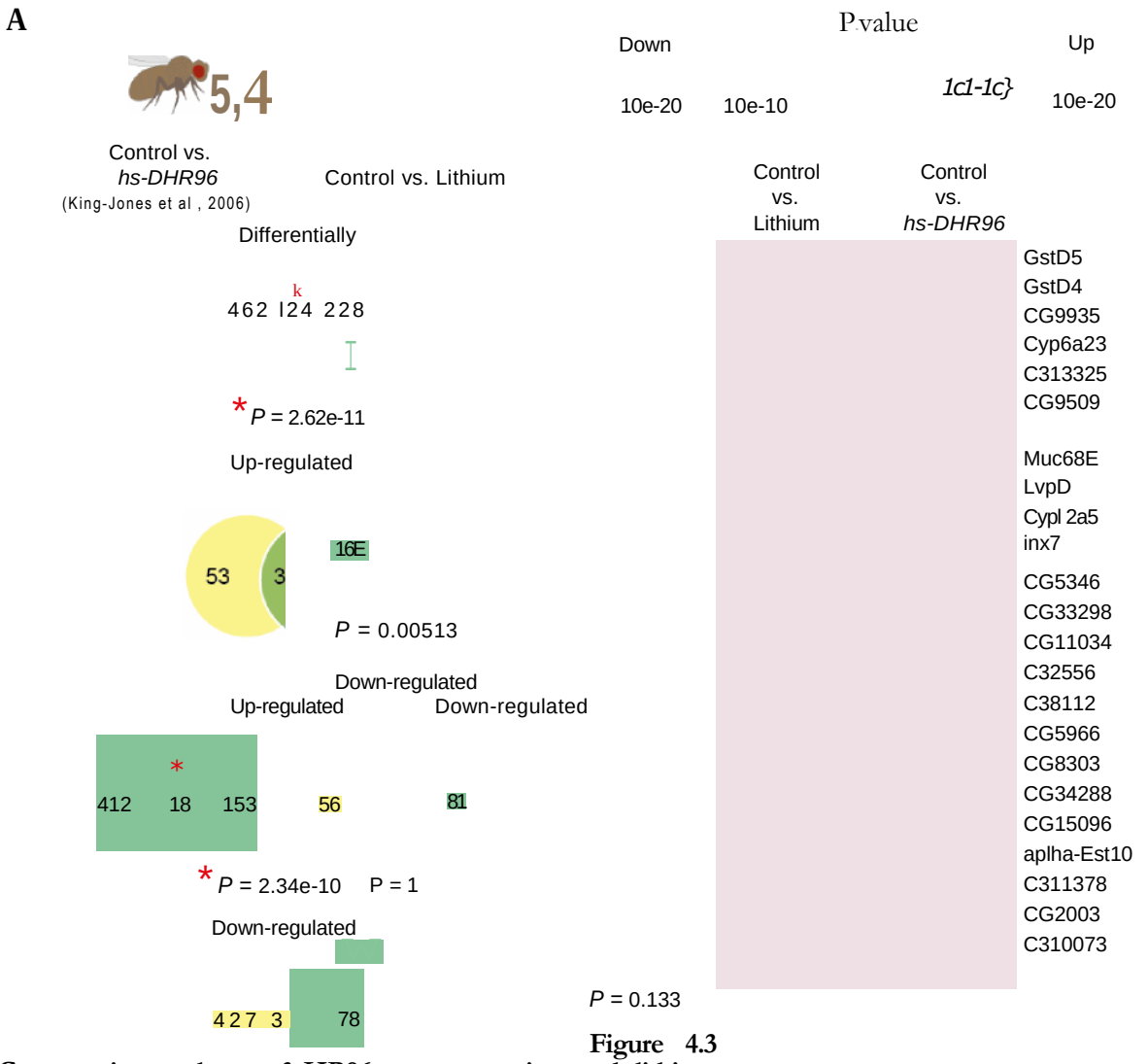


Figure 4.3 Comparative analyses of HR96 over-expression and lithium treatment. (A) Venn diagrams showing overlaps between genes regulated by HR96 over-expression and the transcriptional response to lithium. Significance *P* < **0.001.** (B) Heat map that shows genes that overlapped between the two treatments. Bioinformatics analyses performed by Dr. Dobril Ivanov.

4.4.3 The transcriptional response of cncC/NRF-2 over-expression overlaps with that from lithium

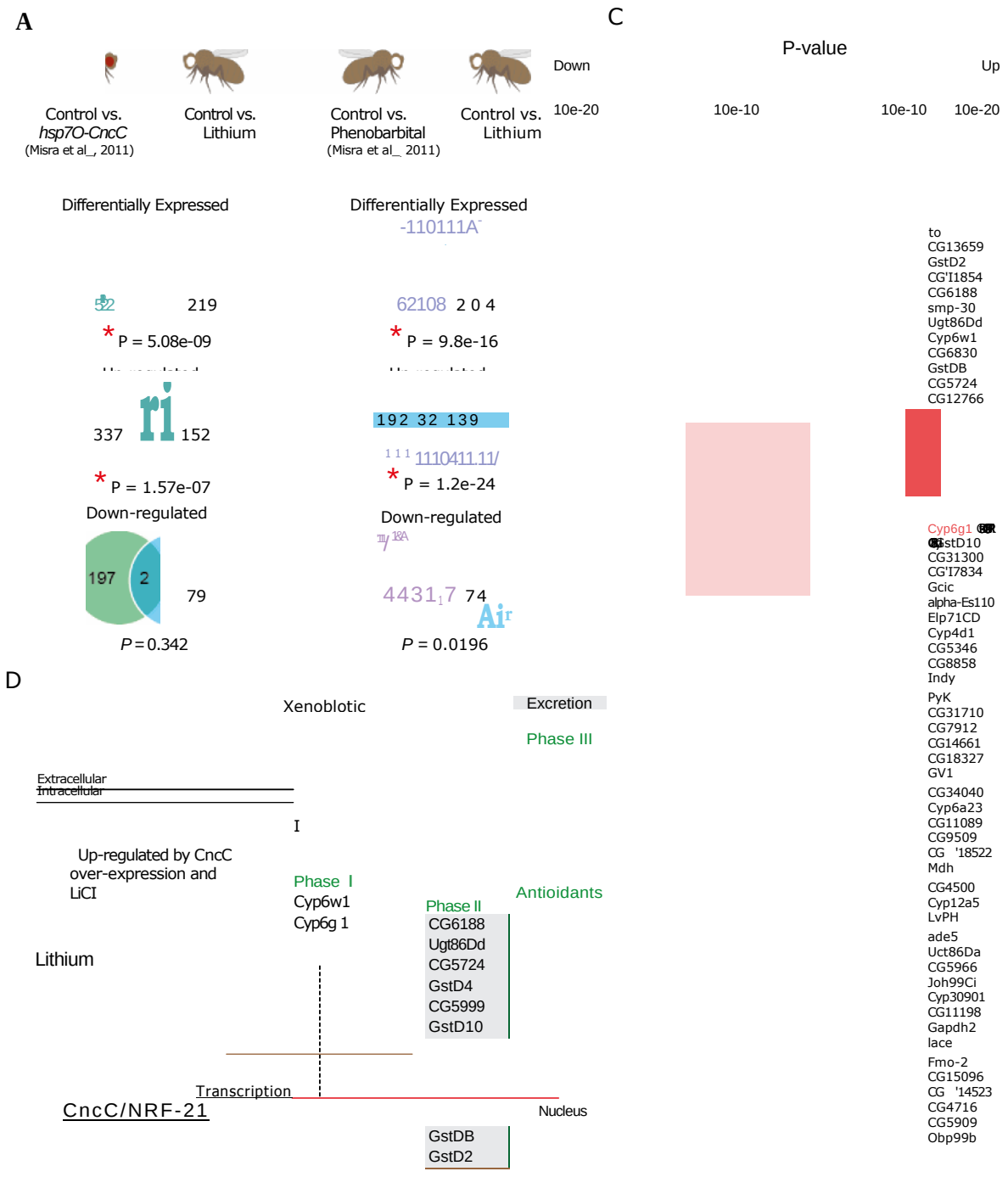


Figure 4.4 Comparative analyses of the transcriptional signature of cncC overexpression and lithium. (A) Venn diagrams showing overlaps between genes regulated by cncC overexpression and the transcriptional response to lithium. (B) Venn diagrams showing the overlap between genes regulated by the xenobiotic phenobarbital and lithium. Significance $P < 0.001$. (C) Heat map that shows genes that overlapped between the three interventions. **(D)** The common transcriptional signature of cncC overexpresion, phenobarbital and lithium treatments map to the xenobiotic detoxification pathway. Bioinformatics analyses performed by Dr. Dobril Ivanov.

In *C. elegans,* stress resistance is dependent on two transcription factors SKN-1/NRF-2 and daf16/FOXO, and over-expression of either extends lifespan and regulates stress resistance (Kwon et al., 2010; Libina et al., 2003; Tullet et al., 2008). In *Drosophila,* dFOXO plays a role in protecting against some forms of stress like xenobiotic, but not oxidative stress, while it is required for lifespan extension under IIS down-regulation (Slack et al., 2011). Protection against oxidative and xenobiotic stress is presumed to be the result of up-regulating enzymes in the xenobiotic detoxification pathway (Alic et al., 2011b; Kaletsky and Murphy, 2010; Schuster et al., 2010). The response to both xenobiotics and oxidative stress in *Drosophila* has been reported to be regulated by the fly homologue of NRF-2, cap'n'collar (CncC) (Sykiotis and Bohmann, 2010). Moreover, activation of CncC by heterozygous loss of its natural repressor Kelch-like ECH-associated protein 1 (Keapl), extends lifespan of male flies (Sykiotis and Bohmann, 2008). The control of the transcriptional signature by CncC has been assessed both pharmacologically and genetically (Misra et al., 2011). We first assessed whether lithium induced a transcriptional response similar to the one obtained when CncC was over-expressed (Misra et al., 2011). When the differentially expressed genes were compared, we found a significant overlap (Figure 4.4A; P = 5.08e-09, Fisher's exact test). This overlap was only significant at the up-regulated level, since it was not significant among genes down-regulated by both treatments (Figure 4.4A; P = 0.342, Fisher's exact test), but they did share a significant overlap of up-regulated genes (Figure 4.4A; P = 1.57e-07, Fisher's exact test).

It was previously reported that the barbiturate anticonvulsive phenobarbital can induce a similar transcriptional response as CncC over-expression (Misra et al., 2011). We therefore explored the possibility that lithium could also induce a similar transcriptional profile as phenobarbital treatment. We therefore analysed the overlap between differentially expressed genes by lithium with those previously reported to change expression upon phenobarbital treatment (Misra et al., 2011). We found that treatment with either lithium or phenobarbital elicited a transcriptional response that significantly overlapped (Figure 4.4B; P = 9.8e-16, Fisher's exact test). The overlap was once more only significant at the up-regulated (P = 1.2e-24), but not at the down-regulated level (Figure 4.4B; P = 0.0196, Fisher's exact test).

We then compared the differentially expressed genes that significantly overlapped with lithium when compared to CncC over-expression or phenobarbital treatment (Figure

4.4C). It was interesting to note that the genes with significant overlap participated in the three phases of xenobiotic metabolism (Figure 4.4C and D).

4.4.4 Lithium activates the cncC/NRF-2 pathway

I also used a GFP reporter to confirm that lithium is indeed activating CncC. For this I used a previously generated GstD-GFP reporter that responds both to chemical inducers of CncC, or to genetic manipulations activating the pathway (Sykiotis and Bohmann, 2008). I treated flies carrying the GstD-GFP reporter with increasing concentrations of lithium. I observed a dose-dependent increase in the levels of GFP as assessed by immunoblot analysis (Figure 4.5; $P < 0.05$, ANOVA post hoc Tukey-Kramer). Taken together, our results suggest that lithium elicits a transcriptional response similar to that of CncC over-expression, which would indicate that lithium activates CncC to up-regulate genes in the detoxification pathway.

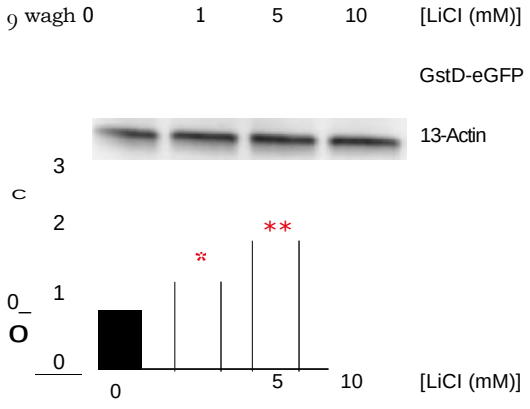


Figure 4.5 Lithium activated the detoxification transcription factor CncC. The activation of the detoxification pathway was determined by treating flies expressing the reporter GstD-eGFP with lithium for 15 days and analysing the expression of GFP by immunoblot analyses. * $P < 0.05$, ** $P < 0.01$ (ANOVA post hoc Tukey Kramer). N = 3 biological replicates of 10 flies each.

4.4.5 Lithium modifies survival in the presence of different stressors

To further confirm that the induction of the xenobiotic metabolising pathway was functional, I challenged flies pre-treated with lithium to pro-oxidants and xenobiotics. Given that phenobarbital has already been described transcriptionally as a xenobiotic, I used a similar concentration as the one used to asses gene regulation (Misra et al., 2011). Flies pre-treated with increasing concentrations of lithium ranging from 1 to 100 mM were significantly resistant to phenobarbital (Figure 4.6A). Doses ranging from 1 to 75 mM LiC1 almost doubled their median lifespan (-80% lifespan extension), and completely doubled their maximum lifespan ($P < 0.001$, log rank test). Even 100 mM of lithium significantly

protected against phenobarbital stress, though its effect was modest in comparison to the other doses of lithium tested (*P* < 0.01, log rank test). These results suggest that even lower doses of lithium are sufficient to induce a robust transcriptional response downstream of CncC to protect against xenobiotics. I also tested a second xenobiotic, the anti-malarial drug chloroquine (Lindblom et al., 2001). Using this xenobiotic, I only observed protection with lower doses of lithium (Figure 4.6B; 1-10 mM *P* < 0.05, log rank test) and 100 mM lithium significantly made the flies sensitive to chloroquine (Figure 4.6B; *P* < 0.05, log rank test).

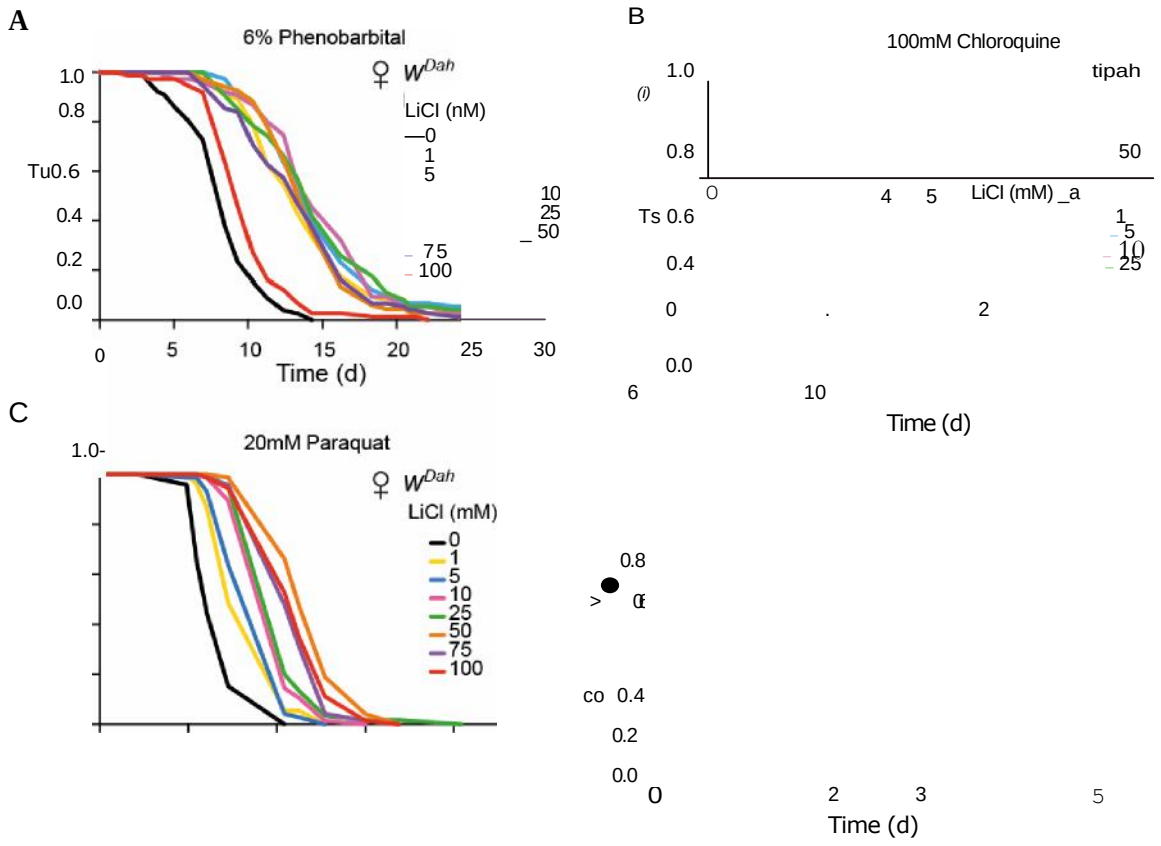


Figure 4.6 Lithium pre-treatment protected against xenobiotic and oxidative stress. For all experiments flies were pre-treated for 15 days before challenged with (A) 6% phenobarbital, (B) 100 mM chloroquine, and (C) 20 mM paraquat.

I also assessed whether lithium could protect against the redox-cycler paraquat. Paraquat gets converted into superoxide in the mitochondria, mostly by acting on complex

I of the respiratory chain (Cocheme and Murphy, 2008). Paraquat also requires the detoxification pathway for metabolism as RNAi-mediated down-regulation of CncC makes flies more sensitive to it (Sykiotis and Bohmann, 2008). When flies pre-treated with lithium (1-100 mM) for 15 days were transferred to sucrose/agar vials containing 20 mM paraquat flies showed a dose-dependent response (Figure 4.6C). Increasing the

— 75
— 100

concentration of lithium from 1 to 50 mM significantly improved survival in a dose-dependent manner ($P < 0.001$, log rank test for all doses compared to control). 75 or 100 mM LiC1 were slightly less beneficial than 50 mM lithium, yet protected significantly more than 25 mM ($P < 0.05$, log rank test). Overall, my experiments show that lithium treatment protected against xenobiotic and oxidative stress, but that the response could vary depending on the dose of lithium tested and also the xenobiotic used.

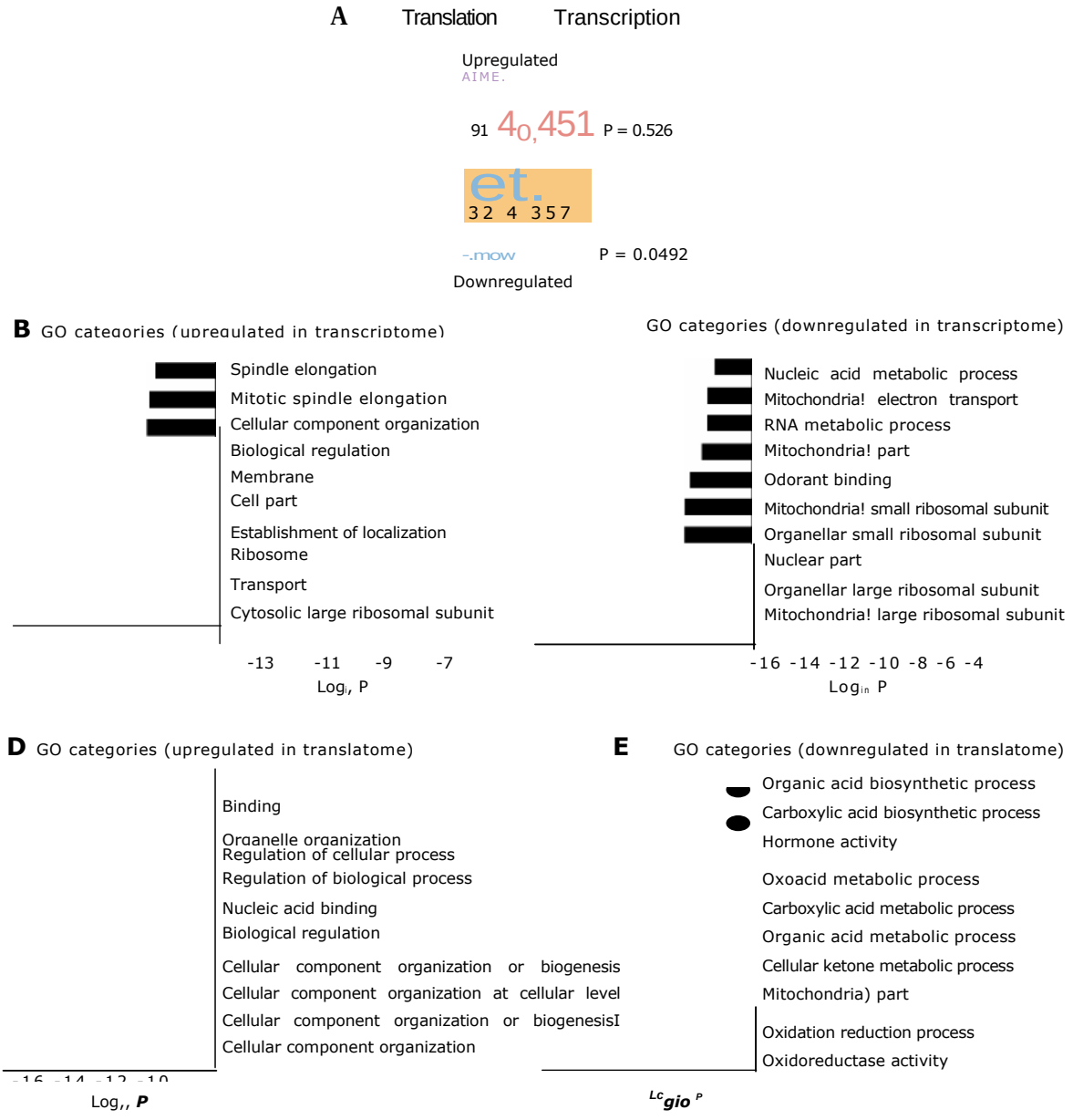


Figure 4.7 Comparison of genes and functional categories regulated by lithium at the transcription and translational level. (A) Venn diagrams showing the overlap between changes elicited by lithium at the translational and transcriptional level (B-E) Top ten Gene Ontology (GO) categories enriched in lithium treated flies at the transcriptional (B and C) and the translational (D and E) level. Bioinformatics analyses performed by Dr. Dobril Ivanov.

4.4.6 Transcriptomic and translatomic response of lithium showed enrichment for mitochondrial complex-1

We recently showed that lithium reduces translation in yeast and flies (Sofola-Adesakin et al., 2014). This is an additional potential mechanism by which lithium could confer longevity benefits, as down-regulation of translation has been shown to extend lifespan (Hansen et al., 2007; Syntichaki et al., 2007). However, the specific genes regulated by translation to extend lifespan have been poorly studied. Our previous analysis of lithium treatment suggested that lithium possibly down-regulated a specific subset of genes rather than globally affecting protein synthesis (Sofola-Adesakin et al., 2014). In order to determine the differential regulation of translated genes we combined polysome profiling with microarray analysis. This allowed us to identify changes in the translatome. We first assessed the overlap of genes differentially regulated at the transcriptional and translational level not obtaining statistical significance (Figure 4.8A). This highlighted that the translational read-out was not a mere representation of the transcriptional response.

We next performed catmap analysis to determine gene-ontology (GO) enrichment and assess functionally relevant changes in expression (Breslin et al., 2004) (Figure 4.8A). We then assessed whether transcriptional and translational responses to lithium could yield expression changes in similar functional categories in spite of not regulating the same genes. To our surprise we observed a significant overlap of GO categories that changed transcriptionally and translationally in the same direction ($P < 0.0001$, Fisher's exact test; Figure 4.8A). Interestingly, there was a clear enrichment of categories involving mitochondrial complex I. The overlap between GO categories down-regulated at the transcriptional and translational level resulted in a significant overlap that only showed functional categories representing complex I of the mitochondrial respiratory chain (Figure 4.8B).

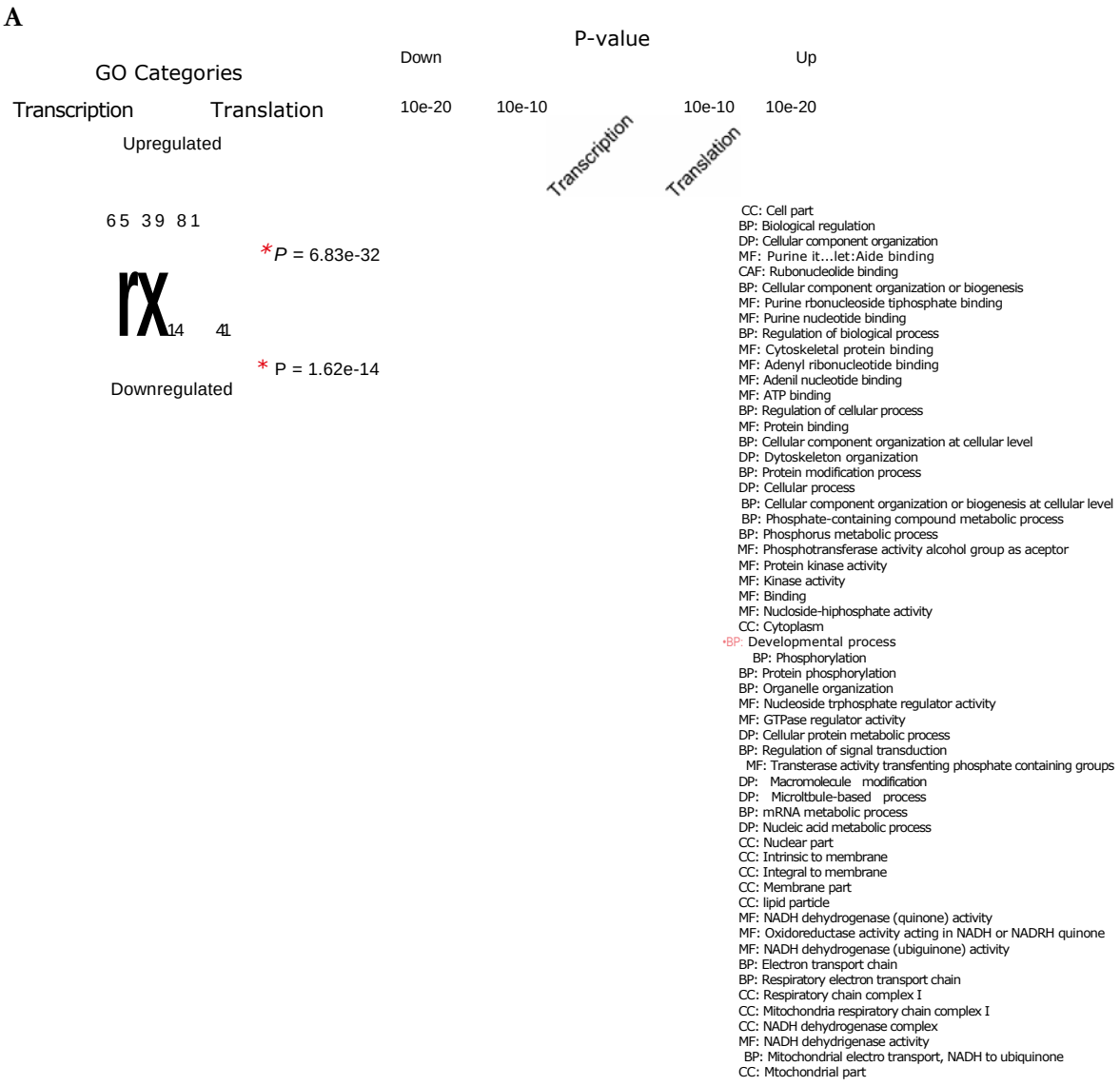


Figure 4.8 Overlapping functional GO categories modulated by lithium at the transcriptional and translational level. (A) Venn diagrams showing overlaps between GO categories changes by lithium. (B) Heatmap showing the overlapped categories. Bioinformatics analyses performed by Dr. Dobril Ivanov.

4.4.7 Lithium uncouples mitochondrial complex-1, but does not alter its oxygen consumption

To assess the physiological relevance of this observation, we analysed mitochondria' respiration. Whole-fly mitochondria were isolated after 15 days of lithium treatment and age-matched untreated control flies. Mitochondria from lithium treated flies showed a reduced RCR by mitochondrial complex I ($P < 0.05$, t-test), but not complex II ($P > 0.05$,

t-test; Figure 4.9A). Lithium had no effect on basal oxygen consumption by either complex I or complex II ($P > 0.05$, t-test; Figure 4.9B).

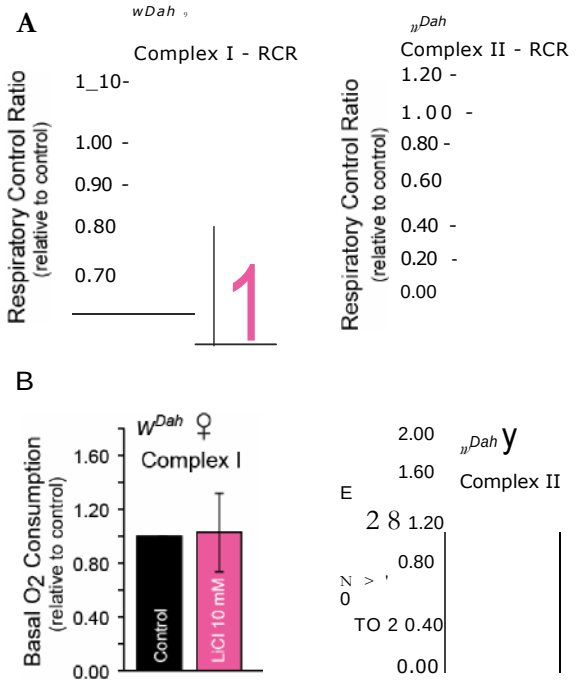


Figure 4.9 Lithium reduced the respiration control ratio (RCR) of mitochondrial complex 1. (A) RCR measurements for complex I and **II** of the mitochondria' respiratory chain of mitochondria isolated from flies treated with lithium for 15 days. (B) Measurements of 02 consumption from the same mitochondrias. * $P < 0.05$. N = 400 (pooled) flies per condition. Mitochondria' physiology measurements were done by Dr. Fernando Bartolome-Robledo.

4.5 Discussion

4.5.1 Lithium elicits a transcriptional signature of detoxification

We have found here that lithium elicits a transcriptional response that does not resemble that of IIS down-regulation. This was not entirely surprising given that we previously showed that lithium did not require dFOXO to extend lifespan and that lithium could further extend lifespan of already long-lived IIS mutant flies. Indeed our results suggest that the transcriptional response to lithium and IIS down-regulation go in opposite directions, which might indicate molecularly why these interventions were additive.

Surprisingly, lithium also elicited a transcriptional response dissimilar to that of dHR96 over-expression. This was in spite of overlapping at genes like GstD4 and GstDS both involved in phase 2 of the detoxification pathway. However, we were able to identify that the transcriptional response of lithium treated flies significantly overlapped with flies over-expressing the transcription factor CncC. Interestingly the overlap was only significant at the up-regulated level, which is in agreement with CncC/NRF-2 being a transcriptional activator. Moreover, we were able to show that lithium up-regulated the

expression of GstD-eGFP by immunoblot analyses further confirming that lithium activates CncC/NRF'-2. Our results showing that lithium activates CncC/NRF-2 are in agreement with a recently published report showing that lithium increases the nuclear fraction of NRF-2 after chronic (5 days) but not acute (6 hrs) treatment in rat pheochromocytoma PC12 cells (Rizak et al., 2014). Lithium has been reported to directly activate NRF-2 in rat astrocytes (Correa et al., 2011) and human hepatocytes (Jiang et al., 2014).

NRF-2 is considered to be mainly an activator of phase 2 of the detoxification pathway (Hayes and Dinkova-Kostova, 2014; Oliveira et al., 2009). However, comparison of our data with previously published microarray data showed that some enzymes of phase 1 and phase 3 are also up-regulated by both CncC over-expression and the treatment of xenobiotics like phenobarbital and lithium. This is not entirely surprising as the detoxification should require enzymes from the three phases to be able to metabolise foreign toxins. We considered that the molecular characterisation showing similarity between lithium and phenobarbital should allow reconsideration of lithium as a xenobiotic.

Interestingly lithium was able to protect flies from the deleterious effect of paraquat, phenobarbital and chloroquine. All of these compounds can be catalogued as xenobiotics, given that they are foreign to *Drosophila's* internal milieu. The mechanism behind a xenobiotic being effective at protecting against another xenobiotic might be trough the "priming" or adaptive mechanism described for hormesis (Rattan, 2008; Yun and Finkel, 2014). For example worms or flies exposed to low-doses of a stressor are subsequently able to cope better to the exposure of the same stressor at higher lethal doses (Khazaeli et al., 1997; Lithgow, 2001; Sarup et al., 2013). It would be interesting to test whether low non-lethal doses of paraquat, phenobarbital or chloroquine would be able to protect flies against very high doses of lithium. For example, we have documented that flies exposed to lithium at 200 mM reach median lifespan within a day or 2 of exposure. Perhaps pre-treatment with low doses of these other compounds could elicit a xenobiotic response capable of instantaneously dealing with the subsequent lethal concentrations of lithium (Sarup et al., 2013). Additionally, testing whether lithium is able of protecting against itself could also prove to be valuable for establish the "priming" mechanism of hormesis for lithium.

An interesting point to raise here is the fact that IIS down-regulation has been shown to up-regulate genes in the detoxification pathway (McElwee et al., 2007),

particularly those with glutathione-S transferase activity. These enzymes are both

regulated by CncC and HR96 (King-Jones et al., 2006; Misra et al., 2011). This could potentially suggest that IIS down-regulation and lithium activate the xenobiotic pathway through different mechanisms, and perhaps therefore elicit a non-overlapping transcriptional response. Moreover, when we overlapped the genes of IIS down-regulation via over-expression of a dominant negative version of the *dInR* (Alic et al., 2011b) we did not detect any similarity between any of the enzymes in the detoxification pathway elicited by lithium. The association of the detoxification pathway and IIS down-regulation was done using heterozygous loss of the insulin receptor substrate CHICO. Perhaps testing whether lithium treatment and loss of CHICO (and also CHICO with CncC and HR96 over-expression) overlap at the transcriptional level could unveil differences between different IIS mutants.

4.5.2 Mitochondrial respiratory chain complex I was down-regulated in flies treated with lithium

Additional to the up-regulation of enzymes in the xenobiotic pathway, we also observed that lithium treatment was enriched for GO categories involving mitochondrial complex I of the respiratory chain. Interestingly, these changes were at the down-regulated level for both the transcriptional and translational response. Given that this effect was not uncovered at the single gene level, suggest that this is rather a concerted response influenced by at least a few genes.

Further examination of the transcriptional and translational arrays revealed that CG9172 and CG9762, genes with NADH dehydrogenase (ubiquinone) activity that encode for *Drosophila* subunits of mitochondrial Complex I, were down-regulated in response to lithium. Individual ubiquitous RNAi knockdown of CG9172 and CG9762 extend *Drosophila* lifespan while also protect against PQ (Copeland et al., 2009). Additionally, reduced levels or reduced activity of complex I associate with healthy ageing in *C. elegans, Drosophila* and mice (Copeland et al., 2009; Dillin et al., 2002; Lapointe and Hekimi, 2008; Lee et al., 2003; Rea et al., 2007).

We tested the significance of the transcriptional and translational response to lithium by analysing the activity of mitochondria isolated from flies that had been treated with 10 mM lithium for 15 days. Interestingly we observed that while lithium did not affect at all the activity of complex II, it reduced the respiratory control ratio of complex I

without affecting its oxygen consumption. We are unable to predict the implication of this

response, but we will further explore it. It will be interesting to test whether this response is capable of producing ROS, for example. If lithium was increasing ROS through complex I inhibition this could suggest that lithium is inducing a mitohormetic effect (Ristow, 2014; Ristow and Zarse, 2010; Yun and Finkel, 2014). Among the responses that cells would elicit to protect themselves against ROS production would be the activation of NRF-2 (Ristow, 2014), which would then provide a mechanistic insight as to how is lithium activating CncC/NRF-2. Mixed results have been reported as to the role of lithium for ROS generation and defense. For example, Eskandari and colleagues showed that lithium increased ROS production in rat hepatocytes, which led to mitochondria' membrane potential collapse and release of cytochrome c with subsequent cytotoxicity (Eskandari et al., 2012). On the other hand, it has been shown that lithium protects against H202-induced cell death in a neuroblastoma cell line (Arraf et al., 2012). Most of these studies are carried out in cell lines where the effects reported could be cell-line specific. We will have to carefully assess the role of lithium for ROS generation and clearance as the response to lithium might be cell type-specific.

Additionally, paraquat is not an oxidative stressor *per se.* Paraquat needs to be converted in the mitochondrial matrix to superoxide. One major site of superoxide production by paraquat is complex I (Cocheme and Murphy, 2008). Given that lithium might be inhibiting complex I, it is also possible that lithium prevents the formation of superoxide by paraquat and other toxins that increase ROS through complex I of the respiratory chain.

4.5.3 Does lithium induce a hormesis response? Is it a hormetin?

One of the big challenges with lithium treatment is its toxicity. Only small doses are recognised to have disease impact before side effects like renal failure and thyroid abnormalities develop. This has forced close monitoring of circulating levels in patients with lithium prescriptions (Freeman and Freeman, 2006; Severus et al., 2008). The biphasic response with lower dose promoting health benefits, while higher doses being toxic, is the signature of a hormetin. Interestingly such a biphasic response for survival was recently reported in a mammalian cell line (Suganthi et al., 2012). I have also discussed that lithium is potentially eliciting an adaptive response to stress and it activates the CncC/NRF-2 pathway which is at the heart of the hormesis response (Calabrese and Mattson, 2011; Mattson, 2008a; Ristow, 2014). Additionally our preliminary data on the —

omics response to lithium and our mitochondrial measurements could suggest that lithium acts also through ROS generation and mitohormesis. Further experiments will be required before lithium can be categorically categorised as a hormetin. However, suggestions that lithium is indeed a hormetin have been provided by others (Allender et al., 1997; Gao et al., 1993; Suganthi et al., 2012), leading to its inclusion in catalogs of chemical inducers of hormesis (Calabrese, 2005b; Calabrese and Baldwin, 2001).

Chapter 5

GSK-3 in ageing and neurodegeneration

"There is nothing more exciting in science than a completely new twist to an area that everyone thought had been solved many years earlier".
Phillip Cohen and Sheelagh Frame

5.1 Abstract

It has been known for the past 20 years that GSK-3 is the main target of lithium. Both GSK-3 and lithium seem to converge in similar cellular pathways, translating into a myriad of potential downstream targets. Additionally non-convergent points have also been described, the most prominent being the regulation of inositol recycling. Lithium seems to regulate myo-inositol levels, which led to the inositol depletion hypothesis as mechanistic insight into its therapeutic value for BPD. Interestingly neither GSK-3 nor inositol monophosphatase (IMPase) have been evaluated for their role in the regulation of the ageing process. In this chapter I aimed to characterize their role in ageing, with particular emphasis on GSK-3. I tested the ubiquitous and tissue-specific effects of GSK-3 by genetic manipulation. Additionally I was able to test the interaction of lithium treatment and GSK-3 in the whole fly and also in specific sub-systems. I observed that at the whole fly level GSK-3 down-regulation extended lifespan and this effect was epistatic with lithium treatment. The converse was also true, i.e., increased ubiquitous expression of GSK-3 shortened lifespan and lithium was able to rescue this effect. However, over-expression of GSK-3 only in neuron extended lifespan and this was additive with lithium treatment. Interestingly the gut and fat body, highly implicated in the regulation of lifespan through HS down-regulation, did not respond to increased expression of GSK-3, suggesting that indeed GSK-3 acts in a tissue-specific manner to regulate the rate of ageing and control longevity. I also analysed the role of GSK-3 and lithium in a Drosophila model of Alzheimer's disease by expression of 4_{1_42}. Lithium and GSK-3 seem to act in different pathways to modulate lifespan of flies expressing 4_{1_42}, though both interventions seem to regulate neurodegeneration. Thus, I have uncovered a complicated relationship by which lithium and GSK3 modulate ageing in different tissues and neurodegeneration.

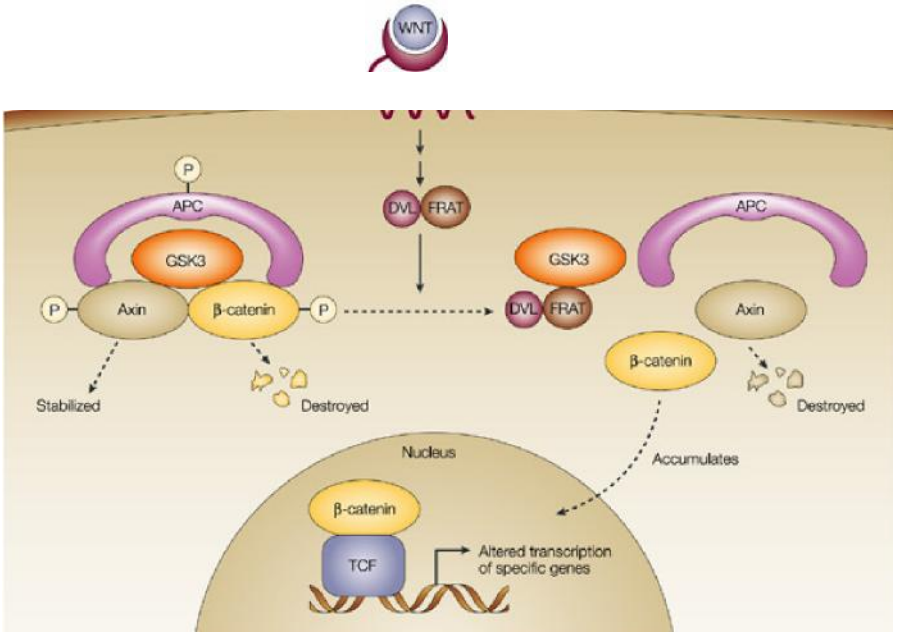
5.2 Introduction

5.2.1 GSK-3 in cellular signalling

Glycogen synthase kinase-3 (GSK-3) is a protein serine/threonine kinase involved in a wide range of physiological actions that regulate development, cell cycle, oncogenesis, gene expression, metabolism, circadian clocks, apoptosis, and neuroprotection, among others. It has been described to participate in two cellular pathways, IIS and the wnt/I3-catenin pathway (Frame and Cohen, 2001; Jope and Johnson, 2004; Rayasam et al., 2009)

The insulin signalling pathway is responsible for cellular glucose uptake providing cells with this substrate to generate adenosine triphosphate, the principal cellular energy source. GSK-3 is downstream of Akt and immediately upstream of GS. GSK-3 functions to supress GS. Therefore, when insulin activates the pathway AKT inhibits GSK-3 allowing GS to synthesise glycogen (Cross et al., 1995, 1997). A detailed description of the role of GSK-3 in the insulin signalling pathway was provided in Chapter 1.

The other cellular pathway in which GSK-3 is involved is the Wnt/I3-catenin pathway (Figure 5.1). f3-catenin acts as a transcription factor. In the absence of stimuli the binding protein axin joins the *adenomatous polyposis colt* (APC) protein along with GSK3 and I3-catenin to form a complex that, after a sequence of phosphorylations, promotes the degradation of I3-catenin. In the presence of a positive stimulus Wnt binds to the transmembrane receptor frizzled activating the protein disheveled by direct binding to frizzled that along with the GSK-3-binding protein frat, allows the disruption of the complex formed by APC, axin and GSK-3. This in turn lowers the phosphorylation rate of I3-catenin allowing it to escape from the degradation complex. Instead, I3-catenin translocates to the nucleus and interacts with LEF-1/TCF family of transcription factors that regulate important proteins for cellular development during embryogenesis (Cohen and Frame, 2001; Jope and Johnson, 2004).



Oturd ROviCekli I Drilg DISCOVErrV

Figure 5.1 Schematic representation of the wnt/P-catenin pathway. GSK-3 plays a major role in this cellular pathway since it controls the rate of transcription by negatively regulating 13-catenin. When Wnt is activated, GSK-3 is inactive allowing translocation of13-catenin to the nucleus. Image taken from (Cohen and Goedert, 2004).

There are two mammalian isoforms of GSK-3, GSK-3a and GSK-30, the former is inactivated by phosphorylation at serine 21, and the latter at serine 9 (Cohen and Frame, 2001; Jope and Johnson, 2004; Rayasam et al., 2009). As mentioned earlier, the phosphorylation and hence inactivation of GSK-3 is primary for the regulation of glycogen synthesis. However, phosphorylation of GSK-3 does not seem to be a fundamental mechanism for inactivation of GSK-3 in the wnt/I3-catenin pathway, and there is evidence to support that the kinase activity of GSK-3 is not affected by Wnt activation (Cohen and Frame, 2001; Cross et al., 1997; Ding et al., 2000; McManus et al., 2005). This is consistent with the fmding that insulin activation and phosphorylation of GSK-3 does not increase I3-catenin levels. On the other hand, GSK-3 assembled with axin, APC and 13-catenin in the degradation complex is inaccessible to Akt (Ding et al., 2000). Therefore, although GSK-3 is common to both signalling pathways, their pools should be different or may function differently, however interaction between these pathways has been reported (Cohen and Frame, 2001; Frame and Cohen, 2001).

GSK-3I3 inactivation is the major cause for GS activity in the muscle, although this has been suggested to be partly due to increased levels (4-fold) of this isoforms compared to GSK-3a. However, inactivation of neither GSK-3 isoform appears to be relevant for GS

activity in the liver and in exercise-induced muscle GS dephosphorylation (McManus et al., 2005).

5.2.2 Regulation of GSK-3 and its inhibition by lithium

GSK-3 exerts its effects through a unique substrate specificity in which most of its substrates need prior phosphorylation (or 'priming phosphorylation') in a consensus recognition sequence (Ser/Thr-[X-X-X]-pSer/pThr). As mentioned earlier, GSK-3 in its constitutively active state, maintains GS inactivated through phosphorylation. However, in order for GSK-3 to phosphorylate GS at Ser652, the latter needs to be phosphorylated by casein kinase 2 (Ser656, the priming phosphorylation site). The initial phosphorylation by GSK-3 may induce phosphorylation of further residues, hence generating 'multiple phosphorylation domains'; in the case of GS a total of 5 (Cohen and Goedert, 2004; Frame and Cohen, 2001; Rayasam et al., 2009). GSK-3 phosphorylates tau and this has been implicated in the generation of neurofibrillary tangles in AD (Cohen and Goedert, 2004).

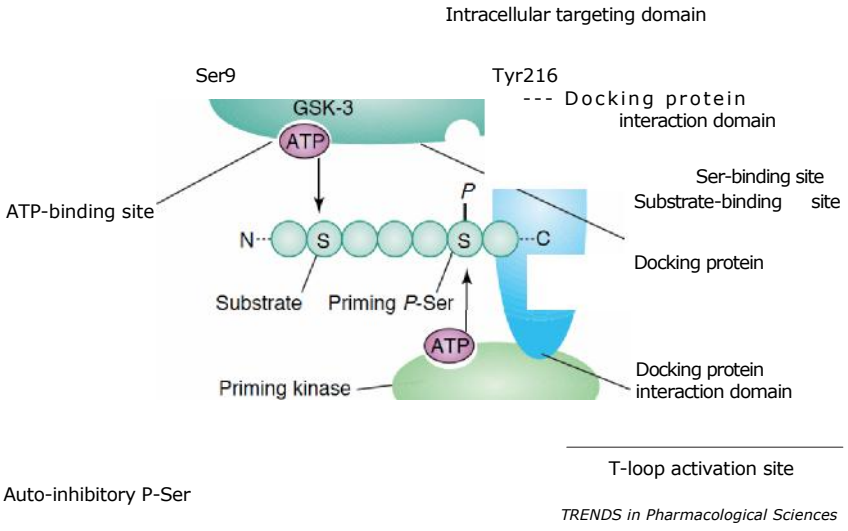


Figure 5.2 Sites of GSK-3 inhibition. GSK-313 is activated by phosphorylation at Tyr216 and inhibited by phosphorylation at Ser9 and also by competing for ATP at the ATP-binding site. Lihium ibhibits GSK-3 by increasing the phosphorylation at Ser9 and also by competing with ATP. Taken from (Meijer et al., 2004)

Lithium is the only available drug recognized to inhibit GSK-3 (Figure 5.2). It is widely prescribed for the treatment of BPD (Freeman and Freeman, 2006; Soares and Gershon, 1998). And although it has been in use for more than fifty years, there is still interest in its pharmacological properties and new therapeutic applications are found

constantly (Chuang et al., 2002; Phiel and Klein, 2001). The first indications that GSK-3 was a direct target of lithium came over a decade ago. Klein and Melton showed, by a peptide substrate assay, that LiC1, within therapeutic concentrations, was able to block GSK-3I3 mediated phosphorylation of protein phosphatase inhibitor-2 and tau. They proposed that lithium mimicked the effect of insulin signalling through inactivation of GSK-3I3 (Klein and Melton, 1996). Stambolic *et al.,* observed a similar substrate phosphorylation effect in *Drosophila* cells where lithium treatment mimicked the effect of Wnt/f3-catenin *(wingless/Armadillo* in *Drosophila)* activation (Stambolic et al., 1996). Nowadays, we know that lithium has a direct and indirect mechanism to inhibit GSK-3. Lithium directly inhibits GSK-3 acting as a competitive inhibitor of Mg^{2+}; on the other hand, it indirectly inhibits GSK-3 by increasing its phosphorylation. Although, the later mechanisms is not fully understood, it is accepted that the indirect effect might be through inactivation of a phosphatase that normally removes phosphate residues from GSK-3 thus allowing its activation (Jope, 2003; Ryves and Harwood, 2001).

Since GSK-3 has been found to be implicated in several neuronal physiological processes, as well as in the pathogenesis of a diverse range of brain-related diseases, lithium is currently studied as a potential treatment option for diseases such as AD and other tauopathies (corticobasal degeneration, Pick's disease), HD, schizophrenia, among others (Cohen and Goedert, 2004; Hooper et al., 2008; Jope and Johnson, 2004; Lovestone et al., 2007).

5.2.3 The role of GSK-3 in ageing

There is no direct evidence of the role of GSK-3 in ageing. However, there are some indirect observations that suggest that GSK-3 function may be of great relevance for lifespan and ageing. The closest observation to date of the role of GSK-3 in ageing comes from a pharmacogenetic study of the effects of lithium in *C. elegans* (McColl et al., 2008). Lithium is the most potent drug, medically available, known to inhibit GSK-3 (Jope, 2003; Phiel and Klein, 2001). They observed that lithium treatment extended lifespan, and when this was correlated with gene-transcripts response to lithium and RNA interference knockdown experiments, they concluded that the effects of lithium could be by modulation of histone methylation and chromatin structure. However, when they expressed a maternally rescued null *gsk-3I3,* they observed a 36% reduction in *C. elegans* lifespan.

Additionally, treatment of *gsk-3I3* null worms with lithium, further decreased lifespan (McColl et al., 2008).

Work in the *klotho* mice has shown that regulation of the Wnt/I3-catenin pathway is relevant for lifespan (Liu et al., 2007). The *klotho* mice were generated serendipitously as a result of an insertional mutation that disrupted the promoter region of an, until then, unknown gene, now named *klotho* (Kuro-o et al., 1997). The *klotho* gene encodes a type-I single-pass transmembrane protein that is an obligatory co-receptor of the fibroblast growth factor 23 (FGF23). As FGF23 regulates phosphate metabolism and it is also related with a premature ageing phenotype, this has unveiled a potential link between ageing and phosphate metabolism (Kuro-o, 2009, 2010). The *klotho* mice developed normally but presented with an early ageing phenotype and extremely reduced lifespan (Kuro-o, 2009; Kuro-o et al., 1997). It was reported an average lifespan of 60.7 days with no mice living longer than 100 days. Moreover, the *klotho* deficient mice developed age-related changes and diseases such as arteriosclerosis, osteoporosis, emphysema, skin atrophy and infertility (Kuro-o et al., 1997). On the other hand, mice overexpressing *klotho* significantly outlived their littermates by 18 to 30% (Kurosu et al., 2005).

The above illustrates that klotho is an important protein involved in the regulation of lifespan and ageing. Its relevance for GSK-3 was established later when Liu and colleagues showed that klotho physically interacts with Wnt, as co-immunoprecipitation experiments evidenced that klotho immunoprecipitates with several Wnt isoforms. Even more, this biophysical interaction was shown to negatively regulate the Wnt/I3-catenin pathway. *Klotho* mice show either reduced (when obtained by insertional mutation) or no (generated by conventional gene targeting) activity, resulting in increased Wnt pathway activity in several tissues. Additionally, *in vitro* studies where primary mouse embryonic fibroblasts were grown in a Wnt3a-conditioned medium, and analyses of skin follicles of mice overexpressing Wntl (increasing in both the activity of the Wnt/(3-catenin pathway), showed a significant augmentation of senescence when compared against controls (Liu et al., 2007). Since increased Wnt/f3-catenin pathway activity would maintain GSK-3 in an inactive state, it is possible to hypothesize that a persistent inactive GSK-3 could be in part responsible for the pro-ageing phenotype of the *Klotho* deficient mice. This is also consistent with the lifespan effect of null *gsk-3/3* mutants in *C. elegans.*

Additional evidence of the role of GSK-3 in ageing has come from the overexpression of the Wnt/I3-catenin pathway in *Drosophila.* Shen *et al.,* over-expressed *wingless,* the fly homolog of Wnt, using the Act-GS conditional expression system

(allowing limited life-cycle expression). Overexpression of *wingless* was lethal to both male and female larvae, but when *wingless* was over-expressed during adulthood, it reduced lifespan up to 42% (Shen et al., 2009).

The available data does not give direct evidence as to what is the role of GSK-3 in ageing. Lithium is recognized as a GSK-3 inhibitor however, the results from McColl are contradictory to some extent to what one would expect from GSK-3 inhibition and lithium treatment (McColl et al., 2008). As mentioned earlier, they found that although lithium treatment increased the lifespan of worms, a null *gsk-3I3* had detrimental survival effects. Moreover, the indirect evidence presented from modulation of the Wnt signalling pathway better supports that inhibition of shaggy/GSK-3 has a pro-ageing effect.

5.2.4 Lithium and the inositol depletion hypothesis

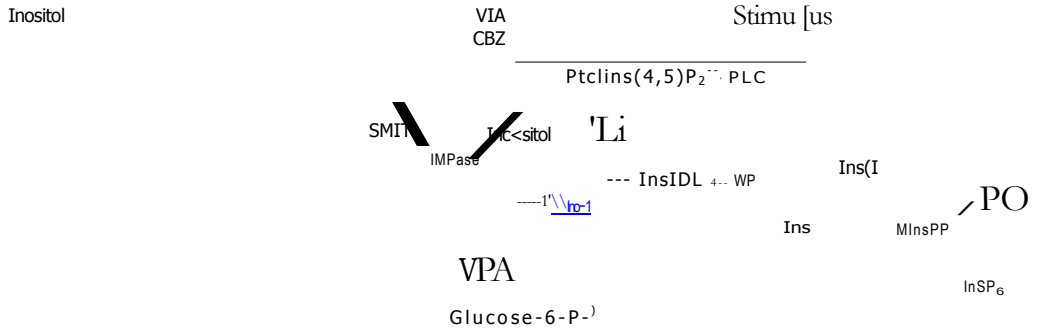


Figure 5.3 Inositol phosphate pathway. Lithium and other mood-stabilising drugs are thought to act by inhibiting specific components of the inositol phosphate pathway. Besides inhibiting recycling of inositol via IPP and IMPase inhibition, lithium also inhibits ***de novo*** synthesis of inositol through IMPase inhibition. However, valproic acid (VPA) most directly inhibits synthesis through inhibition of inositol synthase (ino-1). Additionally lithium, VPA and carbamazepine (CBZ) also inhibit transport across the membrane via the sodium/myo-inositol transporter (SMIT) Taken from (Harwood, 2005).

The inositol depletion hypothesis of lithium proposed by Berridge, Downes and Manley suggests that lithium acts to stabilize mood via inhibition of the recycling of inositol and interfering with inositol 1,4,5-triphosphate (IP3)-mediated cell signalling (Figure 5.3) (Berridge et al., 1989). This followed from the observation of Allison and Stewart who described that lithium-treated rats showed a 30% reduction of myo-inositol levels in cerebral cortex. Importantly they showed that this effect was not reproducible by NaC1 (Allison and Stewart, 1971). However, other studies have called these results into question (Phiel and Klein, 2001). Lithium is presumed to inhibit the recycling of inositol for the re-

synthesis of inositol phospholipids via negatively acting at least on two enzymes, inositol-1,4 biphosphate 1-phosphatase (IPP) and inositol-1(or 4)-monophastase (IMPase) (Berridge, 1985; Chiu and Chuang, 2010; Harwood, 2005).

Myo-inositol is the substrate for the formation of the lipid membrane phosphatidylinositol (PtdIns). Phosphorylation in its inositol carbon ring results in the formation of mono-, bis- and tris- Ptdlns phosphate (PtdInsP). Stimulus activation, for example of G protein coupled receptors, activates the enzyme phospholipase C (PLC) which hydrolyzes Ptdlns(4,5)P2 (or PIP2) to release soluble inositol tris-phosphate (IP_3). This is particularly relevant for calcium homeostasis. Additionally, PLC releases diacyl glycerol (DAG) which activates protein kinase C (PKC) and its downstream targets (Can et al., 2014; Harwood, 2005).

IP3 dephosphorylation by subsequent action of IPP and IMPase recycles inositol (mostly present as the stereoisomer myo-inositol). Lithium inhibits IPP and IMPase within the therapeutic range (0.5 to 1.5 mM) (Can et al., 2014; Phiel and Klein, 2001). Inositol depletion by these and other enzymes has been proposed as a common mechanism of action for lithium, valproic acid and carbamazepine (Figure 5.3) (Williams et al., 2002). Lithium is a non-competitive inhibitor of IMPase, which means that the degree of inhibition is dependent (or more effective) on both the inhibitor and the substrate; the more inositol phosphate, the more inhibition (Phiel and Klein, 2001; Williams and Harwood, 2000). This has often been taken as an explanation why lithium stabilises mood in disease patients but not in non-affected subjects (Phiel and Klein, 2001). It is presumed that patients with BPD and other mood disorders have and increased pool of inositol or signalling through the IP_3 signalling pathway, though evidence for this is scarce (Can et al., 2014). Interestingly, myo-inositol supplementation is capable of blocking the effect on inositol depletion (Williams and Harwood, 2000; Williams et al., 2002).

5.2.5 Alzheimer's disease: clinical and pathological principles

Alzheimer's disease (AD) is the most common neurodegenerative disease; it contributes 60 to 70% of all causes of neurodegeneration (Holtzman et al., 2011). It has been estimated to affect over 35 million people worldwide (Selkoe, 2012). While the contribution to overall mortality from other chronic degenerative diseases has been declining over time (the so called morbidity compression), AD is increasingly becoming a dominant contributor to death (Figure 5.4) (Alzheimer's Association, 2014).

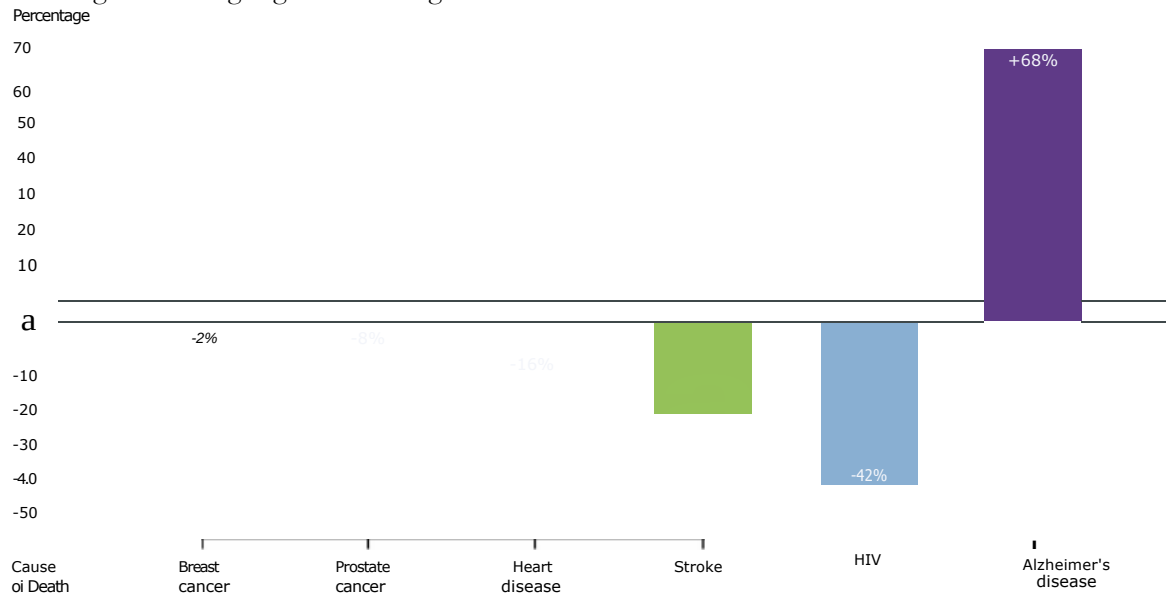


Figure 5.4 Percentage changes in mortality for the major killer diseases between 2000 and 2010 from data from the National Center for Health Statistics, USA. Taken from (Alzheimer's Association, 2014).

The major risk factor for AD is ageing, as the incidence of the disease increases with age (Mayeux and Stern, 2012). Other important risk factors are family history, APOE status, cardiovascular health, education and traumatic brain injury, amongst others (Alzheimer's Association, 2012, 2014). Indeed there is great evidence that altered metabolism of glucose and cholesterol increase the risk of AD, though this connection seem to go in both directions (Castillo-Quan et al., 2008; Luchsinger, 2010). Family history is relevant since AD has a strong genetic component. Most cases of AD develop later in life (LOAD, late onset AD), after the 7th decade, and are thought to be sporadic (with unknown cause, or multigenic in aetiology). However, less than 1% can develop the disease as early as there forties (EOAD, early onset AD). EAOD or familial AD arises secondary to mutations in genes that increase the production of amyloid beta (A(3). AD is characterised by the presence of two pathological hallmarks, extracellular senile plaques consisting of insoluble aggregated Af31_42 (A(3 and Af31_42 [or Af342] can be used interchangeably to denote the full length of the amyloidogenic peptide) and intracellular neurofibrillary tangles composed of a hyperphosphorylated form of the microtubule associated protein tau (George-Hyslop, 2000; Hardy, 2004; Selkoe, 1991; Tanzi, 2012). These pathological hallmarks were recognised early in the 1900's by the German psychiatrist Alois Alzheimer in Auguste D. This was a 51-year-old woman who presented with an atypical form of dementia that included auditory hallucinations, delusions,

paranoia and aggression along with short-term memory loss and language deficits. Alois Alzheimer's description of the pathological features of the disease, later named after him by his mentor Emil Kraeplin, remain to be one of the landmark breakthroughs of the disease (Goate and Hardy, 2012; Ramirez-Bermudez, 2012).

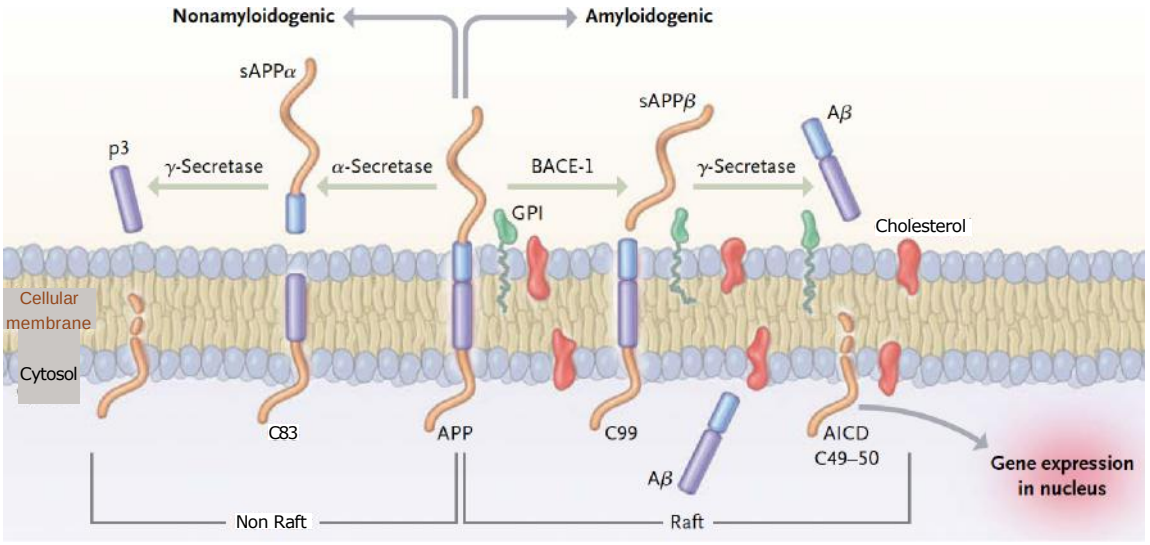


Figure 5.5 Amyloid precursor processing and the formation of 4_{1_42}. For detail description see main text. Taken from (Querfurth and LaFerla, 2010).

AI31_42 derives from the `abnormal' processing of the ubiquitous type I transmembrane protein amyloid precursor protein (APP). APP695, the major neuronal form of APP (other forms like APP751 and APP770 are expressed elsewhere), is cleaved by two metabolic routes, the amyloidogenic or non-amyloidogenic paths (Figure 5.5). In the non-amyloidogenic route APP is sequentially cleaved by a- and y-secretase. Cleavage by a-secretase results in the release of a large N-terminal ectodomain (sAPPa) to the extracellular space, while the remaining C83 fragment is digested by y-secretase to release to the extracellular space a fragment called p3 and the amyloid intracellular domain (AICD) released in the cytoplasm. On the other hand, amyloidogenic processing initiates with processing by (3-secretase (3-site APP-cleaving enzyme 1 (BACE-1) realising a shorter sAPP called sAPP(3. The remaining C99 fragment is processed then by y-secretase liberating A(3 of different lengths, including A(31_38, **A**

A(31_42 and A(31_43 (Cummings and Cole, 2002; LaFerla et al., 2007; Mann et al., 1996; Querfurth and LaFerla, 2010; Welander et al., 2009). A(31_42 is the most common and pathogenic form of A(3 present in plaques, though recently the pathogenicity of $A13_{1\_43}$ has also been described (Saito et al., 2011).

There is always been debate as to which one, Al3 or tau, drive the disease. The amyloid cascade hypothesis offers a conceptual framework for the study of AD (Figure 5.6). This hypothesis proposes that increased Al3 production is an early event of the disease that drives in an almost sequential manner the pathology that leads to neurodegeneration and clinical AD (Goate and Hardy, 2012; Hardy, 2009; Hardy and Selkoe, 2002; Selkoe, 2006). The greatest support for this theory comes from the fact that mutations in APP that increase the production of Af31_42, alter the ratio of Af31_40 to A131-42, or increase the aggregation propensity of A131_42 all lead to familial EOAD (Bertram et al., 2010; Goate et al., 1991; Guerreiro et al., 2013; Hardy, 2004; Nilsberth et al., 2001; Tanzi, 2012). However, mutations in tau do not cause AD and have been linked to other forms of neurodegeneration like fronto-temporal dementia and some forms of parkinsonism (Goedert, 2005; Spillantini and Goedert, 2013; Wolfe, 2009).

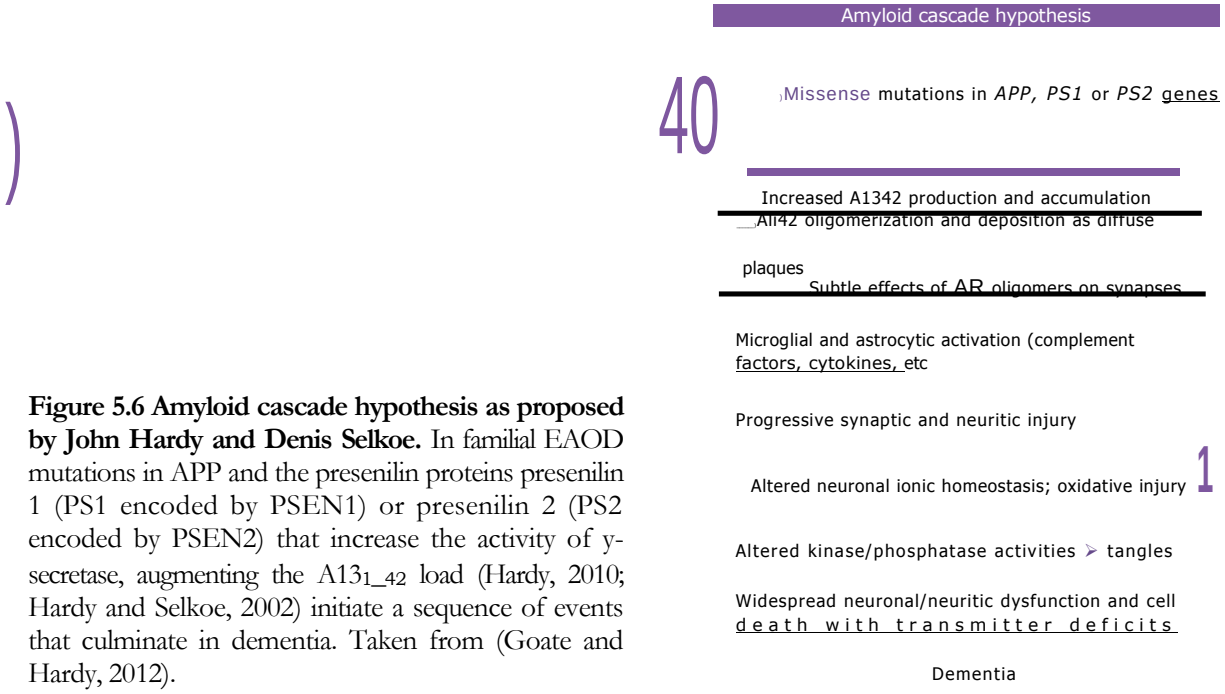


Figure 5.6 Amyloid cascade hypothesis as proposed by John Hardy and Denis Selkoe. In familial EAOD mutations in APP and the presenilin proteins presenilin 1 (PS1 encoded by PSEN1) or presenilin 2 (PS2 encoded by PSEN2) that increase the activity of y-secretase, augmenting the $A13_{1\_42}$ load (Hardy, 2010; Hardy and Selkoe, 2002) initiate a sequence of events that culminate in dementia. Taken from (Goate and Hardy, 2012).

Clinically, the disease has an insidious presentation that often starts with minor short-term memory loss (often dismissed by patients and those surrounding them) and orientation difficulties. Remote or long-term memory is only lost later in the disease. After many years other symptoms like loss of verbal and motor control, judgement and reasoning can also accompany. Less common symptoms include depression, hallucinations

and psychosis; sleep disturbances, agitation, anxiety, etc. (Blennow et al., 2006; Cummings and Cole, 2002; Holtzman et al., 2011; Mayeux, 2003).

Even though we have understood a great deal of the disease, there is very little the community can offer patients in terms of treatments. Pharma and academia have embarked in huge and costly clinical trials just to end up with disappointing outcomes and no therapeutic approach with clinical value. Where are we failing? All therapeutic interventions that have entered clinical trials have done so with a great deal of preclinical data backing them up. Certainly, the effort is being done, yet the fruits are long overdue. This has made several to reconsider the approach. For example, the amyloid cascade hypothesis has been partly blamed for being the main focus of most academics. Yet, clinical data still supports that Af31_42 as an early pathogenic event. Moreover, mutations that increase Af31_42 production translate clinically in AD, which cannot be said by mutations arising in tau. Others have also considered that the use of mouse models that do not recapitulate the disease are also to blame. Although fmger pointing will not bring solutions, serious considerations need to be taken so that we can bring the community to a consensus and efficient approach to tackle this horrible disease (Guerreiro and Hardy, 2011; Hardy, 2009; Lansbury and Lashuel, 2006; Selkoe, 2012).

5.2.6 *Drosophila* models of Alzheimer's disease

Although *Drosophila* possesses an APP like protein, *Appl* (Luo et al., 1992; Rosen et al., 1989), it is contested whether it possess a functional I3-secretase responsible for the cleaving of the amyloidogenic portion, though the fly does possess a functional y-secretase (Carmine-Simmen et al., 2009; Fossgreen et al., 1998). Moreover the amylogogenicity of *Appl* has been shown to be poorly conserved in evolution (Rosen et al., 1989). A viable option for modelling AD in *Drosophila* has been the reconstitution of the system, i.e., inserting in the fly all the components of the amyloidogenic processing pathway that lead to the generation of Af31_42. This has proven to be effective at showing that the processing can indeed happen when these genes are inserted in the fly. Through incorporation of human APP695, a- and y-secretase, the fly has been used to determine toxicity of A131-42 and also to screen for modifiers of Af31_42 production. However, given that the processing is happening because of the artificial introduction of human components, this approach has been regarded as less revealing (Bonner and Boulianne, 2011; Iijima and Iijima-Ando, 2008).

An alternative has been to introduce full length Al3 instead to study the downstream effects that lead to neurodegeneration. By using the Ga14/UAS system (described in Chapter 2) Iijima and colleagues studied behavioural and survival of flies expressing A(31_ ao or A(31_42. They observed that expression of A(31_42 in the fly brain lead to the formation of amyloid deposits, age-dependent learning defects, locomotor defects and neuronal loss. Additionally these flies also showed reduced survival. In contrast, expression of A131_40 did not lead to neurodegeneration, locomotor defects nor reduced survival, though it associated with learning defects (Iijima et al., 2004). Crowther and colleagues used a similar strategy but also expressed A(31_42 carrying the Arctic mutation, a Glu22Gly amino acid substitution that accelerates aggregate formation (Nilsberth et al., 2001). They showed that the Arctic mutation showed more accelerated and severe phenotypes than expressing wild type A(31-42 (Crowther et al., 2005; Kinghorn et al., 2006).

Recently our group used another fly genetic tool, the Gene Switch system (described in Chapter 2) (Osterwalder et al., 2001), to show that the effects of A131_42 are not produced by disrupting the developmental programme of neurons as the expression of the transgenes is only started once the fly was matured. Sofola and colleagues expressed A(31_40 or A(31_42 (with the Arctic mutation) only in adult neurons. While expression of Arctic-A(31_42 reduced survival and accelerated age-related locomotor decline, expression of A(31_40 did not. They also show that the expression of Arctic-A(31_42 lead to the progressive deterioration of the synaptic capacity of the giant fibre system, an escape system in *Drosophila*. Interestingly, they did not observe neuronal cell loss (Sofola et al., 2010). This model can be used to tease out early pathogenic event given that A(31_42 is expressed only when the experimenter provides fly medium supplemented with the GeneSwitch inducer (mifepristone or RU486). Additionally, Rogers and colleagues used it to determine whether older neurons are more susceptible to the toxic effects of Arctic-A(31_ 42. They found that older flies die faster than younger flies when Arctic-A(31_42 is expressed, even when the mRNA or peptide levels are controlled to be similar in the young and old (Rogers et al., 2012). The GeneSwitch system model for the expression of Arctic-A(31_42 has also been used to determine that *Drosophila* tau is downstream of A131_42 in the pathogenic cascade (Sofola et al., 2010), and that drugs that modify Arctic-A(31_42 levels improve the phenotypes observed in this model (Sofola-Adesakin et al., 2014).

5.3 Methodology and experimental design

5.3.1 *Drosophila* strains

All shaggy lines for ageing experiments were crossed into the $^{-14,Dah}$ background. Experiments for neurodegeneration were carried out in the ^{w1118} background. UAS-sgg, UAS-sgg(S9E) and UAS-sgg(S9A) were obtained from Dr. Oyinkan Sofola-Adesakin. UAS-sgg-RNAi and UAS-IMPase-RNAi lines were obtained from Vienna *Drosophila* RNAi Center and UAS-sgg-RNAi(1) and UAS-sgg(KD) were obtained from the Bloomington Stock Center. S1106 driver was obtained from Dr. Ivana Bjedov. UAS-Arctic-A01-42 was a generous gift from Damian Crowther (Cambridge, UK) and UAS-0N4Rtau was obtained from Dr. Fiona Kerr.

5.3.2 Immunoblotting

The following primary antibodies were used: phospho(Ser9)-GSK-3 (#9331 Cell Signaling Technologies: 1:500), total-GSK-3 (#G8170-40 US Biologicals; 1:1000), total human tau (#A0024 Dako; 1:1000), CP13 (phospho(Ser202)-human tau; a generous gift of Dr. Peter Davies to Dr. Fiona Kerr; 1:100), PHF-1 (phospho(Ser396/Ser404)-human tau; a generous gift of Dr. Peter Davies to Dr. Fiona Kerr; 1:100) .

5.3.3 Myo-Inositol supplementation

Myo-inositol (15125 Sigma) was prepared as 20 mM before supplemented to fly medium at the appropriate concentration.

5.4 Results

5.4.1 Lithium inhibited shaggy across a wide range of concentrations

GSK-3 is a well-documented target of lithium (Jope, 2003). I therefore decided to investigate whether the concentrations used in my experiments were able to inhibit GSK-3. I treated flies for 15 days before testing by immunoblot analyses. Lithium increased the inhibitory phosphorylation (Serine 9 or S9) of shaggy *(sgg)* in a dose-dependent manner (Figure 5.7; $P < 0.05$, ANOVA post hoc Tukey- Kramer). Thus, the concentrations used in my experiments, including the ones that extend lifespan, significantly inhibited *sgg*.

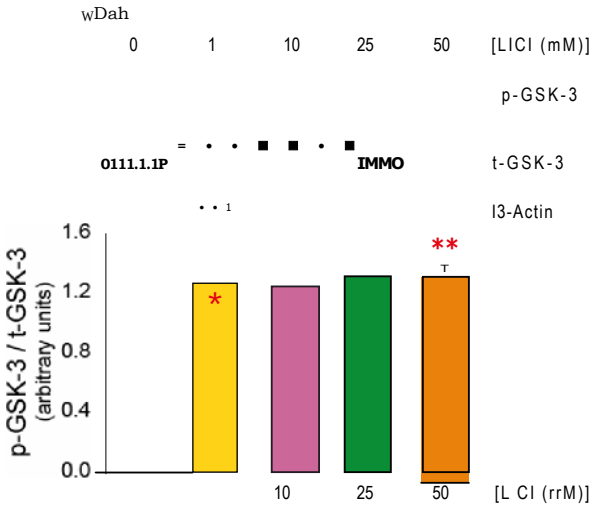


Figure 5.7 Lithium increased the inhibitory Ser9 phosphorylation of shaggy/GSK-3. Female flies were treated with lithium for 15 days after which they were analysed by immunoblot analyses. * $P < 0.05$, ** $P < 0.01$ (ANOVA, post hoc Tukey-Kramer). Bar represents 3 biological replicates of 10 flies each ± SEM.

5.4.2 Different shaggy transgenes modified tau phosphorylation levels

I decided to investigate the role of GSK-3 in *Drosophila* ageing and to evaluate how it interacts with lithium. I first assessed the kinase activity of over-expressing *sgg*, an RNAi line against *sgg* and several mutants that differentially regulate its kinase activity. Given the lack of reagents to test the ability of *sgg* to phosphorylate downstream targets, I evaluated the ability of *sgg* (or mutants) to phosphorylate the human version of the microtubule associated protein tau (hTau, specifically ON4R tau). Tau is a *bona fide* downstream target of GSK-3, which has made it a relevant pharmacological target for the treatment of AD and other relevant diseases where tau is hyperphosphorylated (Hooper et al., 2008; Querfurth and LaFerla, 2010). Moreover, *sgg* has previously been shown to be

able to phosphorylate hTau, which correlated with neurodegeneration in flies (Chatterjee et al., 2009; Mudher et al., 2004). I first made a double mutant fly carrying the ubiquitous actGS driver and the UAS-0N4Rtau. I then crossed this double mutant with control flies or other UAS lines making it possible to have flies carrying a driver and two UAS lines simultaneously. I observed that while over-expression of wild-type *sgg* or the S9A and S9E mutants increased tau phosphorylation (*P* < 0.05, ANOVA post hoc Tukey-Kramer), RNAi-mediated knockdown of *sgg* significantly reduced phosphorylation levels (Figure 5.8; *P* < 0.05, ANOVA post hoc Tukey-Kramer). The S9A *sgg* mutant carries an alanine instead of a serine in position nine, while the S9E mutations carries a glutamic acid substitution in the same position (Bourouis, 2002; Papadopoulou et al., 2004). The S9A mutation should render the enzyme activity constitutively active, while the S9E mutation should act as a phosphomimic, hence acting as a dominant negative. As it will become evident from experiments presented later, none of my experiments suggest that the S9E and S9A mutation are phenotypically different (see discussion). I sequenced the mutations in my fly stocks to corroborate that my flies carried the appropriate mutations, finding that they indeed were S9A or S9E mutants (data not shown).

Over-expression of a kinase dead (sgg-KD) mutant did not significantly modify tau phosphorylation levels (*P* > 0.05, ANOVA post hoc Tukey-Kramer). Thus, I identified *sgg* transgenes with the ability to reduce tau phosphorylation and to increase it.

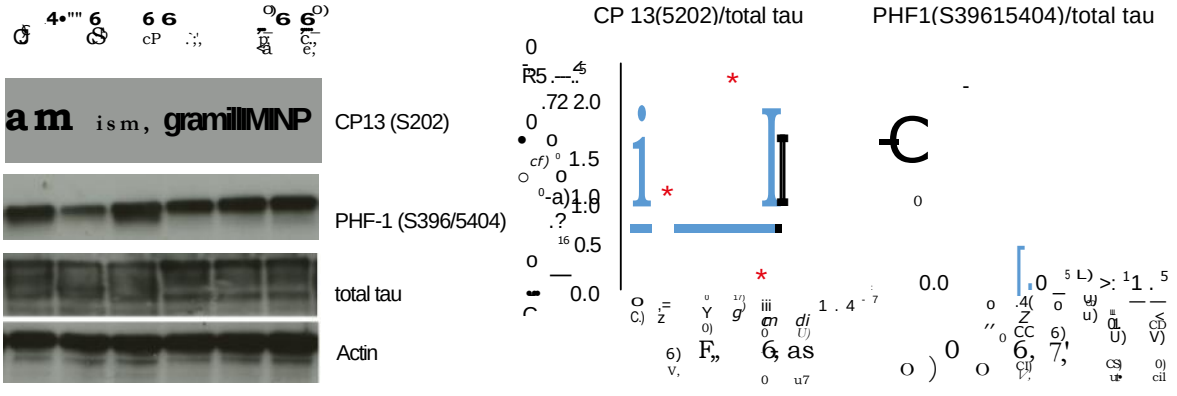


Figure 5.8 *In vivo* phosphorylation of tau by several transgenes of shaggy. Flies expressing tau and one of five UAS-sgg lines were assessed for their ability to phosphorylate human tau by using two antibodies against tau sites known to be phosphorylated by GSK-3 (Ser202 and Ser404). * ***P*** < 0.05 (ANOVA, post hoc Tukey Kramer). N= 4 replicates of 10 flies each ± SEM.

5.4.3 Shaggy transgenes that increased tau phosphorylation reduced lifespan

I first analysed how *sgg* transgenes that increased tau phosphorylation modulate lifespan. For this purpose I over-expressed wild type *sgg*, the S9A or S9E mutant using the actGS driver and fed them the inducer RU486 from day 2 post-eclosion. Over-expression of either of these transgenes shortened lifespan significantly (Figure 5.9; $P > 0.001$, ANOVA post hoc Tukey-Kramer for the induced lines in comparison to their respective control).

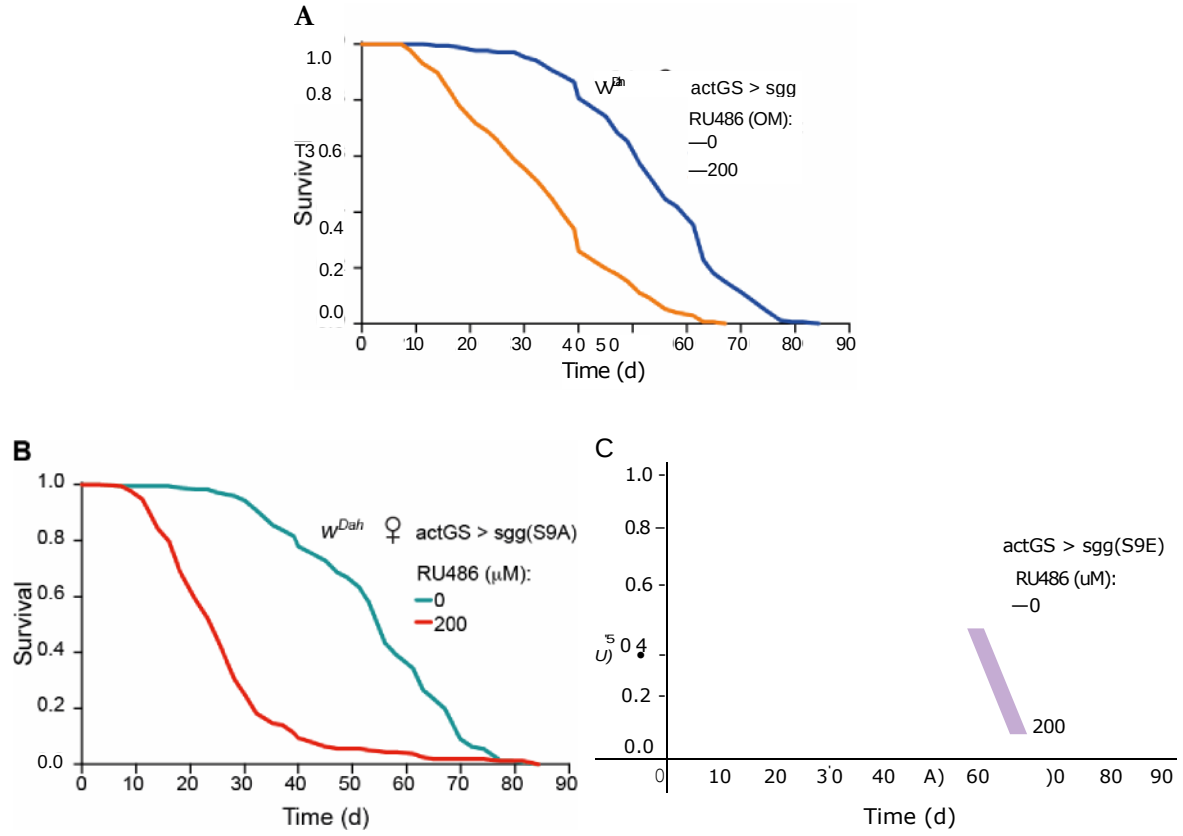


Figure 5.9 Lifespan of GSK-3 UAS lines that increased tau phosphorylation. (A) Over-expression of wild type *sgg* shortened lifespan. **(B)** Over-expression of the S9A *sgg* mutant or the (C) S9E *sgg* mutant reduced lifespan. N= 150 female flies per condition. A set of these experiments was performed in collaboration with Dr. Ivana Bjedov.

Over-expression of wild type *sgg* shortened lifespan by about 30%. Median and maximum lifespan of flies over-expressing *sgg* were 33.7 and 54.5 days respectively. In contrast their uninduced controls showed a median and maximum of 54.5 and 73.1 days, respectively. Flies over-expressing the *sgg* S9A mutant had a median lifespan of 25, while their non-induced counterpart showed a median lifespan of 53 days. Interestingly, both the induced and uninduced lines showed a maximum lifespan of 63 days. In a similar vein, flies over-expressing the S9E mutation showed a median and maximum lifespan of 27 and

56 days, respectively; in contrast to a median and maximum lifespan of 58 and 70 days of the un-induced controls. Thus, *sgg* transgenes that increased tau phosphorylation reduced lifespan when over-expressed in adulthood from 2 days post-eclosion.

5.4.4 Early or late-onset over-expression of shaggy reduced lifespan

I just showed that over-expression of *sgg* increased phosphorylation of human tau *in vivo*. This evidence of increased GSK-3 activity correlated with a significant reduction in lifespan. Interestingly, the survival curves for the S9A and S9E mutants showed an early accelerated death rate, after which the curves plateau making it seem that flies expressing either of these *sgg* mutants could survive as long as the last 10% of the un-induced controls (Figure 5.9B). The plateau effect in the survival curve after the initial high mortality could suggest that over-expression of *sgg* mutants with increased kinase activity has deleterious effects at younger, but not older ages. Therefore, I evaluated the possibility that late-onset expression could have favourable effects in comparison to early onset over-expression. I tested the effect of over-expressing the S9E or S9A *sgg* mutants after 2 days post-eclosion (as in previous experiments) and after 15 or 30 days. For these experiments flies were reared at the same time and randomly separated into 4 groups, one of which started RU treatment at day 2, one at day 15 and another one after 30 days of eclosion. The remaining group was labelled as the un-induced control. Flies that were switched on at later stages (after 2 days) were kept in the same fly medium as the control group until the time of switch. I observed that lifespan was significantly reduced independent of the age at which the over-expression was started (Figure 5.10). When the S9A mutant was induced 2 days post-eclosion the median lifespan was 21.6 days, while median lifespan when induction was started later, 15 and 30 days, was 35.6 and 52 days, respectively (*P* for all comparisons < 0.001, log-rank test; Figure 5.10A). The time required from induction (when RU was supplemented for the first time) to reach median lifespan was very similar being 19.6, 20.6 and 22 days, respectively for inductions starting 2, 15 and 30 days post-eclosion. A similar scenario was observed when the S9E mutant was expressed in a similar time frame (Figure 5.10B). The median lifespan of flies over-expressing the S9E mutant was 21.6, 41 and 50.5 days for initiating gene expression at days 2, 15 and 30 post-eclosion, respectively (*P* for all comparisons < 0.001, log-rank test). The time required for reaching median lifespan from the moment of switching to fly medium supplemented with RU was 19.6, 26 and 20.5 days, respectively.

I also tested the effect of over-expressing these *sgg* mutants under lower (25 piM RU; Figure 5.10C and D) RU concentrations of the inducer observing a consistent and similar result than when induced with 200 piM of the inducer. My results thus suggest that ubiquitous over-expression of wild type *sgg* or *sgg* mutants that increased the phosphorylation of human tau *in vivo* reduced lifespan whether expressed early in adulthood or late in life.

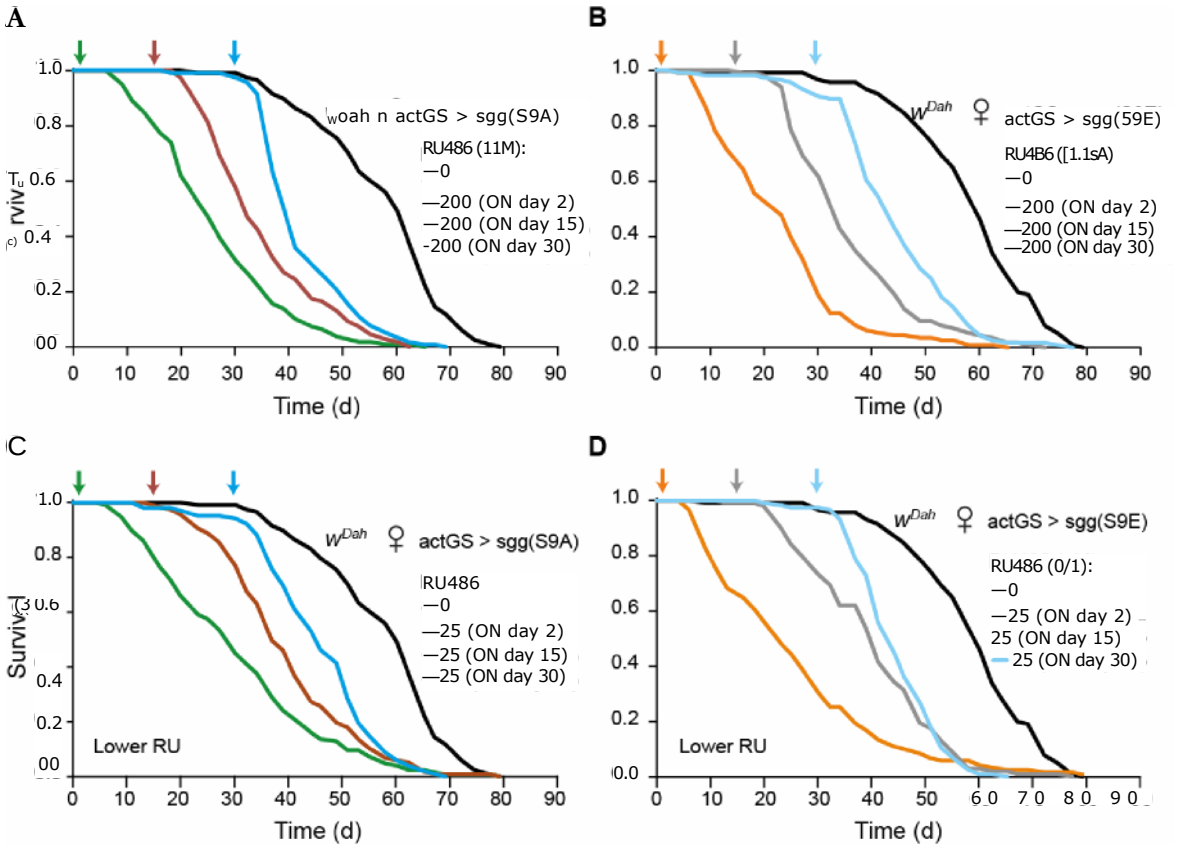


Figure 5.10 Late-onset over-expression of shaggy shortens lifespan. A and B show the over-expression of the (A) S9A or **(B)** S9E mutant induced by 200 11M RU, while C and **D** show over-expression with 25 jiM RU, respectively. Arrows represent time to switch ON expression by supplementing RU. N= 150 female flies per condition.

5.4.5 Shaggy-RNAi extended lifespan and protected against paraquat

Having established that *sgg* mutants that increase tau phosphorylation reduce lifespan I sought out to investigate the role of the line that reduced phospho-tau levels. Hence, I turned my attention to the *sgg* RNAi fly line and tested its role in longevity. Down-regulation of *sgg* significantly increased lifespan *(P* < 0.01, log rank test; Figure 5.11A). I also tested an independent *sgg* RNAi line also observing lifespan extension when induced

(Figure 5.11B; $P < 0.01$, log rank test). Thus, RNAi-mediated knockdown of *sgg* increased lifespan

Given that lithium treatment protects against oxidative stress I explored the possibility that genetic down-regulation of *sgg* could also protect against paraquat. I induced the expression of the RNAi against shaggy for *15* days before transferring flies to medium containing paraquat. Interestingly, RNAi-mediated knockdown of *sgg* significantly protected against paraquat *(P* < 0.01, log rank test; Figure 5.11C). Thus, lifespan extension and paraquat resistance are two features of *sgg* down-regulation.

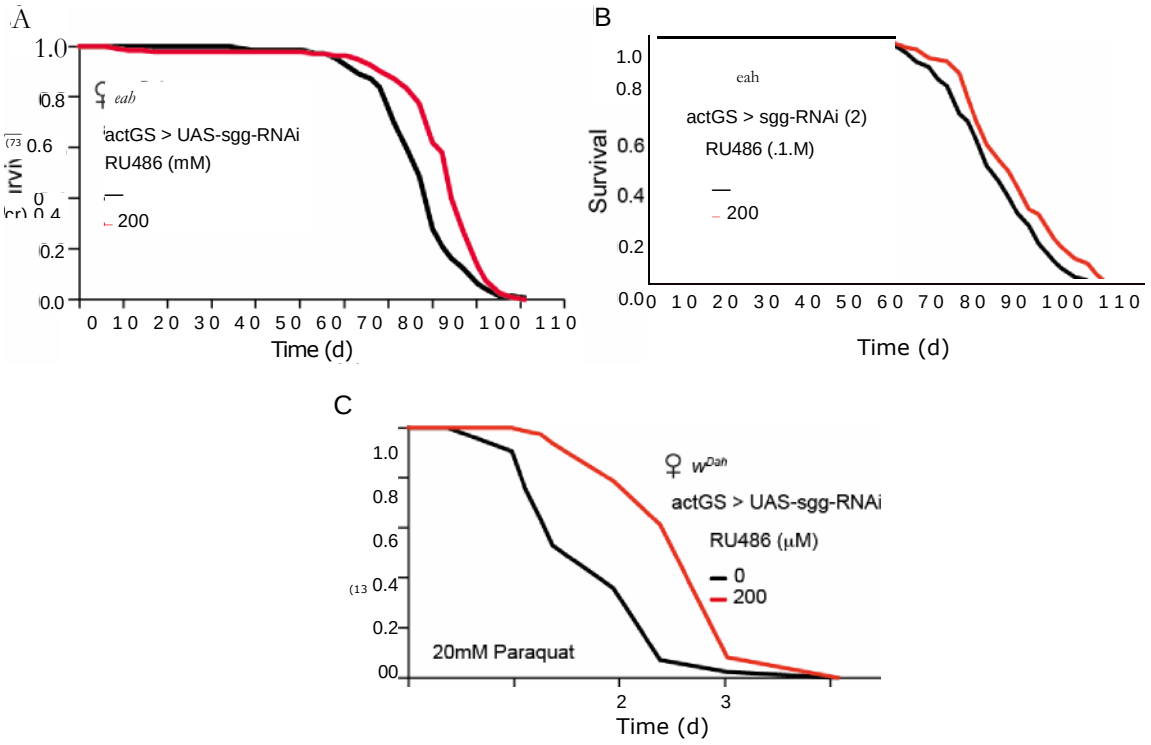


Figure 5.11 RNAi-mediated knockdown extended lifespan and protected against paraquat. A and B show survival curves of two independent *sgg* RNAi lines. N= 160 female flies per conditions. (C) shows the response of knockdown of *sgg* to paraquat. N = 90 flies per condition.

5.4.6 Genetic manipulations of shaggy did not consistently alter hypoxia, armadillo and the IIS pathways

In an attempt to understand the molecular mechanisms behind GSK-3 regulation of lifespan I assessed the expression of genes transcriptionally regulated when certain pathways change. For this I hypothesised that changes having a meaningful impact in the regulation of lifespan due to *sgg* modulation would happen in opposing directions when *sgg* is down-regulated and when *sgg* or the S9A mutant are over-expressed.

One pathway that responds in opposite directions when GSK-3 is manipulated is the hypoxia response pathway. When GSK-3 is down-regulated, genes downstream of the transcription hypoxia-inducible factor-la (HIF-1a) are induced. On the contrary, over-expression of GSK-3 reduces HIF- 1 a (Fliigel et al., 2007). I therefore decided to characterise the mRNA levels of the two most characterised gene targets of HIF-la in *Drosophila, scylla* and *charybdis* (Brugarolas et al., 2004). Surprisingly, I only observed down-regulation of these two genes in the flies over-expressing wild type *sgg* (Figure 5.12A and B; $P < 0.05$; Student's t-test), but not when *sgg* was down-regulated or in the S9A mutant over-expression *(*$P > 0.05$; Student's t-test). As these changes did not follow my genetic manipulations and particularly did not change in flies with sgg-RNAi I consider that the hypoxia response pathway is unlikely to be a major player in the regulation of lifespan due to changes in *sgg* levels.

GSK-3 is known to modulate the arniadillo/13-catenin pathway by targeting armadillo for degradation. This inhibition would reduce signalling to the nucleus, hence lowering the expression of known transcripts (Gordon et al., 2005). I therefore decided to explore two gene targets of the armadillo pathway, namely *Fz3* (which encodes for the receptor frizzled 3) and *naked.* Fz3 mRNA levels were significantly lower (Figure 5.12C; $P < 0.05$, Student's t-test) in flies expressing sgg-RNAi and wild type *sgg,* while no detectable change was observed in flies over-expressing the S9A mutant. mRNA levels of naked only significantly changed in flies over-expressing the S9A mutant (Figure 5.12D; $P < 0.01$, Student's t-test) while no change was detected when *sgg* was down-regulated or over-expressed *(*$P > 0.05$, Student's t-test). Thus, it is unlikely that the armadillo/f3-catenin pathway plays a significant role in the modulation of lifespan through *sgg,* given the inconsistent results between the two gene targets and the genetic manipulations.

I also assessed gene targets of the IIS pathway. Sgg is known to modulate this pathway by targeting IRS-1/chico for degradation, hence down-regulating the pathway (Eldar-Finkelman and Krebs, 1997). I therefore analysed the expression of three known targets of dFOXO, *dInR, chico* and *4E-BP* (Alic et al., 2011b). Once more the results were inconsistent with alterations in the IIS pathway regulating lifespan downstream of *sgg. dInR* and *chico* mRNA levels were down-regulated with expression of sgg-RNAi and over-expression of *sgg* wild type (Figure 5.12E and F; $P < 0.05$; Student's t-test) while no changes were detected in the S9A mutant. In the case of *4E-BP* the only detectable change was observed when wild type *sgg* was over-expressed (Figure 5.12G; $P < 0.05$, Student's t-test), in which it was down-regulated.

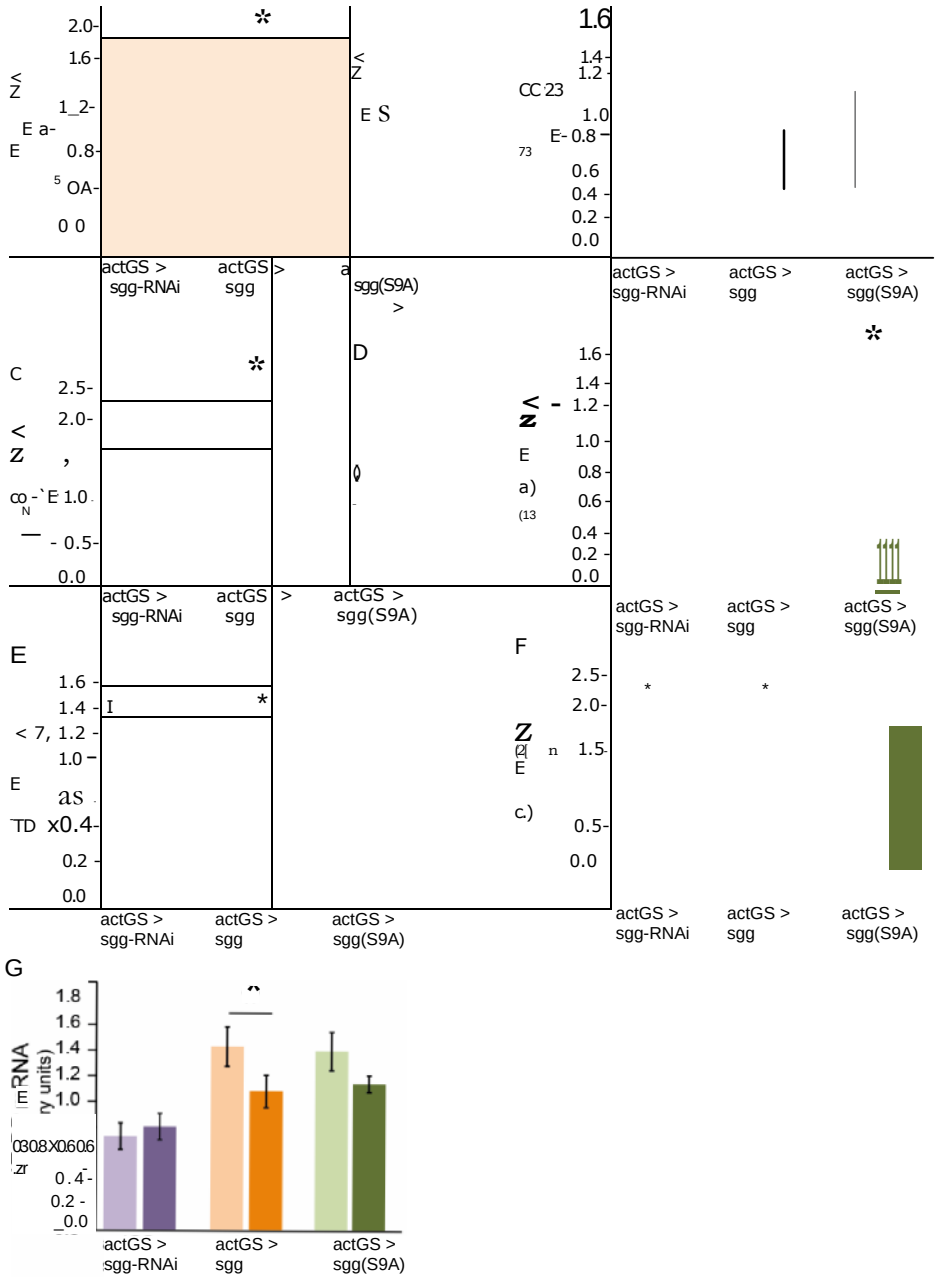


Figure 5.12 mRNA expression levels of gene targets of pathways reported to interact with GSK-3. Expression levels of several genes were quantified by RT-qPCR. Darker shade indicates induction of transgene. These genes are downstream of pathways known to be modulated by GSK-3, namely the hypoxia pathway (A and B), the armadillo/I3-catenin (C and D) pathway and the IIS pathway (E and G). For this experiments mRNA was quantified from quadruplicates of 10 whole flies. * $P < 0.05$. These experiments were performed in collaboration with Dr. Ivana Bjedov.

In summary, these results do not support a role for the hypoxia, armadillo or ITS pathways in the regulation of lifespan by *sgg* modulation.

5.4.7 Lithium partially rescued from the detrimental effects of shaggy over-expression

Next I examined the interaction of different *sgg* transgene lines and lithium for lifespan. First, I over-expressed wild type shaggy and again observed a —30% reduction in median lifespan (*P* < 0.01, log rank test). Interestingly, lithium treatment supplemented in the fly medium at 10 or 25 mM almost completely restored the detrimental lifespan effects of *sgg* over-expression (Figure 5.13A; *P* < 0.01, log rank test). I also assessed the interaction of lithium with the over-expression of the S9A mutant. Over-expression of the constitutively active S9A mutant significantly reduced lifespan by 50% and lithium almost completely rescued the survival detrimental effect (*P* < 0.001, log rank test; Figure 5.13B). These results indicate that lithium treatment can overcome the detrimental lifespan effects of over-expressing forms of *sgg* that increase tau phosphorylation.

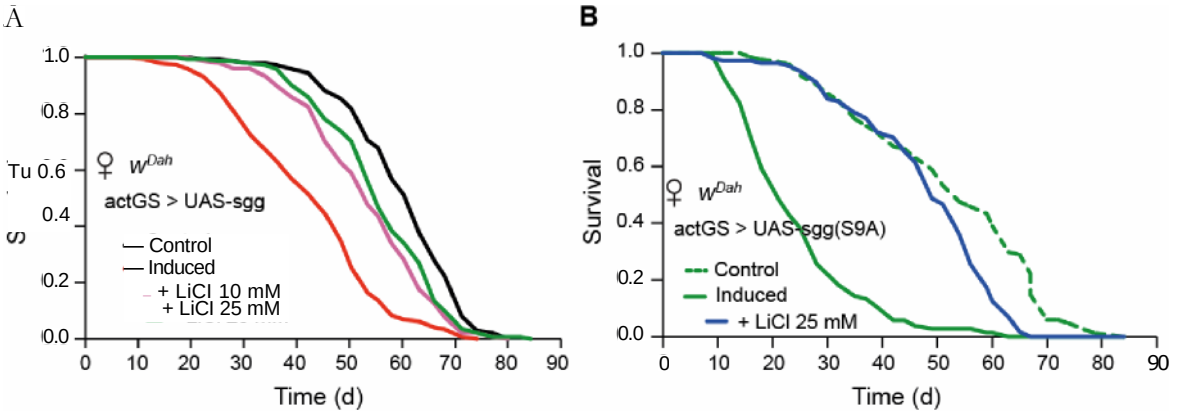


Figure 5.13 Lithium rescued from the lifespan shortening effect of *sgg* over-expression. (A) Lithium 10 or 25 mM rescued from the effects of wild type *sgg* over-expression. **(B)** Lithium almost completely rescued the detrimental effects from over-expressing the *sgg* S9A mutant. N= 150 female flies per condition. Experiment in B was performed in collaboration with Dr. Ivana Bjedov.

5.4.8 Lithium treatment and shaggy-RNAi were epistatic for lifespan

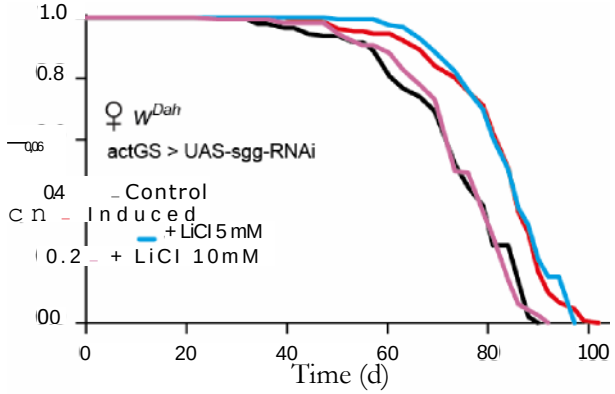


Figure 5.14 Lithium and RNAi against shaggy were epistatic for lifespan extension. Lithium is unable to further extend the lifespan extension conferred by RNAi against *sgg*. N = 150 flies per condition.

I then decided to perform a similar experiment when *sgg* was genetically down-regulated. I was interested to evaluate whether lithium treatment and genetic manipulation of *sgg* were additive or epistatic. Sgg-RNAi-mediated knockdown increased median lifespan by —14% (10 days) and maximum lifespan by —7% (6 days). When lithium was added in the fly medium at a dose of 5 mM, no further lifespan extension was observed (Figure 5.14; $P >$ 0.05, log rank test) as the survival curves of flies with down-regulated *sgg* treated ± 5 mM lithium overlapped. Importantly, doubling the dose of lithium (from 5 to 10 mM) restored the lifespan of the induced sgg-RNAi line to control levels (similar to increasing the concentration of lithium under maximised conditions for longevity; Figure 3.3).

To the best of my knowledge this is the first time an epistatic interaction between GSK-3 genetic down-regulation and lithium has been tested in *Drosophila.* The epistatic effect of the pharmacologic and genetic interventions suggests that inhibition of *sgg* and lithium treatment modulate lifespan by acting on the same pathway. The inhibitory effect of lithium is only partial as shown in Figure 5.7, as is the effect of sgg down-regulation (Figure 5.8), hence when combined a stronger inhibition is achieved. Strong inhibition of sgg obtained with high doses of lithium is detrimental for longevity (Figure 3.3). Hence, though 5 mM LiC1 and sgg-RNAi were epistatic, addition of 10 mM (that would otherwise still be able to extend lifespan) to a fly with optimal down-regulation of *sgg,* restored survival to control levels.

5.4.9 Lithium and myo-inositol were epistatic for lifespan

The inositol depletion hypothesis is an alternative to GSK-3 inhibition for the mechanism of lithium (Harwood, 2005). I therefore tested whether addition of myo-inositol would block the lifespan extension effect of lithium (Figure 5.15A). I observed that supplementation of my-inositol at 10 11M extended lifespan, while 100 ptM shortened lifespan (Figure 5.15B). To my surprise the combination of 1011M myo-inositol and lithium at either 0.5 mM (Figure 5.15C) or 1 mM (Figure 5.15E) were epistatic. However, lithium protected against the detrimental effect of 100 11M myo-inositol supplementation (Figure 5.15D and F). These results do not support that inositol depletion is a mechanism by which lithium promotes longevity, but lithium can protect against increased detrimental effects of myo-inositol.

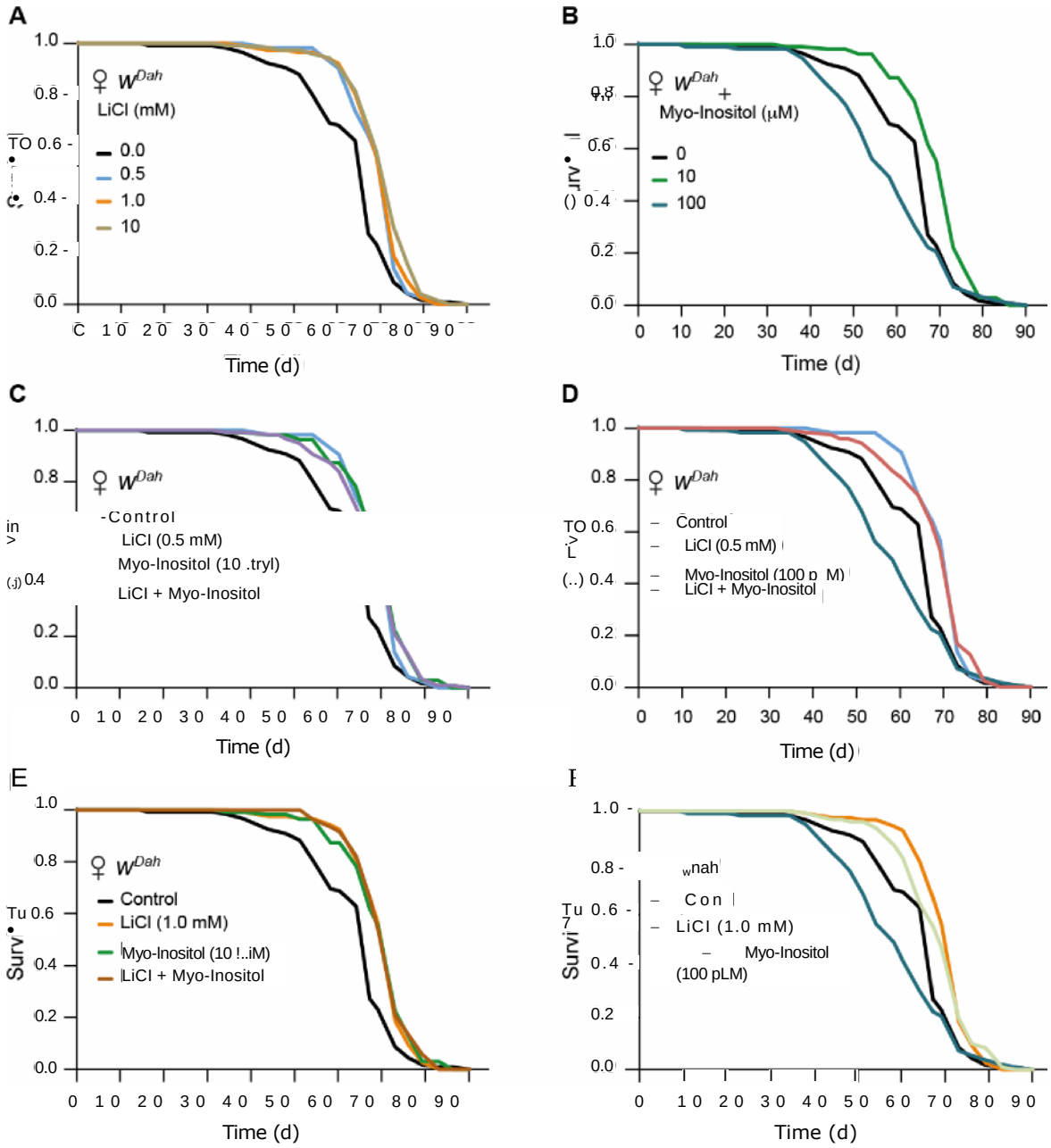


Figure 5.15 Lithium and myo-inositol treatment were epistatic for lifespan. (A) Lithium concentrations from 0.5 to 10 mM extended lifespan. (B). Supplementation of myo-inositol at a concentration of 10 RM extended lifespan, while 100 RM shortened lifespan. (C) Combination of lithium 0.5 mM and myo-inositol at 10 11M were epistatic for lifespan. **(D)** Lithium 0.5 mM can rescue from the detrimental effects of 100 RM myo-inositol. (E) 1 mM lithium and 10 11M myo-inositol were epistatic for lifespan. (F) Lithium 1 mM can rescue from the lifespan shortening of effect of 100 RM myo-inositol.

Activation of autophagy is the proposed mechanism by which inhibition of inositol recycling exerts its effects (Sarkar et al., 2005). I therefore analysed LiCI-induction of autophagy by measuring LC3-I/LC3-II (Atg8 in *Drosophila)* levels, but was not able to detect changes (Figure 5.16A). I indeed observed a trend towards lower levels of both bands yet this did not reach statistical significance *(P* > 0.05, ANOVA, post hoc Tukey Kramer). To further analyse the interaction of autophagy and lithium I treated

heterozygous null *atgl* flies (Lee et al., 2007; Scott et al., 2007) and detected that lithium was still able to extend their lifespan (Figure 5.16B; $P < 0.05$ log rank test). Together my results do not support a role of inositol depletion and autophagy activation as the mediators of the longevity effects of lithium.

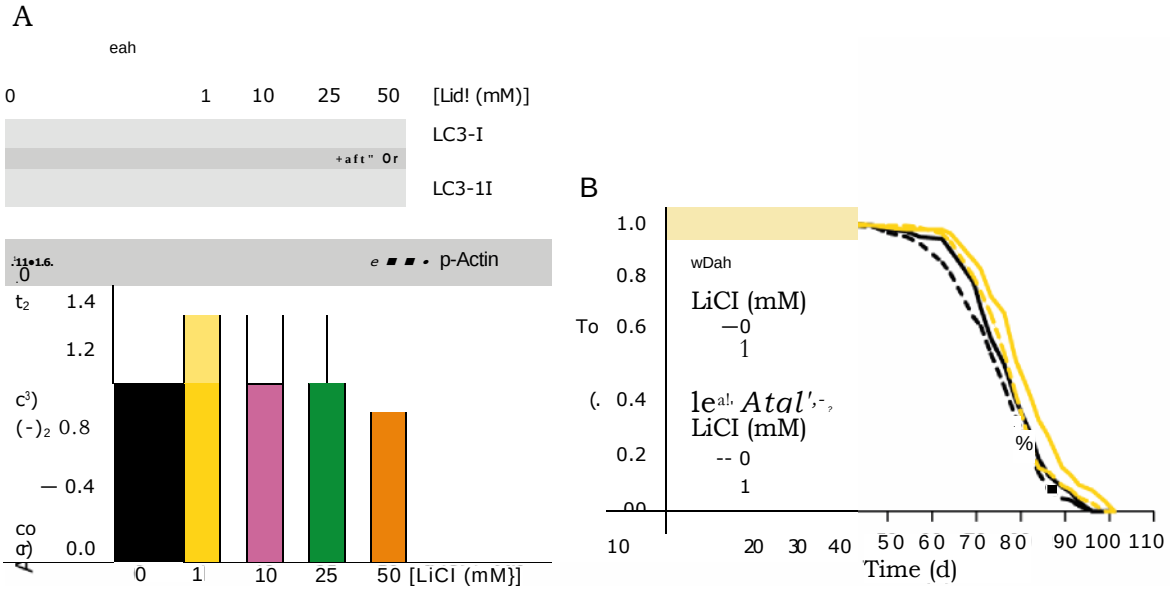


Figure 5.16 Lithium did not induce autophagy and extended lifespan of an autophagy-deficient mutant. (A) I measured the induction of autophagy through measurement of Atg8 (LC3) levels. There was a slight but non-significant reduction in the levels of atg8, not supporting a role for autophagy induction as a mechanism for lifespan extension. Bar represent average detected from triplicates of 10 flies each ± SEM. (B) Lithium extended lifespan of flies with heterozygous loss of atgl. N= 120 flies per condition.

5.4.10 IMPase-RNAi extended lifespan

Drosophila possesses approximately eight uncharacterised genes with predicted IMPase activity. To test whether genetic manipulation of IMPase modulates lifespan I took two of the genes and down-regulated them by RNAi. Interestingly, RNAi-mediated knockdown increased lifespan for both genes (Figure 5.17; $P < 0.01$ log rank test). Thus, IMPase positively regulates lifespan when down-regulated. However, I would require to perform the epistasis experiment to determine how lithium treatment and IMPase down-regulation interact to regulate lifespan.

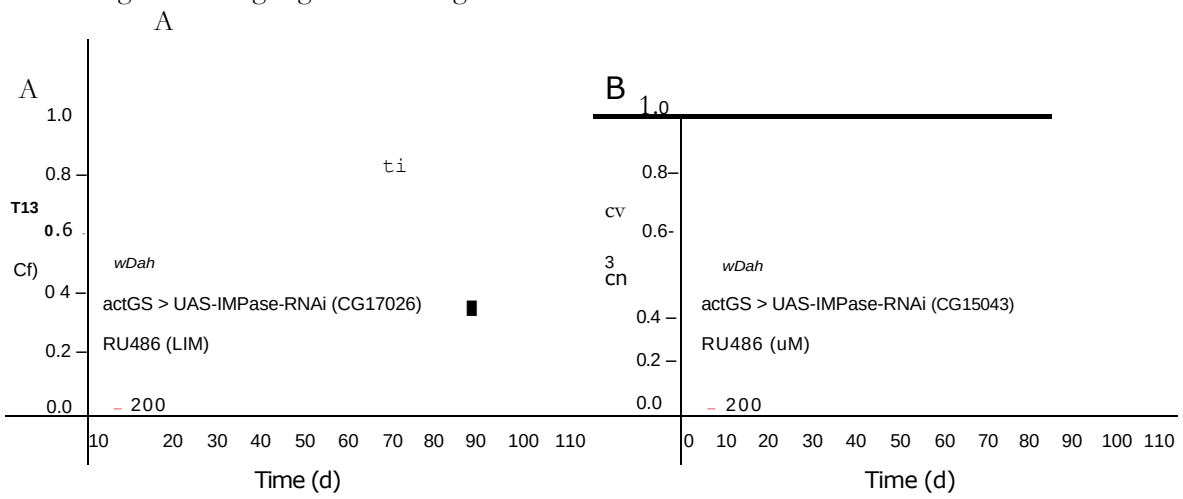


Figure 5.17 RNAi against two putative genes with IMPase activity extended lifespan. Survival was measured from two RNAi lines against two different genes with putative IMPase activity. Both showed lifespan extension. N= 150 flies per condition.

5.4.11 IIS or mTOR down-regulation rescued the deleterious lifespan effects of shaggy over-expression

GSK-3 activity or expression levels are increased in patients suffering from diabetes mellitus and AD (Henriksen and Dokken, 2006; Hooper et al., 2008). Both of these diseases are associated with IIS down-regulation (Boucher et al., 2014; De Felice, 2013). A genetic manipulation that has shown to be evolutionary conserved to extend healthy lifespan is the knockout of the *chico* or IRS-1 in *Drosophila* and mice, respectively (Clancy et al., 2001; Selman et al., 2011). GSK-3 is constitutively active and gets inhibited by insulin stimulation through phosphorylation of its serine-9 by Akt (Doble and Woodgett, 2003; White, 2003). Given that sgg/GSK-3 is downstream of chico/IRS-1 (Figure 5.18A), I speculated that the long-lived *chico*^{i} mutants had either increased expression levels or increased activity (lower phosphorylation of S9) of GSK-3. I analysed the activity and expression levels of *sgg* by immunoblot analyses. To my surprise, I did not identify alterations in expression or activity of *sgg* as measured by total-GSK-3 antibody or for activity levels as measured by the inhibitory phosphorylation site (S9) (Figure 5.18B; $P > 0.05$ ANOVA post-hoc Tukey Kramer). I found interesting that neither GSK-3 activity or levels were differentially regulated under a manipulation upstream of GSK-3. However, others in our group have encountered similar difficulties reproducing *in vitro* data, and only uncovered phosphorylation differences when specific tissues are challenged. For example, loss of CHICO, upstream of Akt, seems to have minimal effects on Akt phosphorylation when the tissue examined is not stimulated with insulin *ex vivo* (Ikeya et al., 2009; and unpublished results from the lab). With this in mind, it would be appropriate

to repeat these experiments with insulin stimulation *ex vivo* to assess whether loss of CHICO has effects on sgg/GSK-3 phosphorylation.

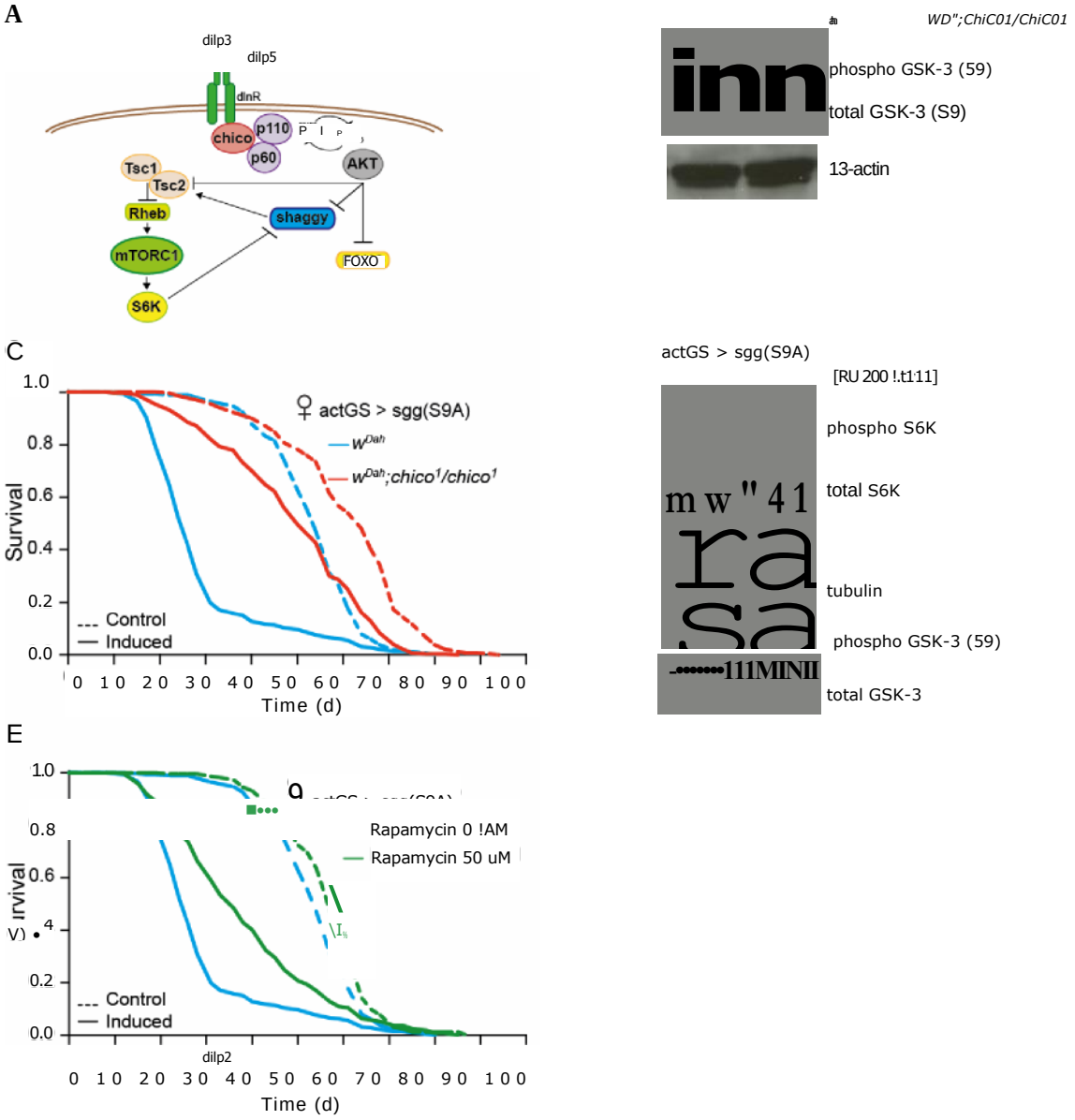


Figure 5.18 Interaction of shaggy and the nutrient sensing network IIS and mTOR. (A) Schematic representation of IIS and mTOR and the role of *sgg* at the centre of the network. (B) Immunoblot analyses for the levels of phospho-GSK-3 and total GSK-3 in *chico* null flies. (C) Survival effects of over-expressing the S9A mutant in flies lacking CHICO. N = 180 flies per condition. (D) mTORC1 activity was analysed in flies over-expressing the S9A mutation. (E) I supplemented flies over-expressing the *sgg* S9A mutant with 50 rapamycin and evaluated the survival effects. Experiments in C and E were performed in collaboration with Dr. Ivana Bjedov.

Next I sought to determine whether IIS down-regulation by homozygous loss of *chico* would be protective against *sgg* over-expression. I therefore used similar

experimental conditions to over-express the S9A *sgg* mutant in a *chico* null background (Figure 5.18C). The induction of *sgg* S9A in the control background reduced median lifespan from 53 to 25 days, without changing the maximum lifespan of 62.5 days in the un-induced controls *(P* < 0.001, log-rank test). In agreement with published data, loss of *chico* extended median lifespan by 9 days (-14%) and maximum lifespan by 16.5 days (-21%) *(P* < 0.001, log-rank test) (Clancy et al., 2001; Yamamoto and Tatar, 2011). Loss of *chico* almost completely rescued the deleterious effect of the *sgg* S9A mutation (Figure 5.18C). When compared to the induction of the S9A mutation in the control background, the loss of *chico* extend median lifespan by 52% (from 25 to 48.5 days; *P* < 0.001, log-rank test).

Taken together these results suggest that although GSK-3 is downstream of *chico* (or the dilps), the activity or expression levels are not changed when IIS is down-regulated, probably secondary to compensatory mechanisms. This has previously been reported for Akt phosphorylation downstream of CHICO (Ikeya et al., 2009). These unknown compensatory mechanisms might allow for the fly to survive longer than controls. Flies over-expressing an active version of *sgg* were not long-lived, suggesting that alterations in GSK-3 (as in mammals) are detrimental in light of IIS down-regulation. Moreover, IIS down-regulation by loss of the only IRS in the fly rescued the survival of flies over-expressing the *sgg* S9A mutant.

I previously showed that lithium, an inhibitor of GSK-3, rescued the deleterious effects of wild type *sgg* or the *sgg* S9A mutant. Lithium did not modify the activity of mTOR (Figure 3.11). However, it has been shown in mammalian cells that GSK-30 (carrying the S9A mutation) directly phosphorylates and activates the tuberous sclerosis complex-2 (TSC2), which leads to inhibition of mTOR. The S9A mutant therefore reduces the phosphorylation of S6 kinase (S6K) *in vitro* (Inoki et al., 2006). In turn, S6K is a negative regulator of GSK-3 (Frame and Cohen, 2001). Rapamycin, an mTOR inhibitor, extends lifespan in evolutionary distant organism ranging from yeast to mice (Johnson et al., 2013). A previous report from our group showed that flies fed rapamycin have reduced levels of S6K phosphorylation, but do not show changes in the expression or activity of *sgg* (Bjedov et al., 2010). I aimed to determine whether the converse is true, i.e., if increased GSK-3 alters the phosphorylation of S6K, downstream of mTOR. I did not identify changes in S6K phosphorylation (Figure *5.18D; P* > 0.05 ANOVA post hoc Tukey Kramer).

I then decided to evaluate whether rapamycin treatment to inhibit mTOR could also rescue the lifespan shortening effect of the S9A mutation (Figure 5.18E). I treated control flies with fly medium containing 50 ptM rapamycin and observed a significant lifespan extension (Figure 5.18E; $P < 0.001$, log-rank test), comparable to published data (Bjedov et al., 2010). When rapamycin was supplemented to flies over-expressing the *sgg* S9A mutation their median and maximum lifespan was significantly extended by —28% and —8%, respectively *(*$P < 0.001$, log-rank test).

My results showed that down-regulation of the nutrient-sensing network (IIS and mTOR) can protect against GSK-3 over-expression, potentially opening an avenue for these interventions in diseases where GSK-3 is up-regulated.

5.4.12 Genetic manipulation of shaggy did not modulate age-related locomotor decline

More than often short-lived mutants show organismal signs of illness, like reduced fecundity, acceleration of age-related anorexia and/or locomotor decline. I aimed to characterise these phenotypes in flies over-expressing wild type *sgg*, the S9A mutation or down-regulation of *sgg* by RNAi to possibly determine toxicity linked to specific tissues.

First I evaluated the organismal effect of modulating *sgg* for age-related locomotor decline using the negative geotaxis paradigm (Gargano et al., 2005; Jones et al., 2009). I first documented that although *sgg* down-regulation extended lifespan (Figure 5.11), it did not protect against locomotor decline (Figure 5.19A). Moreover, over-expression of either the wild type *sgg* or the S9A *sgg* mutant did not show a significant acceleration of age-related locomotor ability before onset of death in comparison to their respective controls (Figure 5.19B and C). A significant reduction on climbing ability *(*$P < 0.01$, t-test) was only detected after 25% of the induced cohort had died in the case of over-expressing wild type *sgg* (Figure 5.19B), and only shortly before the induced cohort expressing the S9A *sgg* mutant reached median lifespan (Figure 5.19C).

These results were surprising given that flies over-expressing either *sgg* or the S9A mutant showed a significant decline in survival early on during the experiment. Thus, my results did not support a role for *sgg* in the regulation of age-related locomotor decline.

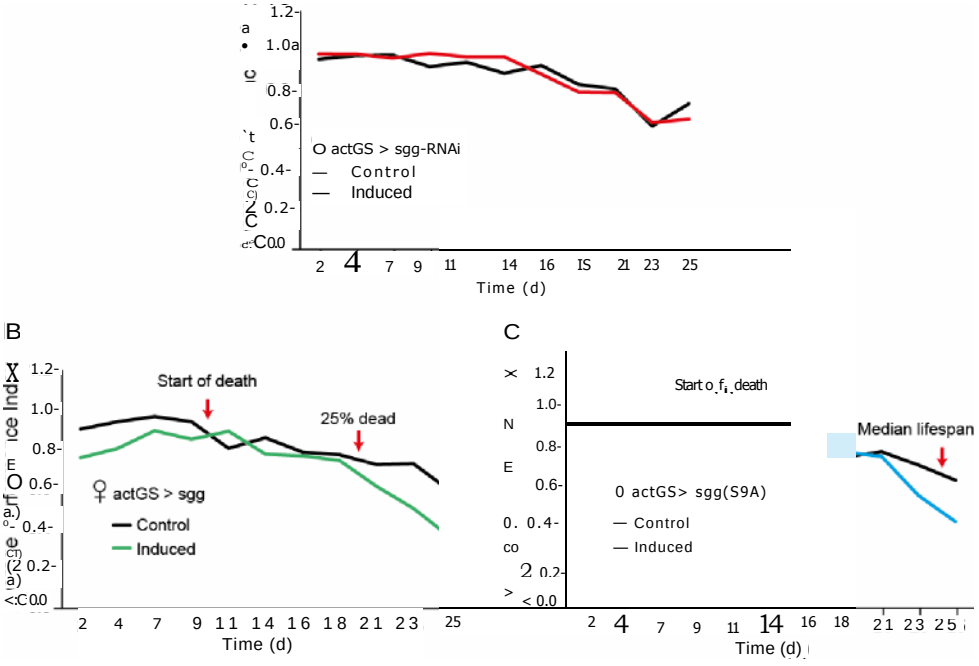


Figure 5.19 Modulation of shaggy levels did not impact age-related locomotor decline. The locomotor ability of flies expressing an RNAi against *sgg* (A), wild type *sgg* **(B)** and the S9A *sgg* mutant. The over-expression of *sgg* **(B** and C) only accelerated locomotor decline once flies were on the steep slope of the survival curve. These experiments were performed in collaboration with Ms. Li Li.

5.4.13 Over-expression of shaggy did not alter feeding behaviour or fecundity

Given the rather surprising result that over-expression of *sgg* did not accelerate age-related locomotor decline in the face of very short lifespans, I aimed to determine whether other phenotypes of generalized health would change when *sgg* is over-expressed.

I first characterized the feeding behaviour of flies over-expressing either wild type or the S9 mutant. Flies show a characteristic decline in feeding behaviour with age (age-related anorexia) (Wong et al., 2009). To determine this behaviour flies are observed during a fixed period of time and the number of flies with their proboscis on the food are counted. As this phenotype changes over time I assessed this phenotype at three different time points, 1, 5 and 15 days after the induction of the transgene. To my surprise feeding frequency did not consistently change in either flies over-expressing wild type sgg (Figure 5.20A) or the S9A mutant (Figure 5.20B). At day 5 flies over-expressing wild type *sgg* showed a significant reduction in feeding behaviour ($P < 0.01$, t-test), but this was restored by day 15. Overall, these results did not indicate that flies over-expressing sgg transgenes with increased kinase activity show age-related anorexia.

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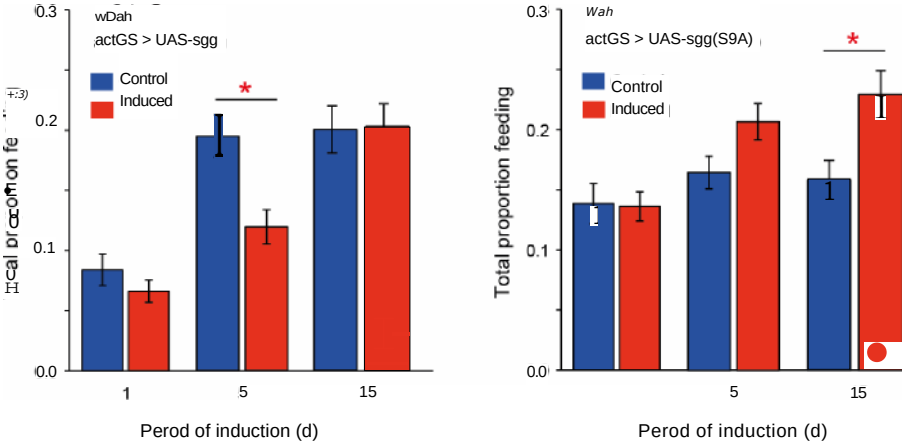


Figure 5.20 Feeding behaviour in flies over-expressing shaggy. Feeding behaviour was analysed by assessing the frequency of proboscis extension in flies expressing wild type *sgg* (A) and the S9A mutant **(B).** * $P < 0.05$. N= 50 flies per condition. These experiments were performed in collaboration with Mr. Michael Shannack.

Next I examined the role of sgg on fecundity. I measured the number of eggs laid per female in 24 hours after 5 days of induction. While there was a slight trend towards reduced fecundity in flies over-expressing either the wild type sgg (Figure 5.21A) or the S9A mutant (Figure 5.21B) after 10 days of induction, the results were not statistically significant ($P > 0.05$, t-test).

These results suggest that over-expression of GSK-3 did not affect general parameters of health as it did not change the locomotor ability, feeding behavior or fecundity of flies even when it shortened lifespan dramatically. I therefore considered the possibility that *sgg* regulated lifespan by influencing specific tissues, or even having tissue specific effects in opposite directions.

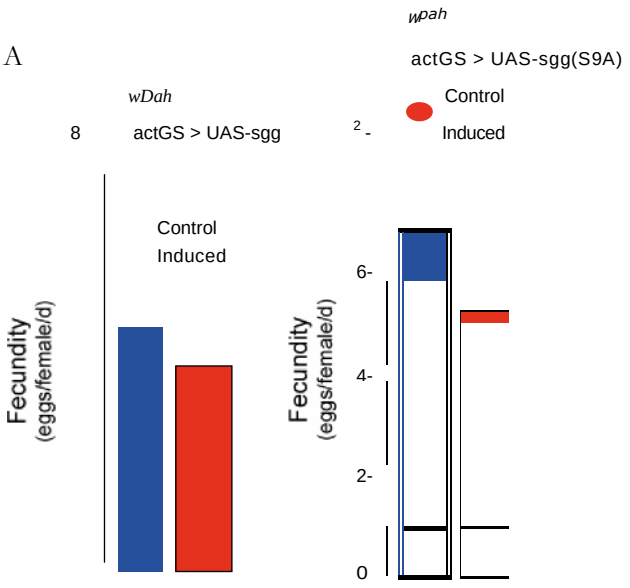


Figure 5.21 Fecundity in short-lived flies over-expressing shaggy. Short-lived flies over-expressing wild type *sgg* (A) or the S9A mutation **(B)** do not show alteration in fecundity. N= 70 flies per condition. These experiments were performed in collaboration with Mr. Michael Shannack.

5.4.14 Over-expression of shaggy in neurons, but not in gut/fat body extended lifespan

I was interested to evaluate the possibility of *sgg* over-expression having different effects in different tissues. Two fly tissues previously involved in the regulation of ageing are the gut/fat body and neurons (Alic et al., 2014b; Hwangbo et al., 2004). Ubiquitous down-regulation of IIS by genetic loss of different components of the IIS pathway extends lifespan in *Drosophila* (Kannan and Fridell, 2013; Partridge et al., 2011; Tatar et al., 2014). This lifespan extension is dependent of the transcription factor *dFOXO* (Slack et al., 2011; Yamamoto and Tatar, 2011), yet over-expression of *dFOXO* has only been reported to extend lifespan in the gut/fat body and neurons (Alic et al., 2014b; Giannakou et al., 2004; Hwangbo et al., 2004). First I decided to explore the possibility that *sgg* over-expression could have milder negative lifespan effects or even be beneficial, when driven in the gut/fat body in comparison to ubiquitous over-expression. I used the Si106 driver to only express *sgg* during adulthood (from day-2 post-eclosion). I over-expressed wild type *sgg* in the gut and fat body using two RU concentrations 50 and 20011M without observing significant differences between the induced and uninduced survival curves (Figure 5.22A; $P > 0.05$, log rank test). When the S9A *sgg* mutant was over-expressed in the gut and fat body (Figure 5.22B), the 50 11M RU induction reduced median lifespan by 4.5 days (from 48 days in the un-induced control to 43.5; $P < 0.01$, log rank test) and maximum lifespan by —2 days (from 62 to 59.7 days). However, the induction with 200 11M RU was not significantly different from the un-induced control ($P > 0.05$, log rank test). I also over-expressed the S9E mutant and did not observe any effect on median or maximum lifespan in either of the RU concentrations used (Figure 5.22C; $P > 0.05$, log rank test).

Collectively these results suggest that the deleterious effects of ubiquitous *sgg* over-expression on lifespan are unlikely mediated by the gut and/or fat body.

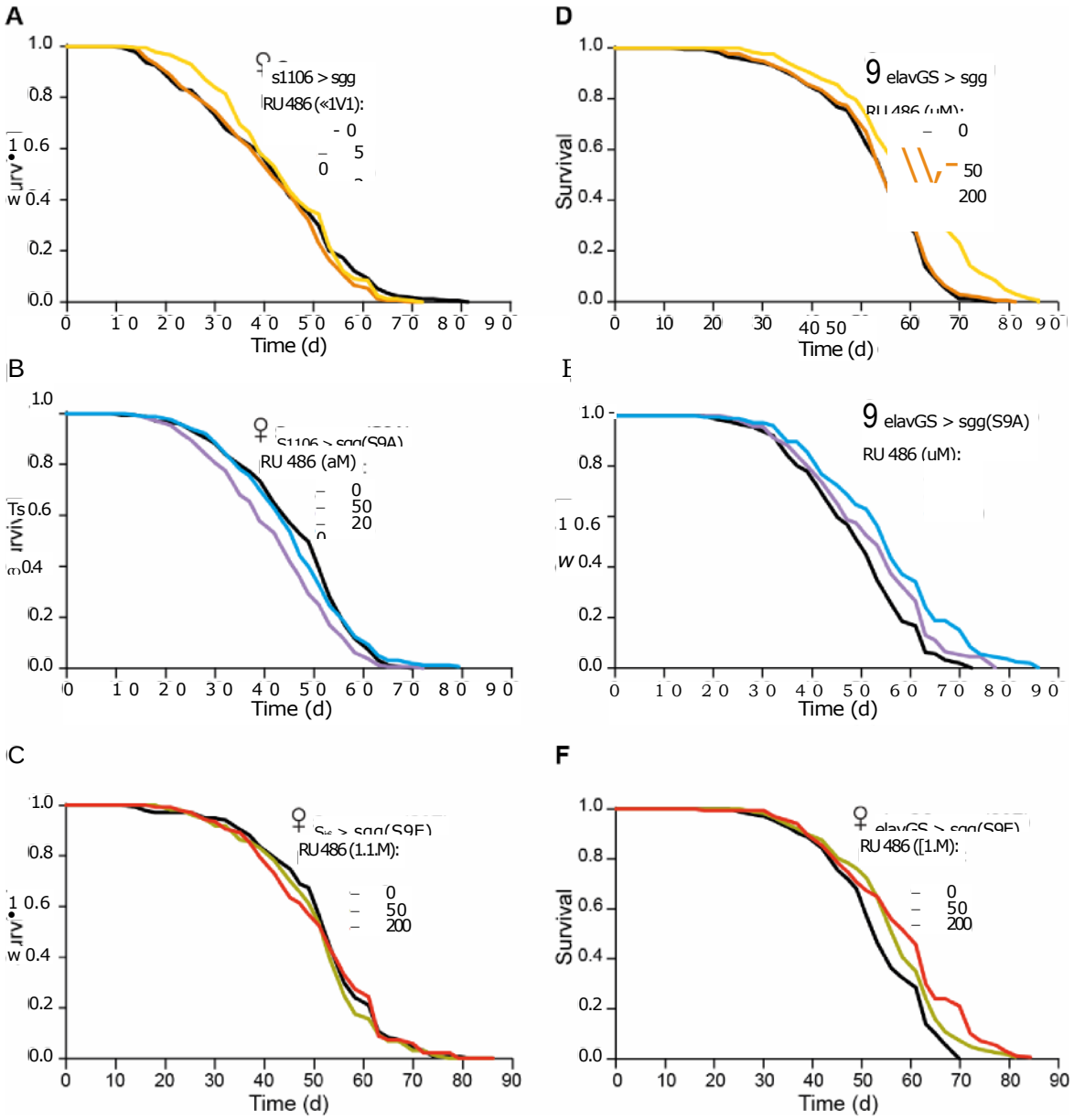


Figure 5.22 Survival of flies over-expressing shaggy transgenes with increased kinase activity in gut/fat body and neurons. Survival effect of the wild type *sgg*, the S9A and S9E mutants was assessed in the gut/fat body (A-C, respectively) or neurons (D-F, respectively). N= 180 females per condition. Experiments were performed in collaboration with Dr. Ivana Bjedov.

I then decided to test the effect of over-expressing ***sgg*** in neurons. I used the pan-neuronal elavGS driver and the same two RU concentrations used to over-express in the gut and fat body. When wild type ***sgg*** was over-expressed only in neurons it extended median lifespan by 5 days (-9%) and maximum lifespan by 9 days (-12.5%), but only when induced with 200 11M RU (Figure 5.22D; $P < 0.001$, log-rank test). No effect was detected when 50 11M RU was supplemented in the fly medium to over-express wild type ***sgg*** ($P > 0.05$, log rank test). In contrast, 50 11M RU was sufficient to significantly extend median (from 49.9 to 52.3 days) and maximum (from 62 to 68.4 days) lifespan when the

S9A mutation was over-expressed pan-neuronally (Figure 5.22B; $P < 0.001$, log-rank test). Induction of the S9A mutant further extended lifespan when 200 11M of the inducer was used. At this concentration the S9A mutant extended median lifespan by —5 days, and maximum by 9 days ($P < 0.001$, log-rank test). When the survival curves of the two RU concentrations were statistically analysed a significant difference was detected ($P = 0.0109$, log-rank test), which suggested a dose-dependent induction effect. I observed a similar effect to the S9A over-expression when the S9E mutant was over-expressed in all neurons (Figure 5.22F). 50 11M RU was sufficient to extend median (by 5 days) and maximum (by 7 days) lifespan ($P < 0.001$, log-rank test). Induction of the S9E mutant with 200 11M RU further extended median (by 7.5 days) and maximum (by 11.6 days) lifespan ($P < 0.001$, log-rank test). A comparison between the survival curves obtained at the two different concentrations of RU also resulted significant ($P = 0.0122$, log-rank test), once more suggesting a dose-dependent effect.

My results showed that *sgg* over-expression could have tissue-specific effects to regulate lifespan. While ubiquitous over-expression of *sgg* or *sgg* mutants reduced lifespan, over-expression in neurons extended lifespan.

5.4.15 Over-expression of shaggy in neurons protected against age-related locomotor decline

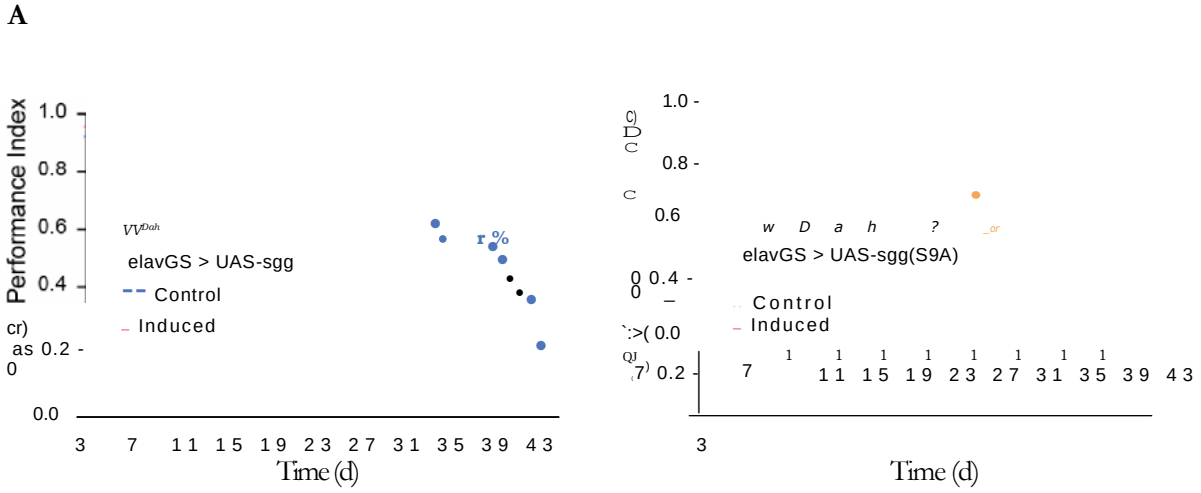


Figure 5.23 Climbing ability of flies over-expressing shaggy in neurons. Age-related locomotor decline was assessed in flies over-expressing the wild type *sgg* (A) or the S9A mutant **(B)** in neurons. N= 60 flies per condition. These experiments were performed in collaboration with Mr. Michael Shannack

I previously showed that over-expression of *sgg* mutants with increased kinase activity did not have effects in the modulation of age-related locomotor decline, in spite of shortening lifespan by almost half (Figure 5.19). As I observed that over-expression of *sgg* in neurons

extended lifespan I wondered whether this genetic manipulation would confer protection against age-related locomotor decline. Over-expression of wild type *sgg* pan-neuronally significantly protected against age-related locomotor decline (Figure 5.23A; $P < 0.001$, two-way ANOVA, post hoc Tukey Kramer). Similarly, over-expression of the S9A mutant significantly improved the locomotor ability of flies during ageing (Figure 5.23B; $P < 0.05$, two-way ANOVA, post hoc Tukey Kramer). In contrast to the over-expression of wild type *sgg*, the S9A mutant did not protect as much, though this effect was rather significant. Thus, over-expression of either the wild type *sgg* of the S9A mutant protected against age-related locomotor decline.

5.4.16 Lithium treatment and neuronal over-expression of shaggy were additive for lifespan

I previously showed that *sgg* down-regulation is epistatic with lithium treatment, suggesting that both interventions modulate ageing by acting on the same pathway (Figure 5.14). Given that over-expression of *sgg* in neurons extended lifespan, I was interested to test whether this genetic manipulation would block the lifespan extension conferred by lithium, or would be additive.

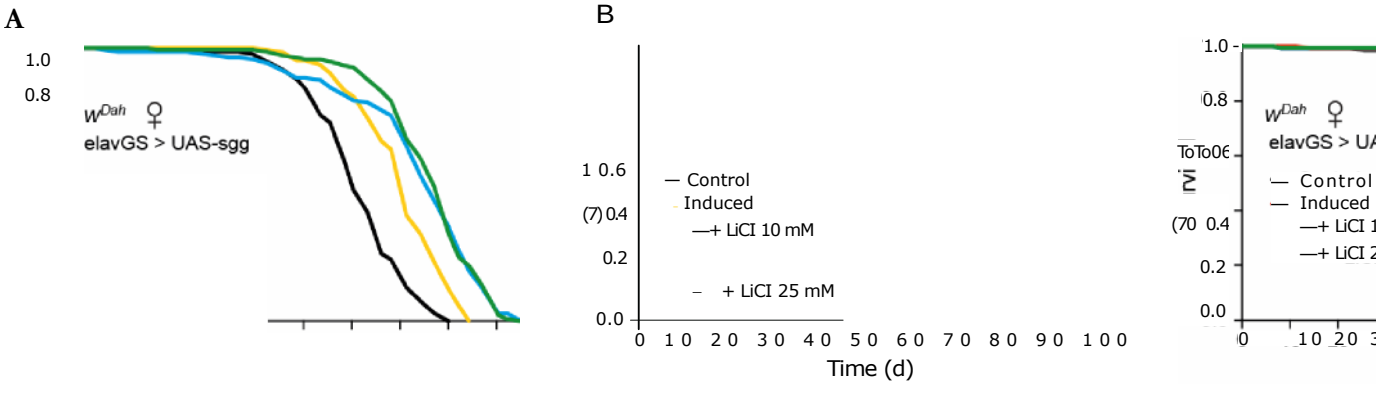


Figure 5.24 Survival analyses of flies over-expressing shaggy in neurons and lithium treatment. I analysed the interaction of over-expressing the wild type (A) or the *sgg* S9A mutant (B) only in neurons and lithium treatment. N= 160 flies per condition.

I over-expressed wild type *sgg* or the S9A mutant in the neuronal tissue (using the elavGS driver), fed them lithium at two concentrations (10 and 25 mM) and evaluated their survival. Over-expression of wild type *sgg* significantly extended median (by 10 days or —15%) and maximum (5.6 days or —7%) lifespan (Figure 5.24A; $P < 0.001$, log-rank test). Lithium supplementation with either dose further extended median lifespan by 6 days and

maximum by 7.5 days *(P* < 0.001, log-rank test). The survival curves of the two lithium concentrations were not statistically different *(P* > 0.05, log rank test). The combination of wild type *sgg* over-expression in neurons and lithium treatment extend median lifespan by 16.2 days (-21.5% from 59.4 days in the un-induced control to 75.6 days in the induced + either lithium treatment) and maximum lifespan by 13.1 days (-15% from 75.6 days in the un-induced control to 88.7 days in the induced + either lithium treatment). I also tested the effect of combining neuronal over-expression of the S9A *sgg* mutant with lithium (Figure 5.24B). The induction of the S9A mutant in neurons significantly extended median lifespan by 11 days (or 15%) and maximum lifespan by 8.4 days (or 10%) *(P* < 0.001, log-rank test). Addition of 10 mM lithium to the fly medium further extended median (by 8.4 days) and maximum (12.5 days) lifespan *(P* < 0.001, log-rank test). The combination of over-expressing the S9A mutant in neurons and supplementation of 10 mM lithium extended median lifespan by 19.4 day or —24% (from 61.8 days in the un-induced control to 81.2 days in the induced + 10 mM lithium) and maximum lifespan by 20.9 days or —22% (from 72.8 days in the un-induced control to 93.7 days in the induced + 10 mM lithium. Supplementation of 25 mM LiC1 to the S9A neuronally over-expressing flies was also additive though the combination was slightly less beneficial, yet the comparison of the two survival curves (+10 mM vs. +25 mM LiC1) was not statistically significant *(P* = 0.0734, log-rank test). The combination of S9A over-expression in neurons and 25 mM lithium extended median lifespan by 22.75% and maximum lifespan by —20% *(P* < 0.001, log-rank test).

Although the effect of over-expressing sgg/GSK-3 in neurons while in parallel inhibiting the kinase activity by lithium supplementation seems counterintuitive, I consider that the key to understand this additive lifespan extension lies in the degree of GSK-3 inhibition achieved by lithium supplementation. I previously showed that lithium dose-dependently increases the inhibitory phosphorylation of GSK-3 (Figure 5.7). However, the degree of inhibition was not maximised by the concentrations of lithium used in the epistasis experiments (Figure 5.24), hence the dose of lithium provided was not enough to inhibit the activity of GSK-3 when over-expressed. This hypothesis should be further explored through combination of western blot analyses comparing GSK-3 and htau phosphorylation in brains (where GSK-3 is over-expressed) to that in the remaining body (where only endogenous sgg/GSK-3 is expressed).

In conclusion over-expression of *sgg* in *Drosophila* neurons is beneficial for lifespan and the combination with lithium treatment can further extend lifespan. These results

suggest that, at least in neurons, lithium and GSK-3 independently control the rate of ageing.

5.4.17 Over-expression of a kinase dead shaggy did not affect lifespan when expressed ubiquitously or in neurons.

My previous result can lead to the interpretation that while lithium inhibits the kinase activity of shaggy this kinase activity is not required for lifespan extension in neurons, as the combination of over-expressing *sgg* in neurons and lithium treatment were additive for lifespan. Thus, offering a conflicting interpretation as to the role of the kinase activity of *sgg* for the regulation of lifespan. I therefore decided to evaluate the role of the kinase dead (KD) mutant that when over-expressed together with hTAU, did not modify tau phosphorylation levels. I ubiquitously over-expressed the KD mutant *sgg* by using the actGS driver (Figure 5.25A), and pan-neuronally by using the elavGS driver (Figure 5.25B). As expected I was unable to obtain a lifespan effect with either intervention $P > 0.05$, log-rank test).

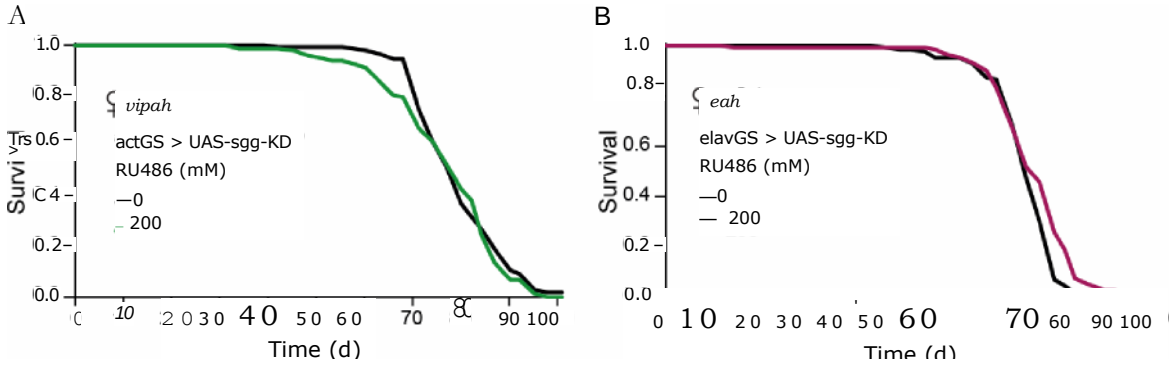


Figure 5.25 Survival analyses of flies expressing a kinase dead mutant of shaggy. Expression of a kinase dead mutant did not modify lifespan when expressed ubiquitously (A) or in neurons (B). N = 150 flies per condition.

Taken together these results suggest that the kinase activity of *sgg* over-expression is required to shorten lifespan when expressed ubiquitously, and to extend lifespan when expressed just in neurons. Hence, additional interactions (perhaps in different tissues) should be acting in concert to allow the additive effect of lithium treatment when the kinase activity of *sgg* is increased in neurons.

5.4.18 Lithium and shaggy over-expression additively extended lifespan of flies expressing AP1_42

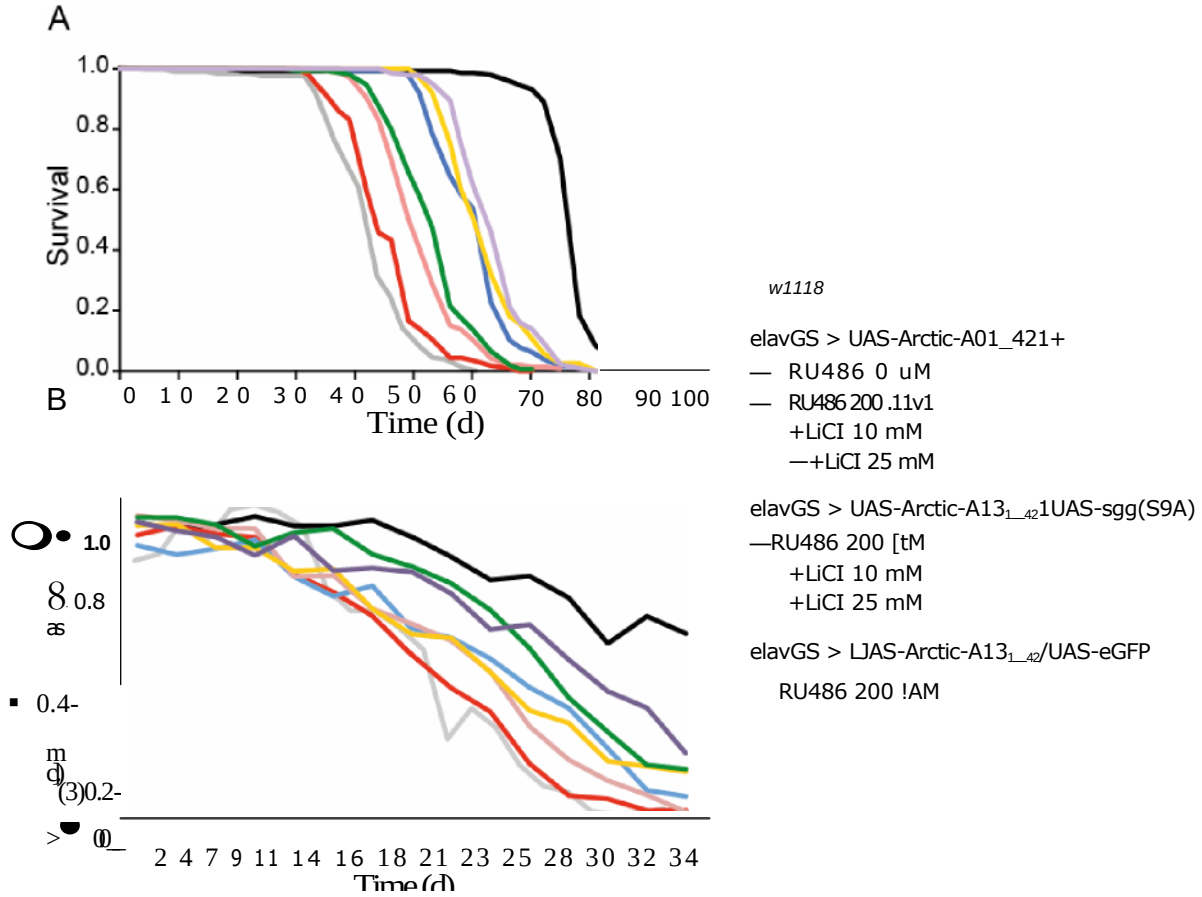


Figure 5.26 Over-expression of the S9A mutant in flies expressing 4_{1_42} improves lifespan and climbing and is additive to lithium treatment. (A) Survival analyses. N= 150 flies per condition. **(B)** Negative geotaxis. N = 60 flies per condition. Experiment performed in collaboration with Ms. Li Li.

Our group recently characterised a late-onset model of AD in flies by using the GeneSwitch system and the over-expression of Af31_42 with the pro-aggregation Arctic mutation (Sofola et al., 2010). Flies expressing Arctic-Af31_42 have a significantly reduced lifespan, accelerated age-related locomotor decline and synaptic defects (Rogers et al., 2012; Sofola et al., 2010). Previous results from our group have shown that lithium treatment can improve the locomotor ability (Sofola et al., 2010) and lifespan (Sofola-Adesakin et al., 2014) of these flies. Interestingly, over-expression of the S9E *sgg* mutant also improved the locomotor ability and lifespan of flies expressing Arctic-Af31_42 (Sofola et al., 2010). The S9E was used as dominant negative *sgg* mutant, hence the interpretation of these results was that inhibition of pan-neuronal *sgg* and lithium have similar effects. However, their interaction was not tested. Given that I have shown in multiple assays that

over-expression of wild-type *sgg*, the S9E and S9A mutants behave similarly and that the S9E increases tau phosphorylation (Figure 5.8), I do not consider that the S9E mutant acts as a dominant negative. To eliminate conflicting interpretation I did not use the S9E mutant, but used the S9A mutant and combined the genetic manipulation with lithium treatment.

As previously shown, lithium treatment at either 10 or 25 mM extended the lifespan of flies expressing $Af3_{1\_42}$ (Figure 5.26A; $P < 0.001$, log-rank test). Over-expression of sgg(S9A) in flies expressing A131_42 significantly extended lifespan (Figure 5.26A; $P < 0.001$, log-rank test). To control for dilution of Ga14 I also co-expressed Af31_42 in the presence of GFP only detecting a mild but significant ($P < 0.05$, log-rank test) lifespan shortening effect. Moreover, addition of either 10 or 25 mM lithium further extended lifespan of flies over-expressing the S9A mutant *sgg* and Af31_42 in neurons (Figure 5.26A).

Taken together our results indicate that lithium and *sgg* act in an additive manner to extend lifespan in flies expressing A01-42.

5.4.19 Lithium and shaggy over-expression additively improved locomotor function of flies expressing AP1_42

Next I analysed whether the additive effects of lithium and *sgg* over-expression also impacted locomotor activity. I over-expressed the S9A *sgg* mutant in flies expressing $A131\_42$ in neurons and observed that indeed over-expression of sgg(S9A) significantly improved the locomotor ability of flies expressing $AP1_{\_42}$ ($P < 0.01$, two-way ANOVA; Figure 5.26B). Once more, co-expression of AP1_42 with GFP was performed ruling out any confounding effect of Ga14 dilution ($P > 0.05$, two-way ANOVA). When lithium was supplemented in the media at either 10 or 25 mM, there was a further protection to that conferred by the S9A mutation on its own ($P < 0.05$, two-way ANOVA; Figure 5.26B). Thus, the effects of over-expressing *sgg* and lithium were additive to improve the altered locomotor ability of flies expressing Af31_42.

5.5 Discussion

5.5.1 Shaggy/GSK-3 regulates ageing in *Drosophila*

The first interesting result was that modulation of the kinase activity of shaggy is achievable with different transgenes and mutants. I was able to confirm that down-regulation of shaggy by RNAi reduces the phosphorylation of human tau at two phospho-sites known to be phosphorylated by GSK-3 (Querfurth and LaFerla, 2010). I was also able to show that the overexpression of wild type shaggy increases the phosphorylation of tau. This is in agreement with a previous report that demonstrated that wild-type sgg/GSK3 is able to increase the phosphorylation of human tau (Chatterjee et al., 2009). Unexpectedly, I consistently observed that the dominant negative GSK-3 mutant S9E hyperphosphorylates tau. The S9E mutant carries a single point mutation in the highly regulated serine 9. Serine 9 phosphorylation inactivates the enzyme. In the S9E mutants, the serine at position 9 has been changed to a glutamic acid (hence the S9E) that should act as a phosphomic not allowing activation of the kinase activity. However, I have consistently seen that the S9E behaves in a similar fashion to that seen when wild type shaggy is over-expressed in the fly, or when the constitutively active shaggy mutant is expressed. The constitutively active mutant S9A carries a substitution of serine for alanine at position 9. This change should prevent phosphorylation at this site and therefore should render the protein enzymatically active. The discrepancy between the theoretical function of S9E (considered a dominant negative mutant) and its kinase activity has been documented with mammalian GSK-3. Eldar-Finkelman et al showed in an *in vitro* kinase assay that the S9E and S9A mutants were able to either autophosphorylate or to phosphorylate inhibitor 2 to a similar extent (Eldar-Finkelman et al., 1996). This is, therefore, in agreement with the results of my *in vivo* kinase activity experiments. Since the S9E and S9A mutants behave similarly and also phenocopy the over-expression of wild type shaggy, it is very unlikely that the S9E is a real dominant negative mutant fly line and it should be considered instead as an active mutant.

My observation that three of the transgenic lines (S9E, S9A and wild type *sgg)* were able to hyperphosphorylate tau when expressed ubiquitously in the adult fly highly suggests that they would produce similar effects in the lifespan of flies. Indeed both of the shaggy mutant transgenes (S9E and S9A) and the wild type *sgg* transgene dramatically reduced lifespan when they were ubiquitously expressed. Furthermore, the lifespan reductions seen in flies expressing either the S9E or S9A mutant transgenes lifespan were

of similar magnitude, suggesting that these mutants have comparable enzymatic activities. Moreover, the effects seen with these mutants were greater than that produced by the over-expression of wild type *sgg*.

The fmding that shaggy mutants are able to hyperphosphorylate tau (and hence have increased kinase activity) in association with a reduction in lifespan is novel and has not been previously reported. Hyperactivity of GSK-3 has been found in several diseases such as diabetes mellitus, AD, HD, and schizophrenia among others (Cohen and Goedert, 2004; Jope and Johnson, 2004; Lovestone et al., 2007; Hooper et al., 2008). As several of these diseases are age-related, it is not entirely surprising that hyperactivation of *sgg* is involved in a pro-ageing phenotype. However, to the best of my knowledge, this is the first time that constitutively active *sgg* mutants have shown to lead to lifespan shortening when expressed throughout the fly.

Conversely, and in agreement with the *in vivo* kinase activity measurements, ubiquitous down-regulation of GSK-3 by RNAi significantly increased lifespan in the fly. This result also supports my previous conclusion that the S9E mutant does not behave in a dominant negative manner If the S9E was inhibiting the function of the endogenous *sgg*, or reducing its kinase activity, it would behave similarly to the GSK-3 RNAi line.

To the best of my knowledge this is the first time that lifespan extension has been achieved by down-regulation of *sgg*. The positive effects of GSK-3 inhibition have been extensively documented in a diverse range of diseases (Lovestone et al., 2007; Hooper et al., 2008), including age-related disorders such as AD and PD, diabetes mellitus and cardiovascular disease (Cohen and Goedert, 2004; Jope and Johnson, 2004; Lovestone et al., 2007; Hooper et al., 2008). However, its involvement in ageing had remained unexplored until recently. A recent paper showing that global knockout of GSK-3a in mice shortens lifespan and drives the development of broad-spectrum abnormalities, complicates the interpretation of my results (Zhou et al., 2013). However, given that complete knockout of GSK-3I3 is lethal (Hooper et al., 2008), together these results might suggest that complete inhibition (or in these cases knockout) is detrimental. Interestingly, the global GSK-3a model showed activation of mTORC1, which is in disagreement with my data of the interaction of GSK-3 and mTOR. It would be worth exploring what are the mechanisms that lead to increased mTORC1 activation. I have been unable to detect alterations in mTORC1 either by lithium treatment or *sgg* down-regulation (or inhibition). Moreover, though increased GSK-3 should inhibit mTORC1, as it activates TSC2 (Inoki et al., 2006), I have been unable to detect changes in flies over-expressing the S9A mutant

(or wild type *sgg)*. Moreover, I was able to show that mTOR inhibition by rapamycin treatment was able to partially rescue from the deleterious effect of *sgg* over-expression. It would be worth exploring whether the interaction of mTOR and GSK-3 is cell or tissue-specific.

Additionally I was able to show that loss of *chico* does not lead to alterations in *sgg* as neither the total protein nor the phosphorylation levels were changed. Diabetes, which is characterized by reduced insulin signaling, is associated with increased levels and/or activity of GSK-3. Given that flies lacking CHICO have reduced insulin signaling, I was expecting for some change in *sgg*. This could potentially suggest that compensatory mechanisms might be acting to prevent this dysfunction in the long-lived *chico* null flies. Moreover, over-expression of the S9A mutant in the loss of *chico* background significantly restored lifespan. However, an alternative explanation can be proposed. Loss of *chico* in the context of increased GSK-3 activity does not allow for lifespan extension. Perhaps a fly model with reduced IIS and increased GSK-3 would more closely resemble a diabetes model than a healthy ageing intervention. These speculations should be approached experimentally to gain insight into the biology of GSK-3 as a modulator of the ageing process.

In conclusion I have shown that over-expression of wild type *sgg* or mutants with increased kinase activity reduce lifespan while down-regulation of shaggy extends lifespan in the fly.

5.5.2 Shaggy/GSK-3 and lithium act in the same molecular pathway

As mentioned before lithium is known to inhibit GSK-3 and I was able to show here that lithium indeed inhibited *sgg* as it increased the inhibitory phosphorylation at relevant doses. Moreover, my genetic data strongly supports that GSK-3 regulates ageing as ubiquitous over-expression shortened lifespan, while RNAi-mediated knockdown extend lifespan. The epistasis experiments combining the genetic manipulation and lithium treatment showed that while lithium can rescue from the deleterious effects of *sgg* over-expression, it is unable to further extend the lifespan when *sgg* is knocked down. This result strongly suggest that lithium and *sgg* down-regulation act in the same molecular pathway.

5.5.3 Shaggy regulates ageing in a tissue-specific manner

Lithium exerts positive effects in the brain (Eldar-Finkelman, 2002; Hooper et al., 2008). Sofola and colleagues have shown that lithium reduces AP1_42 levels in an AD fly model (Sofola et al., 2010). Therefore, I performed experiments to analyse whether inhibition of *sgg* specifically within the neural tissue could be partially responsible for the lifespan extending effects of ubiquitous down-regulation of *sgg* in the fly. Unfortunately, *sgg* down-regulation in the brain was unable to extend lifespan (not shown). I also tested whether over-expression of *sgg* in the brain would shorten lifespan. Unexpectedly, I found that over-expression of wild type *sgg* or either the S9E or S9A *sgg* mutants in the fly brain were able to extend lifespan. This result was rather surprising, especially in the light that none of these interventions showed changes in lifespan when performed only in the gut/fat body. I have preliminary data to suggest that some of the negative effects of *sgg* over-expression are mediated in the heart tissue. Taken together these results strongly suggest that *sgg* regulates the ageing process in a tissue-specific manner

I additionally showed that lithium treatment of neuronal over-expression of *sgg* and lithium treatment are additive for lifespan. Even when my other data suggest that *sgg* down-regulation and lithium treatment act in the same molecular pathway, these results strongly suggest that lithium does not extend lifespan via the inhibition of neuronal *sgg*.

These results have interesting implications for biomedical research. For a long time pharma and academia have been interested in developing GSK-3 inhibitors for the treatment of a wide range of pathological conditions including, neurodegeneration and altered metabolism (Avrahami et al., 2013; Kramer et al., 2012; Meijer et al., 2004; Takahashi-Yanaga, 2013). However, we are still far from understanding how GSK-3 modulates metabolism, stress response and ageing in different tissues and cell populations. Although my data suggests that global inhibition of GSK-3 would be beneficial for stress-response and ageing, the combination of active GSK-3 in neurons and inhibition elsewhere could be even more effective for extending healthy lifespan. Perhaps teasing out the specific tissues that require active GSK-3 during ageing, will contribute in understanding how relevant would systemic GSK-3 inhibition be for the prolongation of life and the recovery of a youthful state.

5.5.4 Shaggy and lithium modulate neurodegeneration: overlapping mechanisms?

As mentioned earlier, lithium reduces A131_42 levels in the brain. Furthermore, Sofola et al. showed that the active S9E mutant was able to mimic lithium's Al3 lowering effect (Sofola et al., 2010). Since my work suggests that the S9E construct is an active *sgg* mutant I decided to over-express wild type *sgg* and the S9A *sgg* mutant in the neuronal tissue of the AD fly model. In agreement with S9E being an active mutant, I was able to show that neuronal over-expression of either wild type shaggy or the S9A mutant extended the lifespan of the AD fly model.

My results demonstrate unexplained discrepancies between the effects of lithium and over-expression of shaggy in the fly. Lithium is known to inhibit *sgg* and although lithium reduces the activity of shaggy in the AD model (Sofola et al., 2010), over-expression of *sgg* can mimic the protective effects of lithium. These results suggest that although lithium can inhibit *sgg* in the brain, the mechanism by which it confers protection to the AD fly model is independent of *sgg* inhibition. Sarkar and colleagues have shown that indeed lithium and activation of GSK-3 can have mimicking effects without acting through the same pathway (Sarkar et al., 2005). Since the AD fly model used in my experiments does not involve the production of Af31_42, as it expresses A131_42 and bypasses the processing of APP, it is unlikely that the mechanism of lowering Al3 levels is mediated by APP processing as has been reported (Phiel et al., 2003). However, it is very likely this effect is mediated by a degradation mechanism. Lithium has been considered an autophagy inducer (Heiseke et al., 2009; Sarkar et al., 2005) and this could potentially contribute to the lowering effects of AP1_42. However, I was unable to show up-regulation of autophagy in the previous Chapter. Yet, it is still possible that the up-regulation of autophagy is tissue-specific. The next step will be to test whether lithium does indeed activate autophagy in *Drosophila* brains and to show if its Af31_42 lowering effects can be blocked by autophagy inhibition. Alternatively, GSK-3 has also been shown to reduce translation (Shin et al., 2014). It would be appropriate to study whether the effects of lowering GSK-3 are through lowering protein synthesis.

5.5.5 GSK-3/NRF2 pathway: is there a connection?

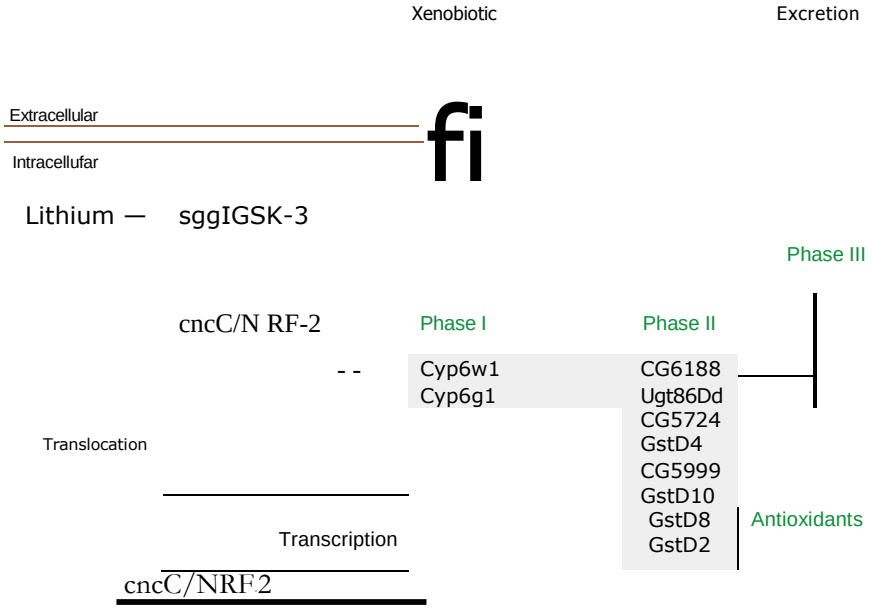


Figure 5.27 Proposed mechanism of action for lithium in ageing and stress-resistance. This is similar to Figure **4.4D** with the addition of GSK-3. Lithium inhibits GSK-3 to de-repress CncCNRF-2 which then activates a transcriptional response of detoxification and protection against stress.

In the previous chapter I showed that lithium up-regulates a transcriptional response mediated by the transcription factor CncC/NRF-2. How do my finding that sgg/GSK-3 and lithium act in the same molecular pathway fit into one story? Interestingly, NRF-2 is regulated by two complementary, but independent pathways, one mediated by the Kelch-like ECH-associated protein 1 (Keap 1), and the other by GSK-3 (Hayes and Dinkova-Kostova, 2014; Ma, 2013; Motohashi and Yamamoto, 2004). Both repress the activity of NRF-2 by sequestering it in the cytosolic compartment. Data in *C. elegans* and mammalian cells have shown that GSK-3 directly represses NRF-2 (An et al., 2005; Rada et al., 2012; Salazar et al., 2006). This would favour a model whereby lithium inhibition of GSK-3 would allow the activation of CncC and the regulation of the transcriptional signature of xenobiotic metabolism. Given that lifespan can be manipulated through the activation of this transcription factor (Sykiotis and Bohmann, 2008; Tullet et al., 2008), my data would suggest that indeed GSK-3 inhibition and the subsequent activation of CncC could be mediating the pro-longevity and stress-resistant effects of lithium (Figure 5.27).

5.5.6 Circadian clocks in ageing: the role of lithium and GSK-3

An interesting and understudied mechanism to regulate longevity and metabolism is the alteration of the circadian rhythms. Circadian rhythms control many physiological processes including feeding behaviour (which will become relevant in Chapter 6), metabolic regulation of hormonal status, xenobiotic metabolism, amongst others (Beaver et al., 2010, 2012; Takahashi et al., 2008; Xu et al., 2008). These particular traits have a great influence in longevity and some of the phenotypes I have presented could be a direct consequence of their regulation by circadian clocks. In *Drosophila*, where clock genes were first identified (Konopka and Benzer, 1971), sgg plays an important role in the regulation of nuclear shuttling of two important proteins involved in the regulation of the length of circadian periods (Stanewsky, 2003). The molecular components of the circadian clock include a cell-autonomous transcription-translation feedback loop encoded by a set of core genes (Reppert and Weaver, 2002; Takahashi et al., 2008). The transcription factors *Clock* and *cycle* induce the expression of *period* and *timeless*. Nuclear accumulation of PERIOD and TIMELESS inhibit the transcriptional activity of the CLOCK-CYCLE dimer. Phosphorylation of PERIOD and TIMELESS delays the cystoplasmic accumulation and nuclear translocation of these proteins, allowing CLOCK and CYCLE to continuously activate transcription. PERIOD is phosphorylated by DOUBLE-TIME, while TIMELESS is phosphorylated by sgg (Harms et al., 2003; Stanewsky, 2003). Flies over-expressing sgg in TIMELESS-expressing neurons show increased entry of PERIOD and TIMELESS to the nucleus and shorter free-running periods (Stanewsky, 2003). Although the role of GSK-3 in mammalian clocks has not been fully elucidated (Takahashi et al., 2008), recent evidence suggests that it plays a similar role in the regulation of circadian rhythms as in flies. Paul and colleagues showed that double transgenic knock-in mice for constitutively active versions of both isoforms of GSK-3 showed increased free-running periods with fragmentation of their wheel-running rhythms (increased activity bouts), decreased amplitude in circadian behaviour, and longer activity period (Paul et al., 2012). As they analysed the master clock in mammals, the suprachiasmatic nucelus (Takahashi et al., 2008), they found that the typical day/night variation in neuronal activity was lost as evidenced by higher spike rates during the night (Paul et al., 2012). The role of sgg/GSK-3 still needs clarification, as evidenced by the contradiction between the fly and mammalian data (i.e., opposite effects on period-length after GSK-3 over-expression). However, the manipulations of sgg/GSK-3 were not similar in both model organisms. Perhaps a detailed examination of circadian rhythms in flies

over-expressing sgg pan-neuronally ((only during adulthood to avoid developmental effects) vs. only in clock neurons (this could be done in subset of neurons) vs. a ubiquitous (adult-only) over-expression, could be informative to more clearly define the role of sgg/GSK-3 in the regulation of central and peripheral clocks. Additionally, the interaction of these genetic manipulations could be analysed in the context of lithium treatment.

The effect of lithium in the regulation of circadian clocks has been known for decades (Klemfuss, 1992). Lithium is known to lengthen the circadian period. For example, mice treated with lithium chronically (approximately for a month) and achieving brain concentrations of 1 mM showed a mild but significant increase in the locomotor activity period (Li et al., 2012). Concomitantly, lithium was also shown to lengthen the period of Period-2 oscillation at the protein level in the suprachiasmatic nucleus and lung tissue, by up-regulating its protein expression. Interestingly, a more potent GSK-3 inhibitor shortened the circadian period in lung fibroblasts, though it also up-regulated the transcription of Period-2 (Li et al., 2012). These rather contradicting results could be more easily resolved by the combination of genetic manipulations to down-regulate GSK-3 and the addition of lithium. As with my previous suggestion, the genetic intervention could be done in a tissue and cell type specific manner, allowing for a more comprehensive understanding of the role of GSK-3 and lithium.

An additional challenge will be to determine the level at which circadian clock regulation integrates with my model of GSK-3 modulation of CncC/NRF-2 (Figure 5.27). A recent report suggests that modulation of the clock might be downstream of sgg/GSK-3, but upstream of CncC/NRF-2 (Pekovic-Vaughan et al., 2014). Pekovic-Vaughan and colleagues found that NRF-2 transcriptional activity is regulated by clock genes, and that arrhythmic mice show poor levels of transcriptional regulation downstream of NRF-2, suggesting that the clock is an upstream regulator of NRF-2 (Pekovic-Vaughan et al., 2014). In a revised model I would place lithium at the top inhibiting GSK-3, which would then directly modulate CncC/NRF-2 (An et al., 2005; Rada et al., 2011; Rojo et al., 2008), and additionally GSK-3 could regulate the circadian clock proteins to modulate the transcriptional activity of CncC/NRF-2 (Pekovic-Vaughan et al., 2014). This would suggest that circadian clock regulation by lithium and sgg/GSK-3 would be at the heart of the regulation of perhaps the longevity, metabolic and stress-responses observed in this work. In the next chapter (Chapter 6) I show how flies expressing A131_42 show reduced feeding behaviour, altered metabolism and stress response. This could indeed all be consequence of a dysfunctional clock. I have data to show that altered GSK-3 activity

(serine-9 phosphorylation) occurs as early as the feeding alterations (data not shown), maybe pointing toward a molecular link between altered behaviour and abnormal clock function. This is especially relevant as flies expressing $A13_{1\_42}$ were recently reported to show abnormal circadian behaviours (Chen et al., 2014). Exploring the role of altered rhythmic behaviour in the context of these phenotypes could resolve the role of the clock alterations in flies expressing $A13_{1\_42}$, however these hypothesis will require extensive exploration.

Chapter 6

The anorexic and stressed fly: neurodegeneration in an Alzheimer's *Drosophila* model

"How slight a thing will disturb the equanimity of our frail minds"

Charles Dickens, *Oliver Twist*

6.1 Abstract

AD has been successfully modelled in Drosophila through the neuronal expression of the Al $_{l\text{-}40}$ and Afl $_{1\_42}$ peptides, which accumulate in AD brains. Several studies have shown that when the 4$_{1_42}$ peptide is expressed in the neuronal tissue of the fruit fly they die earlier than controls and show abnormalities in locomotor activity and synaptic dysfunction. Although these phenotypes are useful for genetic and pharmacological screens, I attempted to characterise earlier phenotypic events. My results evidenced that flies expressing 4$_{1_42}$ show reduced feeding behaviour at a very early age, before the well-characterised locomotor phenotypes are observed. This feeding alteration modified the response to toxins delivered in the food. However, when flies were subjected to sources of stress that do not depend on food intake, they were, as expected, more sensitive than control flies. I also observed that flies expressing 4$_{1_42}$ display a significant resistance to starvation stress, which was unexpected given their reduced feeding behaviour. They appeared to compensate for the reduction in food intake by increasing their levels of lipid storage, which correlated with their starvation resistance. I hypothesise that the reduced feeding of Afl 1-42 expressing flies renders them into a starvation-like state that switches on a storing phenotype. Nutrient supplementation (particularly essential amino acids) restored the altered metabolism, the response to starvation, and even extended the lifespan of flies expressing 4$_{1_42}$. Thus, insufficient nutritional intake secondary to reduced feeding behaviour leads to altered stress-responses and changes in metabolism in flies expressing Afl 1-42.

6.2 Introduction

Protein aggregation is a key feature of the most common forms of neurodegeneration including AD, PD and HD, which are all characterized by deposits of abnormally aggregated forms of specific proteins. Although their role in the pathogenesis of the disease is not completely understood, it is well established that they play major roles in

altered neuronal function and cell death, what is overall considered as neurodegeneration (Cohen and Dillin, 2008; Labbadia and Morimoto, 2014; Taylor et al., 2014; Walker et al.,

2013).

Neurons, and in general eukaryotic cells, are armed with a wide range of response mechanisms to extrinsic and intrinsic stressors. Most of these mechanisms deteriorate with age, which would explain why ageing is such a major risk factor for diseases of protein aggregation, proteinopathies. However, even old postmitotic cells are able to respond to stressors by activating and/or inactivating processes that enable their viability (Cuervo, 2008; Labbadia and Morimoto, 2014; Morimoto and Cuervo, 2014). In the case of proteinopathies, the defence mechanisms most commonly studied are those responsible of clearing damaged organelles and insoluble aggregates. As mentioned in Chapter 1 these mechanisms include the UPS and AL system. However, other response pathways also aid cells during proteotoxic stress and have been shown to ameliorate disease progression in cases of proteostasis collapse.

6.2.1 The role of oxidative stress in Alzheimer's disease

Diseases are often associated with abnormal responses to stress with inappropriate activation of homeostatic responses. This can be because the tissue (or organism) cannot up-regulate the appropriate response, or because there is over activation and/or loss of the ability to self-regulate to end the response (McEwen, 1998). AD has been associated with oxidative stress (Kondo et al., 2013; Moreira et al., 2006). Indeed increased oxidative stress has been shown to be an early sign of disease progression (Nunomura et al., 2001). This has made oxidative stress and pathological hallmark of AD (PraticO, 2013).

Amyloid pathology associates with oxidative stress in three forms. First, amyloid has been shown to increase the production of 11202 and lipid peroxidation, both in cell culture and in transgenic mouse models that produce $Af3_{1\_42}$. It is not entirely understood how Af31_42 increases oxidative stress. One possibility is altered mitochondrial function. $A0_{1\_42}$ inhibits mitochondrial complex I (Hauptmann et al., 2006; Querfurth and LaFerla, 2010; Reddy and Beal, 2008; Zhao and Zhao, 2013). As discussed in Chapter 1, altered mitochondrial function can be both a driver and protective mechanism against ageing. Second, increased oxidative stress can drive disease progression as it increases A131-42 deposition. One mechanism described has been increased expression of enzymes that promote APP cleave into the amyloidogenic pathway (Chen et al., 2008; Oda et al., 2010;

Quiroz-Baez et al., 2009). Third, amyloid production is a response to oxidative stress, which has led to the suggestion that amyloid can act as an antioxidant (Nunomura et al.,

2010; Perry et al., 2002). This last point is less clear, but it would propose that Af31_42 could act as a hormetic molecule. Interestingly, NRF-2, a transcription factor potentially involved in the hormetic response, has been reported to be expressed at lower levels in brains of patients with AD (Ramsey et al., 2007). Moreover, expression of NRF-2 and its target genes have been reported to be lower in transgenic mouse models of AD (Kanninen et al., 2008). When NRF-2 levels are increased in these models they show improvement in memory tasks (Kanninen et al., 2009).

6.2.2 Age-related anorexia in flies and humans

Reduced feeding or anorexia is a common problem of old age. It has been estimated that 5-12% of homebound patients, 15% of community-dwelling older people, 65% of hospitalised patients, and 5-85% of institutionalised elderly patients present protein-energy malnutrition. Though this was often considered to only be a problem in nursing homes and hospitalised patients, it has become increasingly evident that anorexia of ageing or age-related anorexia with the subsequent malnourishment is common in older people in the community (Mir et al., 2013; Morley, 1997).

The drive to eat is primal; it allows the continuation of the species. During the first part of the life cycle of most species feeding and appropriate nutrition allows sexual maturation and reproduction. From birth until mid adulthood there is a steady increase in feeding and biomass accumulation (Morely and Silver, 1998; Morley, 1997). However, it is well documented that food intake declines with age. It is assumed that most of the decrease in energy intake derives from the decline on energy expenditure. Estimates have considered than between the age of 20 and 90 there is a 20% decrease in food intake in females and 43% in males (Mir et al., 2013). The decrease in energy intake has been observed to be greater than the decrease in energy expenditure, which translates into weight loss. This has indeed been documented; lean subjects are more prone to loose weight (Chapman, 2004).

Decreased food intake and the ensuing weight loss are associated with a plethora of health problems like impaired muscle function, altered immune response, cognitive decline, loss of bone mass, anaemia, altered wound healing, delayed recovery from surgery and ultimately death (Chapman, 2004; Macintosh et al., 2000). In a longitudinal

observational cohort study in the US, it was documented that weight loss was more common than weight gain in the older population (those over 65 years of age). Interestingly, weight loss of 5% or more increased the risk of mortality by 65%, and those with initial lower body mass had the highest crude mortality rate (Newman et al., 2001). This of course poses an interesting question as to the benefits of DR late in life. Recently the involvement of protein intake and healthspan was analysed in a cross sectional study involving 6,381 adults (83,308 person-years over 18 years) over the age of 50 years from the United States. Moderate to high protein consumption was associated with higher diabetes-related mortality at all ages. High protein intake (animal fat, not plant fat) at ages between 50 and 65 years was associated with a 74% increase in overall mortality, and these individuals were four times more likely to die of cancer in comparisson to low protein consumers (Levine et al., 2014). Moderate protein intake was also associated with a 3-fold higher cancer mortality. Interestingly, these associations were not significant when analysed against caloric intake. However, higher protein intake amongst individuals above 66 years of age was associated with a 21-28% reduction in mortality for all causes, and a 60% reduction in cancer mortality (Levine et al., 2014). Taken together these studies suggest that at older ages reduced food intake (or protein inatke specifically) is detrimental for health.

Interestingly, *Drosophila* also show age-realted anorexia, actually food intake decreases sharply early in life and remains low throughout the rest of the adult life of the fly (Wong et al., 2009). However, flies seem to respond positively to reduced nutrients as flies switched from full feeding to DR change mortality trajectories withing 2 days to adopt a similar mortality trajectory as flies under DR (Mair et al., 2003). The switches however, were performed very early on in comparisson to the lifespan showed here. It would be interesting to analyse wether increased concentration of food supplemented late in life adds benefits to lifespan and mortality.

6.3 Methodology and experimental design

6.3.1 ATP measurement

ATP concentrations were determined according to the Roche ATP Bioluminesence Assay Kit HS II (Roche, West Sussex, UK). For headless bodies 2 live flies were decapitated and homogenized in 100 ilL ice-cold lysis buffer (provided in the kit) for 1 min using a Kontes pellet pestle. A similar procedure was adjusted using 10 heads per sample. The lysate was then boiled for 5 min and centrifuged at 20,000 g for 1 min. 2.5 ilL of cleared lysate was added to 187.5 ilL dilution buffer and 10 ilL luciferase, and the luminescence was immediately measured using a Tecan Infinite M2000 microplate reader and Magellan V6.5 software. Each reading was converted to the amount of ATP per fly based on the standard curve generated with ATP standards.

6.3.2 Paraquat injections

Paraquat (Sigma 856177) was delivered at a dose of 50 ng/mg according to previously described (Bjedov et al., 2010). To deliver the compound injection pipettes were prepared out of 10 mm glass capillaries. We used the Flaming-Brown micropipette puller (Programme 2). A home-built microinjection machine was used for injections. In parallel flies were also injected with control Ringer's solution. Both injected solutions contained blue dye for visualization of injections (FD&C Blue No.1). Paraquat injections were performed by Dr. Helena Cocheme.

6.3.3 Essential amino acids supplementation

Essential amino acids were prepared according to (Grandison et al., 2009). A 50 mL solution (see Appendix 9) was prepared and then added to 1 L of 1 SY. To control for the extra solution, all other conditions were supplemented with 50 mL of ddH_2O.

6.3.4 Holidic medium

Chemically-defined medium was prepared according to (Piper et al., 2014). Briefly, for 1 L of holidic medium ingredients and amounts described in Appendix 10 were mixed in a 1

L glass bottle in a total volume of 883.7 mL (topped with ddH2O). The mix was autoclaved and kept between 50 and 55 °C after autoclaving. The second set of ingredients in Appendix 10 were added and mixed (an essential amino acid (EAA) stock solution, and non-EAA stock solution are shown in Appendix 11) after which the medium was ready for dispensing (or for adding additional ingredients) into vials.

6.3.5 Immunoblotting

Immunoblot analyses was performed according to protocol described in chapter 2. Primary antibodies used were Cu/Zn SOD (#ab13498 Abcam; 1:1000), Mn SOD (#ab13534 Abcam; 1:1000), catalase (#ab16731 Abcam; 1:2500).

6.4 Results

6.4.1 $AI3_{1\_42}$ elicited a transcriptionally response enriched for oxidative stress and metabolism

To guide my search for other phenotypes to explore, I interrogated the transcriptional profile of fly heads expressing Af31_42. For this we took a recently published data set of microarrays that explored the transcriptional response to Af31_42 (Favrin et al., 2013) and performed catmap analyses (Breslin et al., 2004) to determine functional categories enriched in the data set. Amongst the ten most enriched categories, we identified processes involved with oxidation-reduction and metabolism, which appeared at least twice (Table 3).

Table 3. Enriched GO categories[3] in fly heads expressing 4_{1_42}[4].

GO ID	Functional Category	*P* value
GO:0016491	Oxidoreductase activity	1.63E-17
GO:0005811	Lipid particle	4.14E-16
GO:0044444	Cytoplasmic part	8.71E-12
GO:0055114	Oxidation-reduction process	2.57E-11
GO:0048037	Cofactor binding	8.38E-10
GO:0003824	Catalytic activity	5.46E-09
GO:0009055	Electron carrier activity	6.09E-09
GO:0016769	Transferase activity, transferring nitrogenous groups	1.40E-08
GO:0008483	Transaminase activity	2.64E-08
GO:0008152	Metabolic process	2.07E-07

Thus, the transcriptional response to A131_42 showed that the most enriched category was oxidoreductase activity. Interestingly, also lipid particle and metabolism seemed to be enriched amongst the top ten GO categories. I therefore decided to investigate the response of flies expressing Af31_42 to different forms of oxidative stressors.

[3] Data reanalysed from Favrin et al., 2013.

[4] The analysis was performed by Dr. Dobril Ivanov.

6.4.2 AP1_42 protected against H202 and paraquat when delivered orally

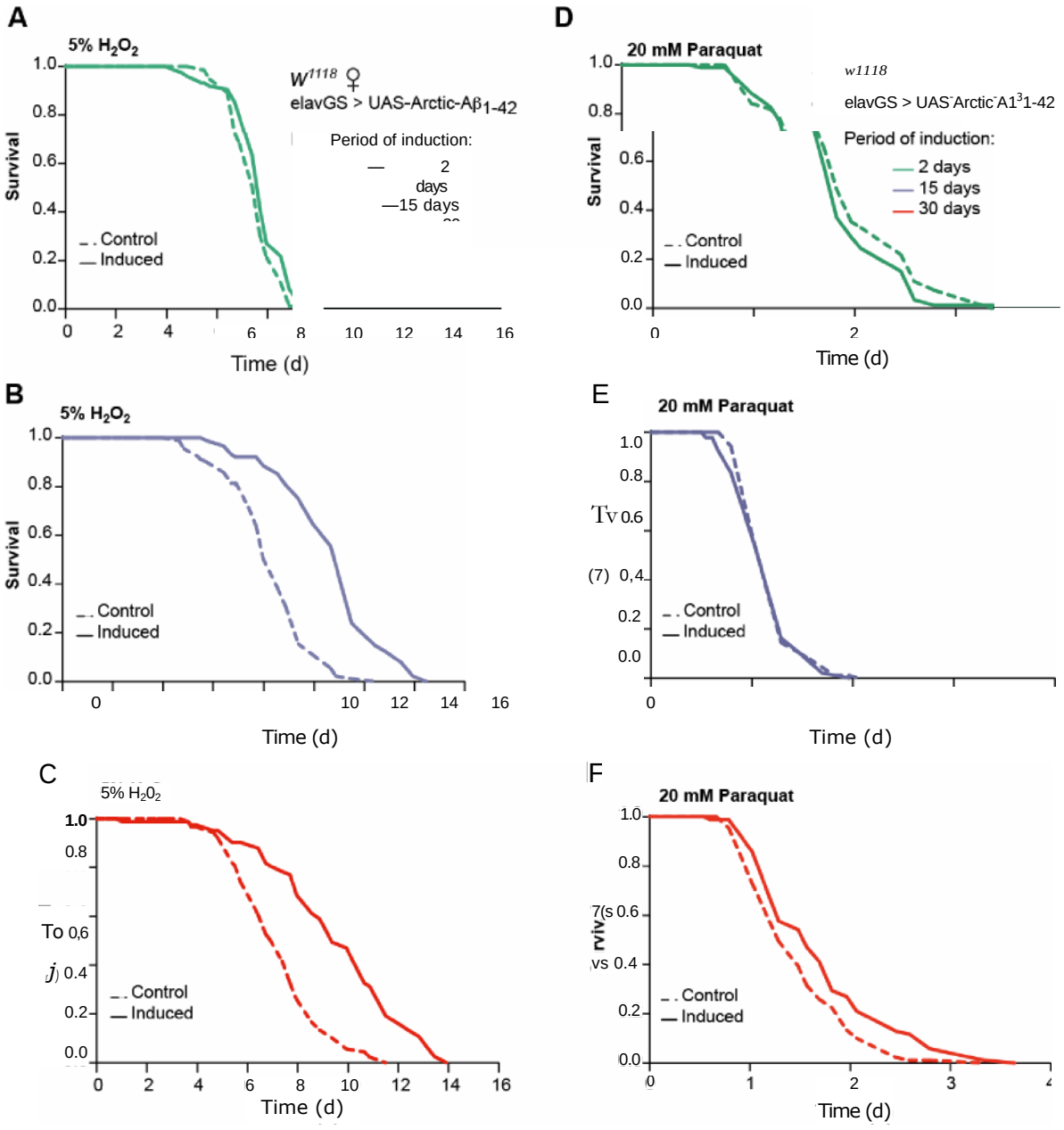


Figure 6.1 Response of 4_{1_42}-expressing flies to 11_20_2 and PQ. (A-C) Response of flies expressing $A13_{1\_42}$ to H_20_2 at different periods of $A13_{1\_42}$ induction: (A) 2 days, (B) 15 days, and (C) 30 days. (D-F) Response of flies expressing $A13_{1\_42}$ to PQ at different periods of $A13_{1\_42}$ induction: **(D)** 2 days, (E) 15 days, and (F) 30 days. Experimental analyses of survivals was performed simultaneously for the three induction periods. N = 90 flies per condition.

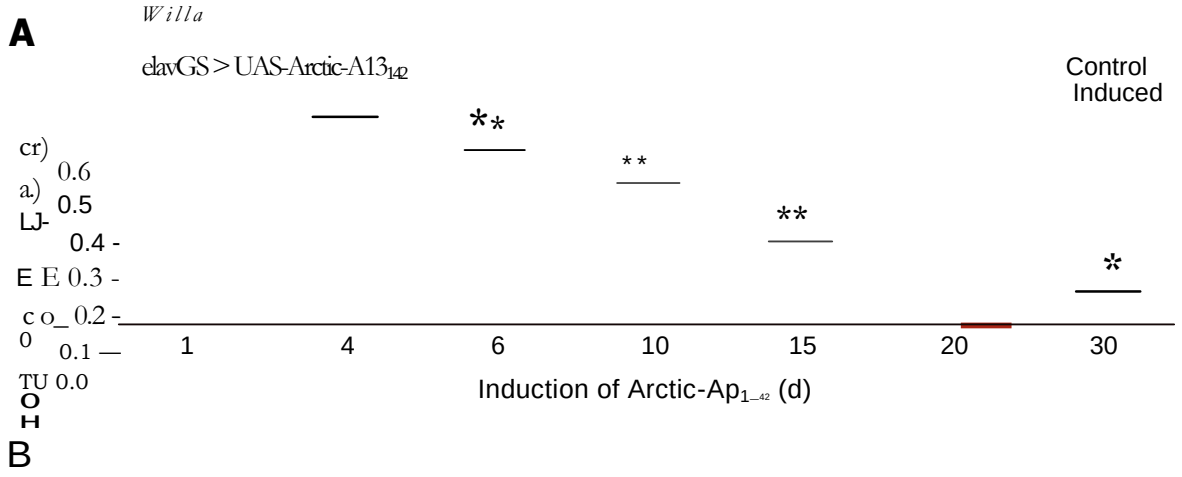
In vitro studies have shown that the presence of Af31_42 leads to free radical formation with subsequent cellular and tissue damage (Hensley et al., 1994; Varadarajan et al., 2000). Moreover this is considered an important mediator of neurodegeneration. To analyse the response of flies expressing $A13_{1\_42}$ to oxidative stressors I analysed their response to 11_20_2

and paraquat (PQ). A cohort of flies expressing Af31_42 carrying the familial Arctic mutation (Nilsberth et al., 2001) in the adult nervous tissue using the UAS/Ga14-GeneSwitch system (UAS-Arctic-Ab$_{i_42}$/+;elav-GS/+) (Osterwalder et al., 2001), were randomly allocated to fly media supplemented with either the GeneSwitch inducer mifepristone (RU486) or vehicle (100% ethanol) (Brand and Perrimon, 1993; Crowther et al., 2005; Osterwalder et al., 2001; Sofola et al., 2010). Given that the GALA protein carries a modification that only allows binding to UAS and expression of the AP$_{1_42}$ in the presence of RU486, the control and study groups have the same genetic background (Rogers et al., 2012; Sofola et al., 2010). I evaluated the response to I-1202 following 2, 15 or 30 days of Af31_42 expression. The survival response to I-1202 was not significantly different from controls after only 2 days of AP1_42 expression (Figure 6.1A; $P > 0.05$, log rank test), but flies became resistant to I-1202 after 15 and 30 days of induction (Figure 6.1B and C; $P < 0.01$, log rank test). I performed a similar experiment using PQ and observed that flies expressing A13$_{1_42}$ for 30 days were resistant to PQ (Figure 6.1F; $P < 0.01$, log rank test), but their response to PQ was indistinguishable from controls following only 2 or 15 days of Af31_42 expression (Figure 6.1D and E; $P > 0.05$, log rank test). Although the response to I-1$_2$0$_2$ was consistent and reproducible, I found that the response to PQ was less consistent and in some trials A131_42-expressing flies were sensitive to PQ. Resistance to PQ was more evident and significant when the stress assays were performed using medium containing yeast (see section 2.6.2), perhaps indicating an interaction between the dietary components and the response to PQ. The results, however, support a trend towards resistance to PQ exposure in aged Af31_42 expressing flies, but highlights the variability of stress responses in Alzheimer's flies using this assay.

6.4.3 Flies expressing A131-42 showed acceleration of age-related anorexia

These assays require or assume equal amount of food intake. To address the bioavailability of toxic stress-activating compounds under our experimental conditions I studied the feeding behaviour of flies expressing A131_42 in their neural tissue. Flies in either group were followed over a 30-day period with regular feeding behaviour recordings (Figure 6.2A). Control flies (fed vehicle) displayed the previously described age-dependent deterioration in feeding behaviour (Wong et al., 2009). To my surprise, however, flies fed with the genetic inducer RU486 expressing the A13$_{1_42}$ peptide, showed a dramatic reduction in feeding behaviour that worsened over time. Furthermore the reduction in

feeding was apparent after only 4 days of A131_42 expression (Figure 6.2A; ***P*** < 0.01, t-test). Indeed these flies barely made any attempt to feed after 15 days.



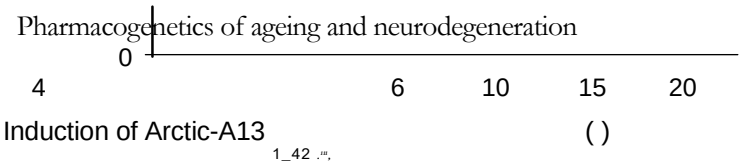
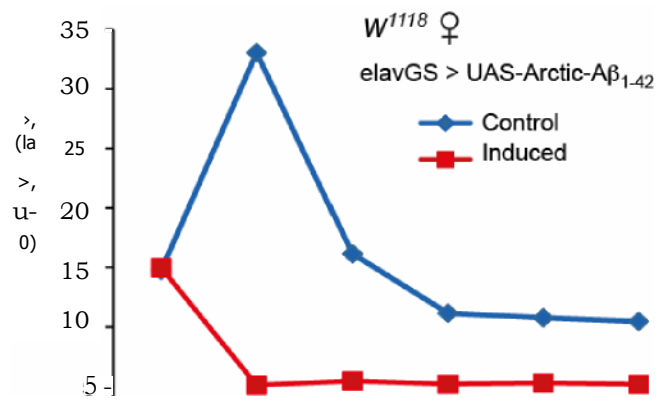


Figure 6.2 Feeding behaviour and fecundity of flies expressing 41_42. (A) The proboscis extension assay was performed over time to analyse feeding behaviour of flies expressing or not A131_42. **(B)** Fecundity was assessed in the same group of flies as in (A). N = 100 flies followed over time. * $P < 0.05$, ** $P <$ 0.001.

A measurable outcome of reduced nutrient supply is the subsequent reduction in the egg-laying ability of *Drosophila* (Chapman and Partridge, 1996; Terashima and Bownes, 2004). I therefore evaluated the number of eggs laid per female fly over 24 hours at similar time points as the feeding behaviour was assessed. Control flies displayed an early increase in egg-laying behaviour that mirrored the increase in feeding, after which the number of eggs laid declined with age. Flies expressing A131_42 however did not show this early rise in egg-laying, rather a dramatic reduction after 4 days of Af31-42 expression



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that remained unchanged for the rest of the trial period (Figure 6.2B; $P < 0.05$ for all points after 4 days of induction, t-test).

6.4.4 AI3$_{1_42}$ sensitized flies against injected PQ

To eliminate the cofounding effects of altered feeding and to further explore the *in vivo* response to oxidative stress I exposed Alzheimer's flies to PQ by means of a single intrathoracic injection (Cocheme et al., 2011, 2012). Flies expressing Af3$_{1_42}$ were more sensitive than controls to injected PQ *(*$P < 0.001$, log rank test). Furthermore there was a positive correlation between longer A131_42 expression periods and sensitivity to PQ exposure, as flies induced for 15 days were shorted lived than flies induced only for 7 days (Figure 6.3; $P < 0.01$, log rank test). To rule out any mortality due to the injection or increased intrathoracic pressure, we injected Ringer's solution to either expressing or non-expressing Af31_42 flies and noted no difference in their survival times *(*$P > 0.05$, log rank test). Thus, Af31_42 expression increases the vulnerability to the redox cycler PQ. The vulnerability to PQ is evident after only 7 days of induction.

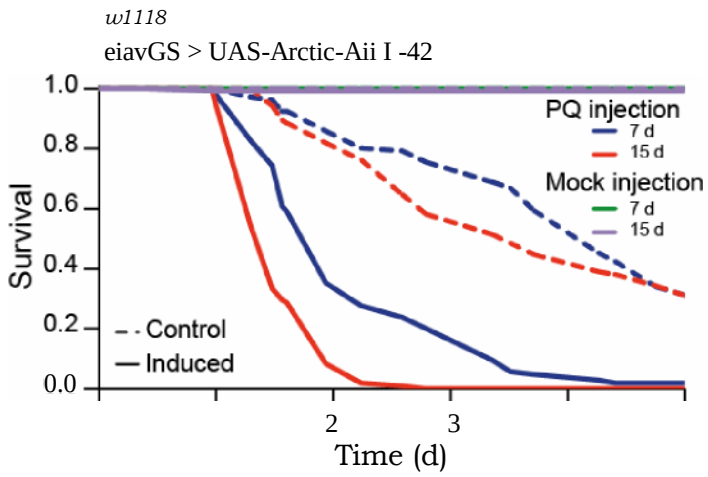

Figure 6.3 Response of flies expressing 4$_{1_42}$ to injected PQ. Flies were induced for either 7 or **15** days and then injected with 2 mM PQ or control Ringer's solution. Survival was analysed. N= 100 flies per condition. Injections were performed by Dr. Helena Cocheme.

6.4.5 $_{\text{Apt-42-induced}}$ anorexia did not modify sensitivity to xenobiotics

In light of my observation that flies expressing AP1_42 show reduced food intake, I cautiously assessed whether this would modify the response to xenobiotics. Several compounds are used to determine resistance to xenobiotics in model organisms (Lindblom et al., 2001; Misra et al., 2011; Yang et al., 2007). I examined whether the A(31-42-expressing flies were non-responsive or resistant to xenobiotic compounds administered in the fly medium. Surprisingly, flies expressing A131_42 were consistently sensitive to the

organochlorine insecticide dichlorodiphenyltrichloroethane (DDT; Figure 6.4A; $P < 0.05$, log rank test). To determine whether this effect was specific to DDT, I also evaluated the response to another xenobiotic, chloroquine (Figure 6.4B). Flies expressing $Af3_{1\_42}$ were significantly more sensitive to exposure with chloroquine compared to non-expressing control flies (Figure 6.4B; $P < 0.05$, log rank test).

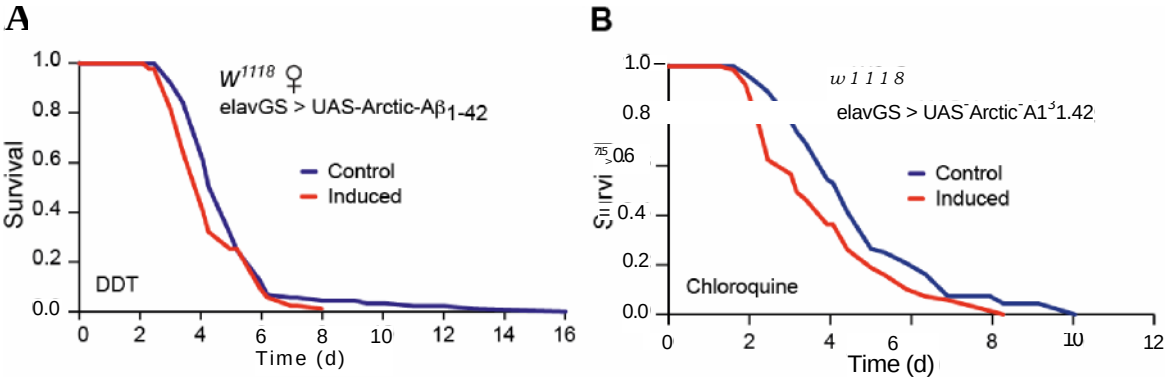

Figure 6.4 Flies expressing 4_{1_42} were sensitive to orally-delivered xenobiotics. (A) Survival response to **DDT** was analysed after 15 days of induction. Experiment performed by Dr. Kerni Kinghorn. **(B)** Response to chloroquine was assessed after 15 days of induction. N = 90 flies per condition.

In view of the fact that flies expressing A131_42 have significantly (2 to 4 fold) reduced feeding intake at day 15 (when these stress assays were performed; see Figure 6.2A), I hypothesised that neuronal expression of A131_42 renders flies sensitive to xenobiotics despite a reduction in food intake. Ingestion of small amounts of these compounds must therefore be sufficient for toxicity to occur.

Given the mixed results when oxidative stressors are supplemented in the fly medium, I conclude that the reduction in food intake, secondary to Af31_42 expression, modifies sensitivity to orally delivered oxidative stressors. This has obvious implications for the use of Af31_42 expressing models in screening for efficacy of orally delivered potential therapeutic compounds.

6.4.6 Ap_{1-42} repressed genes involved in oxidative and xenobiotic stress

To better understand the changes that might yield cells more sensitive to oxidative and xenobiotic stress when exposed to A131_42, I tested expression levels of different proteins involved in the detoxification of free radicals. I immunoblotted against superoxide dismutase 1 (Cu/Zn SOD), superoxide dismutase 2 (Mn SOD), and catalase after several induction periods. The expression levels of these proteins were not significantly altered in

the heads of flies expressing Af31_42 compared to control flies at any of the induction periods tested (Figure 6.5A; $P > 0.05$, log rank test).

Genes responsible for xenobiotic detoxification have been studied at the transcriptional level, revealing a genetic signature in *Drosophila* (King-Jones et al., 2006; Misra et al., 2011; Yang et al., 2007). Interestingly a transcriptional signature of detoxification has also been implicated in lifespan extension (McElwee et al., 2007; Steinbaugh et al., 2012). To investigate whether Af31_42 alters gene expression of enzymes important for detoxification I interrogated a transcriptional data set recently published (Favrin et al., 2013). Importantly, several genes with cytochrome P450 activity showed altered expression in this dataset, six of these genes were down-regulated, while two were up-regulated. These genes have been implicated in oxidative and xenobiotic metabolism (Daborn et al., 2001; Festucci-Buselli et al., 2005; Pedra et al., 2004). To gain insight into the nature of the particular sensitivity to orally-delivered xenobiotics we assessed the overlap of the transcriptional response to Af31_42 (Favrin et al., 2013) and that of the over-expression of CncC (Misra et al., 2011). Differentially expressed genes by Af31_42 or CncC over-expression significantly overlapped over the Bonferroni corrected P-value threshold of 0.0001 (A; P = 8.59-07; Fisher's exact test). However, analyses of the directionality of the differentially expressed genes did not yield significance in any direction. Just above the required threshold were genes down-regulated by A131_42 that were up-regulated by CncC (P = 0.000595). Interestingly, several genes with potential detoxification properties like Cyp6a8, GstD9 and GstD3 were up-regulated by AP1_42 (Figure 6.5C), but not in the common transcriptional signature we previously established between cncC, phenobarbital and lithium (Figure 4.4C). One GST enzymes that was up-regulated by these three interventions was GstD2, therefore we analysed the expression levels of this gene in flies expressing Af31_42 without detecting significant changes (Figure 6.5D: $P > 0.05$, t-test). However, when we analysed whether the expression of this gene changed in the rest of the body (where Af31_42 is not expressed), we found that it was significantly up-regulated. (Figure 6.5D; $P < 0.05$, t-test).

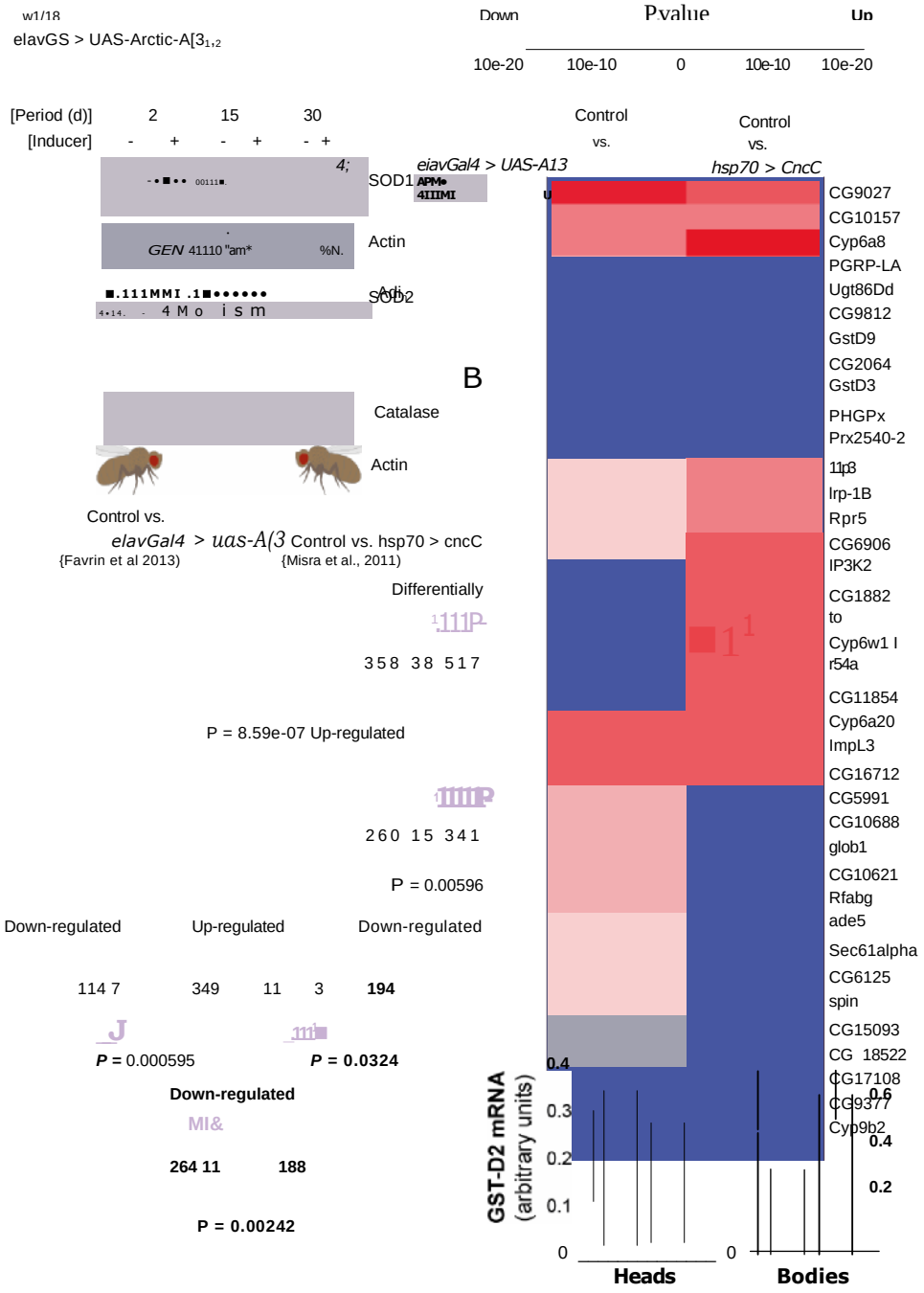

Figure 6.5 Regulation of detoxification enzymes in flies expressing 4_{1_42}. (A) Enzymes involved in protection against oxidative stress were analysed by immunoblot analyses without detecting significant alterations in protein expression levels. These images are representative of 5 repeats (2 of which were performed by Ms. Michelle Briffa) of 10 fly heads per repeat. (B) Comparative analyses of the transcriptional responses to $A13_{1\_42}$ and cncC over-expression. (C) Heat map depicting the differentially expressed genes that overlapped in (B). Analysis in (B and C) was done by Dr. Dobril Ivanov. **(D)** mRNA quantification of Gst-D2 by RT-qPCR using 4 repeats of 10 heads or 5 bodies per repeat. Experiment performed in collaboration with Ms. Li Li. * $\boldsymbol{P} < 0.05$, t-test.

These results highlight the complicated nature of the xenobiotic response in flies expressing Af31_42. It seems that the transcriptional response is similar to that regulated by CncC, yet genes that have been correlated with protection from stress are not precisely the

ones changing. Even more, though we did not find significant difference in the directionality of the response, it could be suggested that the transcriptional profiles go in opposing directions, and that the head and the rest of the body might be coping differently with A(31_42 toxicity.

6.4.7 Chronic expression of AI31_42 correlated with starvation resistance

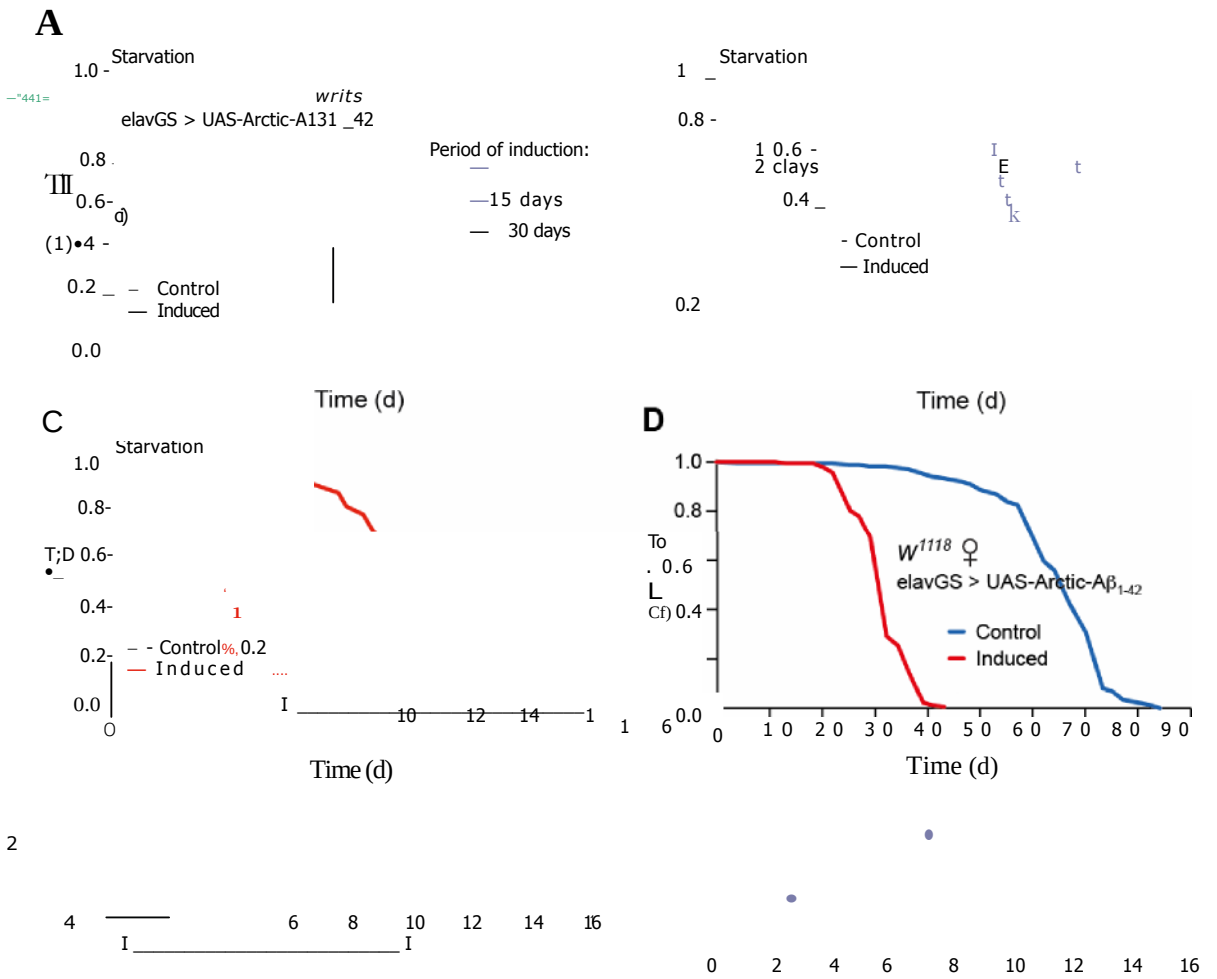

Figure 6.6 Starvation response and regular survival of flies expressing 4_{1_42}. Response to starvation of flies expressing $A13_{1\_42}$ after different periods of induction: 2 days (A), 15 days (B) and 30 days (C). N = 90 flies per condition. All lifespan were recorded simultaneously. (D) Lifespan under basal conditions of flies expressing $A13_{1\_42}$. N = 150 flies per condition.

Given the reduced feeding in flies expressing A131_42, I tested their response to starvation stress. I hypothesised that their reduced food intake would render them susceptible to starvation, especially at later ages, when food intake is almost non-existent. Older control flies were significantly more susceptible to starvation than young flies, as previously

reported (Burger et al., 2007). Contrary to expectation, flies expressing Af31_42 were found to be significantly resistant to starvation after 15 and 30 days of Af31_42 induction (Figure

6.6B and C; $P < 0.001$, log rank test), but not after 2 days ($P > 0.05$, log rank test; Figure 6.6A). Interestingly, the survival response to starvation doubled after 30 days of induction in comparison to 15 days ($P < 0.001$, log rank test; Figure 6.6B and C), which suggest either a chronicity or ageing-related effect. It is interesting to notice that the lifespan of Af31_42 expressing flies under starvation conditions at 30 days of age matches the lifespan of flies expressing AP1_42 under optimal nutritional conditions (Figure 6.6D). This suggests that Af31_42-expressing flies aged for 30 days eat so few nutrients that the food they are housed on in old age does not affect survival and they probably die in a starvation-like state.

6.4.8 Ap1_42- induced starvation resistance was secondary to a nutrition deficiency

My data suggested that the expression of Af31_42 leads to an opposite response to starvation stress to that observed in aging control flies; with Af31_42-expressing flies becoming increasingly resistant to starvation stress with age. Dietary restriction, as well as pharmacological and genetic down-regulation of the nutrient sensing pathways, makes flies resistant to starvation (Bjedov et al., 2010; Broughton et al., 2005; Burger et al., 2007). I therefore hypothesized that the starvation resistance observed in Af31_42 expressing flies is likely dependent on nutrition. I decided to explore three complementary possibilities that could explain this starvation resistance. Firstly, I varied the yeast concentration (the major source of nutrients in the media) from none to 50% more its regular content in our media (Bass et al., 2007; Mair et al., 2005). Secondly, I tested the effect of EAA supplementation, since it is the nutritional component with the biggest effect on *Drosophila* survival (Grandison et al., 2009a). Third, I assessed the induction of the starvation phenotype in a holidic medium (Piper et al., 2014), avoiding any co-founding effect of the yeast in our medium.

I first induced the expression of Af31_42 in flies for a brief initial period of time (5 days; to exclude any chronicity effects of Af31_42 expression) under varying yeast concentrations, as previously described (Bass et al., 2007; Grandison et al., 2009b; Mair et al., 2005) and then transferred them to food lacking any sugar or yeast (starvation conditions). As expected, flies expressing A131_42 responded differently under different yeast concentrations compared to control flies (Figure 6.7A). Flies expressing A01-42 peptide were more sensitive to starvation than non-induced control flies when yeast was

absent from the initial fly medium ($P < 0.01$, log rank test). As the yeast concentration increased the response of A131_42-expressing flies to starvation became first indistinguishable from their controls ($P > 0.05$, log rank test for 0.02X and 0.5X) after which they became resistant to starvation ($P < 0.05$, log rank test). Interestingly, the resistance to starvation was blunted when the yeast concentration was increased by 50% compared to standard fly media (Figure 6.7A; $P > 0.05$, log rank test). These results suggest that the resistance to starvation that Alzheimer's flies acquire over time is dependent on their nutritional status (especially a non-sugar component of the yeast), since they appear to require a yeast component to develop the resistance to starvation.

Secondly, I compared the response to starvation of Af31_42-expressing flies that had been induced in our standard medium to those where EAA had been supplemented in the food. This supplementation has been shown to mimic the effect of full feeding (Grandison et al., 2009a). I evaluated the survival response to starvation after 15 and 30 days of A131-42 induction. Under starvation conditions flies expressing Af31_42 for 15 days were significantly longer lived than non-expressing control flies ($P < 0.01$, log rank test), as shown earlier. When flies expressing $A13_{1\_42}$ were supplemented with EAA, the resistance to starvation was significantly reduced by approximately 50% (Figure 6.7B; $P < 0.001$, log rank test). In a similar experiment, the resistance to starvation of flies expressing Af31_42 for 30 days was compared to those where EAA had been supplemented. Interestingly, though supplementation did indeed significantly reduce the starvation resistance after 30 days ($P < 0.05$, log rank test), the effect seemed minimal in light of the starvation phenotype of $A(3_{1\_42}$-expressing flies at this point (Figure 6.7C).

Third, to determine whether the starvation phenotype was dependent on our yeast laboratory conditions I evaluated the phenotype under non-yeast conditions by using a holidic medium where the concentrations of nutrients can be easily manipulated (Piper et al., 2014). Flies were reared regularly and then induced either in the defined medium or in our regular SY medium for 15 days before switching the flies to the starvation conditions. After 15 days flies induced to express A131_42 on the defined diet showed a slight resistance to starvation that did not reach statistical significance (Figure 6.7D; $P > 0.05$, log rank test). However, when the induction was prolonged to 30 days, flies expressing A131_42 were significantly more resistant than their non-induced controls (Figure 6.7E; $P < 0.01$, log rank test). Thus, the starvation phenotype is unlikely to require the presence of yeast in the medium for its induction and is more likely to be related to the anorexia induced by A01-42. Interestingly, flies that were maintained in the defined medium were significantly more

sensitive to starvation ($P < 0.001$, log rank test) than those kept in the SY medium. This effect was independent of the induction of A131-42.

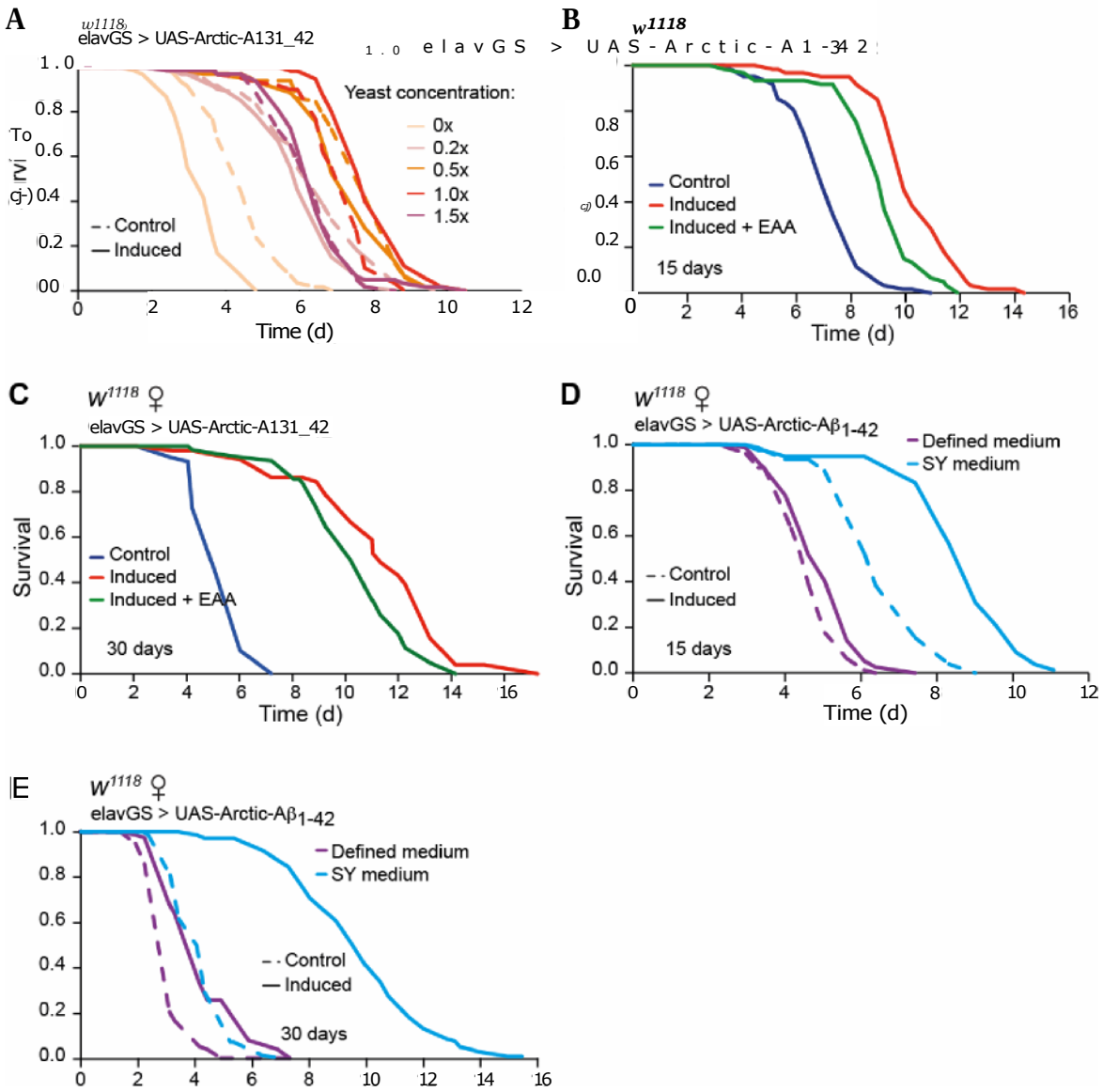

Figure 6.7 The starvation-resistance response of flies expressing At$_{142}$ depends on a nutrition deficiency developed over time. (A) A13$_{1_42}$ expression was induced for five days on five yeast dilutions ranging from no yeast to 1.5 the standard yeast concentration in our medium (100 g/L) after which all nutrients were removed and survival analysed. (B) A13$_{1_42}$ expression was induced for 15 days on medium supplemented or not with EAA and their response to starvation was evaluated. (C) Similar to (B), but flies were induced for 30 days. **(D)** A13$_{1_42}$ expression was induced for in either a defined diet or our standard SY medium for 15 days before analysing the response to starvation. (E) Similar to **(D),** but flies were induced for 30 days. For all of these experiments N = 90 flies per condition.

Take together these results suggest that the starvation-like phenotype induced by Af31_42 switches on a metabolic programme that allows flies to cope when nutrient intake is poor (low yeast or reduced feeding behaviour), but is restored as nutrients are supplied in optimal conditions (as when the yeast content in the media is increased or EAA are

supplemented). However, the switch that modulates the response to starvation seems to be dependent on the balance of nutrients provided by the yeast/EAA and not by carbohydrates. Interestingly, the phenotype is clearly time dependent.

6.4.9 Reduced feeding behaviour lead to impaired lipid metabolism

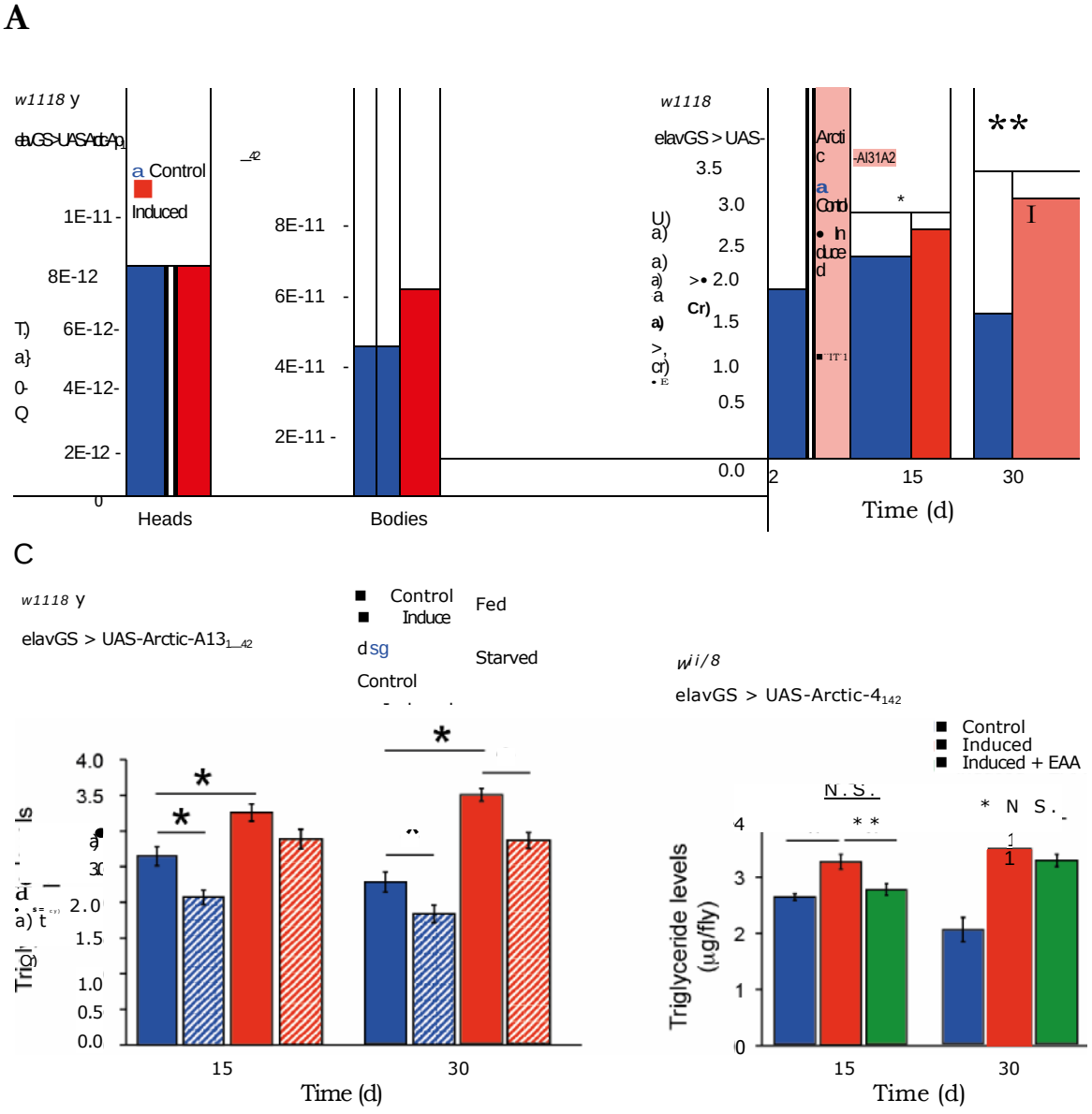

Figure 6.8 ATP and triglyceride levels of flies expressing 4_{1_42} and its modification by starvation and EAA supplementation. (A) ATP measurements in fresh flies after 15 days of induction N= 10 flies per three biological repeats. Protocol performed by Dr. Kerni Kinghorn. (B) Measurement triglycerides after flies had been induced for 15 or 30 days. (C) Measurement of triglycerides after 15 and 30 days of induction under basal conditions and after 48 hrs of starvation. **(D).** Triglycerides measurements after 15 or 30 days of induction in medium supplemented or not with EAA. For all triglyceride measurements 5 biological replicates of 5 flies each were measured per condition tested. * $P < 0.05$, ** $P < 0.01$, t-test or ANOVA post hoc Tukey-Kramer.

To further investigate the nature of the resistance to starvation I first analysed the ATP levels in flies expressing A131_42. ATP levels have been reported to be affected in fly models of neurodegeneration (Chang et al., 2011; Clark et al., 2006). To my surprise flies expressing Af31_42 did not show significant differences in ATP levels when measured in heads and rest of the fly separately (Figure 6.8A; $P > 0.05$, t-test). This suggests that changes in ATP levels are not playing an important role in the stress responses of A131-42 flies.

Flies expressing A131_42 are feeding less and therefore eating fewer nutrients, which would be expected to translate into an imbalance in energy levels. Since ATP levels were indistinguishable from controls, I hypothesised that flies must be maintaining their ATP levels through energy storage despite their poor feeding, in order to survive under starvation conditions. To test this I analysed the levels of triglycerides, the main energy store in *Drosophila* (Baker and Thummel, 2007; Kiihnlein, 2012). Non-expressing control flies showed a slight increase in whole body triglyceride levels after 15 days of feeding with standard fly media and a significant decrease in triglyceride levels after 30 days (Figure 6.8B). Conversely Af31_42-expressing flies displayed a significant age-dependent increase in levels of triglycerides ($P < 0.05$, t-test). These results suggest that flies expressing Af31_42 switch on a metabolic programme to accumulate energy in the form of triglycerides. This reconfiguration of their metabolism would allow for a robust coping mechanism when nutrients are limited in the wild. Indeed flies expressing Af31_42 use triglycerides to a similar extent as control flies when challenged by starvation conditions (Figure 6.8C). Thus, A131_42 leads to the accumulation of triglycerides, as the lipid content is higher in flies expressing $A0_{1\_42}$ allowing them to cope for longer under no nutrient supply.

6.4.10 Supplementation of EAA partially restored the lipid profile

My previous results suggest that the modified responses to starvation are dependent on limited nutrient availability. Therefore I tested whether increased nutrient availability could restore triglyceride levels. Flies expressing A131_42 for 15 days were compared to flies that in addition had been supplemented with EAA. I previously observed that 15 days of EAA supplementation was sufficient to partially suppress the protection against starvation (Figure 6.7B and C). As expected this effect correlated with a complete restoration of the triglyceride levels. Flies expressing A131_42 and supplemented with EAA were not

statistically different from non-expressing control flies after 15 days of induction (Figure 6.8D; $P > 0.05$, ANOVA post hoc Tukey Kramer). However, when the measurements were done after 30 days of AP1_42 expression, the supplementation with EAA was insufficient to significantly lower the increased triglyceride levels, as they remained similarly high as the induced non-supplemented flies (Figure 6.8D; $P > 0.05$, ANOVA post hoc Tukey Kramer).

Taken together my results suggest that the acceleration of age-related anorexia induced by AP1_42 reduces nutrient intake that leads to a starvation-like state. Low nutrient availability in turn seems to promote a switch in metabolism to store energy, whereby flies increase triglyceride levels to help cope with starvation conditions.

6.4.11 Supplementation of EAA increased the lifespan of flies expressing AN-42•

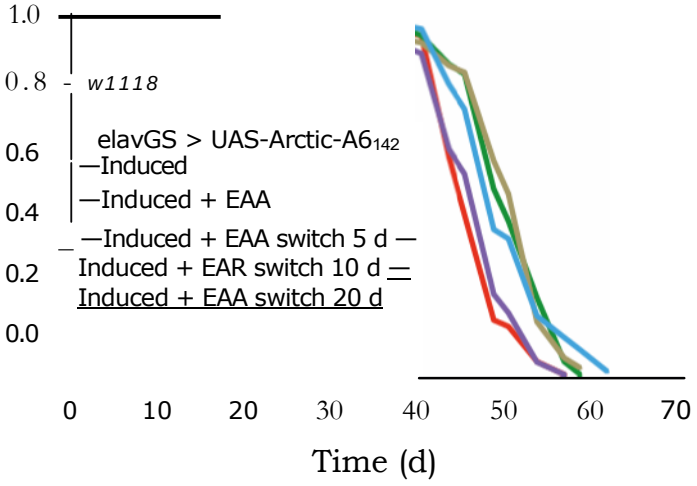

Figure 6.9 Survival analyses of flies supplemented with EAA at different time points during 41_42 induction. Flies were induced to express A131-42 in medium supplemented or not with EAA. From the group of flies induced without EAA supplementation, groups of flies were switched to the supplemented medium after 5, 10 or 20 days of the initial induction. N = 150 flies per condition.

EAA supplementation to A131_42-expressing flies was able to restore triglyceride and the response to starvation (Figure 6.8D and Figure 6.7B). Therefore, I explored the possibility that supplementation of EAA would extend the lifespan of flies expressing $A13_{1\_42}$ in spite of the fact that the normal effect of this intervention is to reduce lifespan (Grandison et al., 2009a). To explore whether EAA supplementation was able to prevent or rescue the lifespan, I either started the induction of A131_42 expression in food supplemented with EAA or vehicle alone. In addition, I switched flies that had been initially induced in our standard medium (SY) to one in which EAA had been added. I did the latter at different time points (Figure 6.9). Induction on media supplemented with EAA showed a significant median lifespan extension of 5 days (11%) in comparison to flies induced on regular media (*P* <

0.01, log rank test). When flies were first induced in our standard media and transferred to food supplemented with EAA after 5 days of induction (when feeding alterations are evident, Figure 6.2A), I observed a median lifespan extension of 7.6 days (17% in comparison to controls; Figure 6.9; P <0.001, log rank test). When the change from regular to supplemented medium was done after 10 days of AP1_42 expression, EAA supplementation was still able to extend median lifespan, though to a lesser extent (only 2.6 days or 6%; P < 0.05, log rank test). However, after 20 days of induction the supplementation of EAA had no effect on lifespan (P > 0.05, log rank test).

These results highlight firstly that flies expressing $Af3_{1\_42}$ are indeed malnourished, and that macronutrient supplementation is necessary to compensate for their poor food intake. Secondly, as the starvation-like state accentuates over time (with Af31_42 chronicity) the nutritional requirements are likely to increase correspondingly, and therefore the same supplementation is unable to rescue their survival and to restores their lipid and starvation response.

6.4.12 AP1_42 and starvation share a common transcriptional response

Finally, we decided to explore whether the transcriptional profile of flies expressing Af31_42 shared similarities with the transcriptional response of flies that had been starved. For this we used a head transcriptional dataset of flies starved for 24 hrs (Farhadian et al., 2012) and an Af31_42 dataset (Favrin et al., 2013). When both transcriptional responses were overlapped we observed that there was significant overlap between differentially expressed genes elicited by A131_42 and starved heads (Figure 6.10A; $P < 0.0001$; Fisher's exact test). Further analyses revealed that the overlap of these transcriptional datasets was also significant at the down-regulated levels (P = 1.39e-05; Fisher's exact test), which suggests that a similar set of genes are regulated by starvation and Af31_42. However, a strong correlation was also detected between genes up-regulated by A131_42 and down-regulated by starvation (P = 1.8E-09), which suggest that Af31_42 differentially regulates them in the opposite direction (Figure 6.10B). These results suggest that part of the transcriptional response elicited by A131_42 is similarly modified by starvation, but most of the response went in opposite directions. Perhaps Af31_42 and starvation have common molecular mechanisms that are regulated accordingly to ensure survival. Careful examinations of the genes involved in the transcriptional response by both interventions could enrich our

understanding of why flies expressing AP1_42 reduced their feeding behaviour and are resistant to starvation.

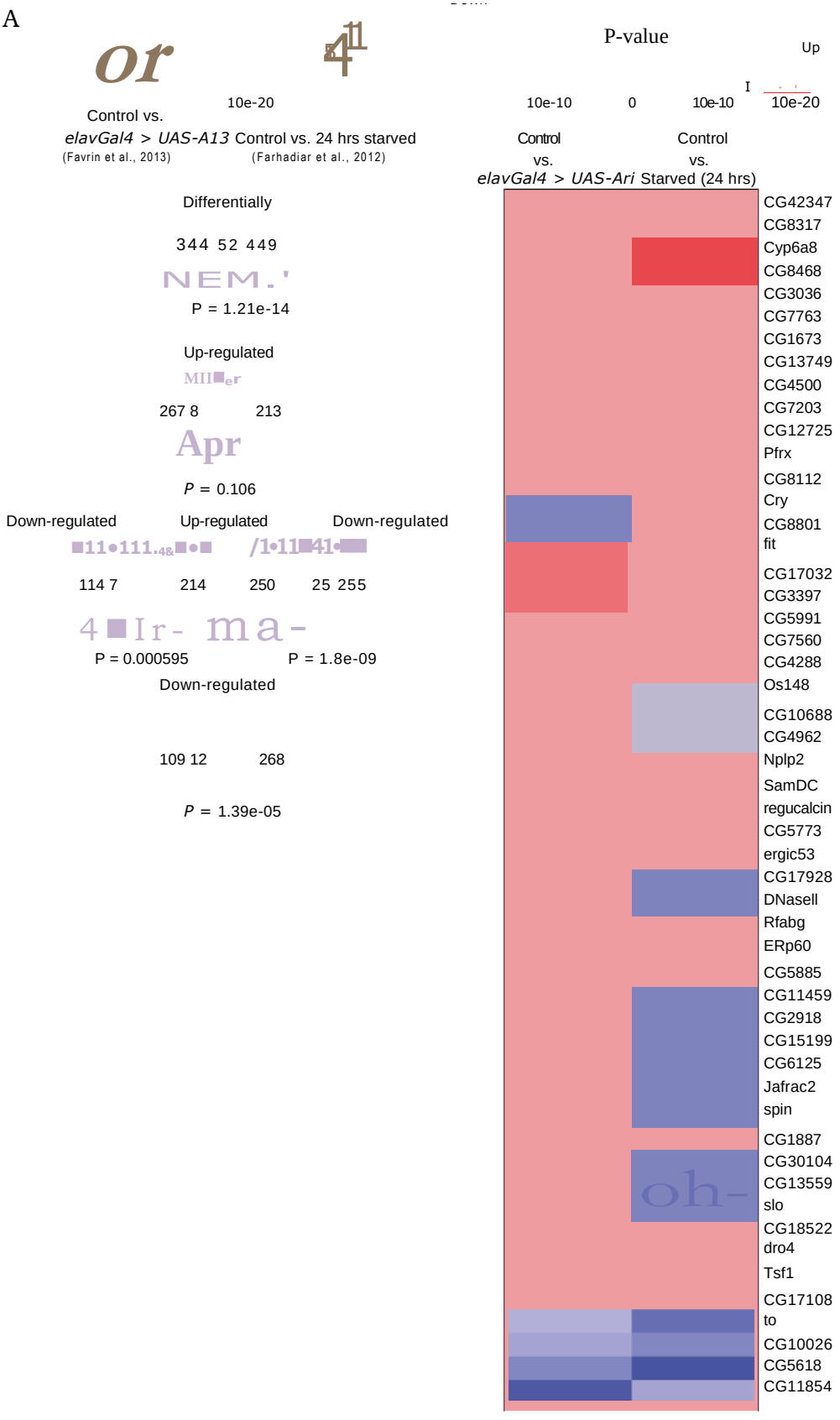

Figure 6.10 Transcriptional overlap between flies expressing $A\beta_{1\_42}$ and flies that had been starved for 24 hrs. (A) Overlap of genes regulated by $A\beta_{1\_42}$ or starvation. (B) Heat map of the differentially expressed genes that overlapped. Analyses for these experiments were done by Dr. Dobril Ivanov.

6.5 Discussion

6.5.1 Anorexia is the earliest sign of neurodegeneration due to $A\beta_{1\_42}$

Adaptations to stress are amongst the most evolutionary conserved mechanisms to ensure survival (Fulda et al., 2010; Kroemer et al., 2010). As a result of evolutionary development, multicellular organisms have acquired endocrinological messengers that enable communication between distant organs, allowing a systemic response that would ensure either a quick restoration of homeostasis, a chronic adaptation (with or without increased survival) or death (Coburn et al., 2013; Durieux et al., 2011; Leopold and Perrimon, 2007). In trying to decipher the response to chemically induced forms of stress in *Drosophila* expressing the Af31_42 peptide in neurons, I observed that the earliest sign of neurodegeneration is reduced feeding behaviour. Reduced feeding with elderly age is a common feature between flies and humans (Morley, 2001; Payette and Gray-Donald, 1995; Wong et al., 2009). Therefore, the reduction in feeding secondary to the presence of the Af31_42 peptide can be considered an accelerated ageing phenotype, as is the effect of A131_42 on *Drosophila's* locomotor ability (Gargano et al., 2005; Kerr et al., 2011; Sofola et al., 2010). However, the reduced feeding behaviour precedes the locomotor decline in both control flies and in the AD fly model. Feeding alterations have also been reported in a mouse model of AD (Adebakin et al., 2012), although this model shows increased feeding behaviour possibly because of a gut-brain signalling malfunction. Reduced feeding behaviour or caloric intake have been observed in patients with AD, commonly in advanced stages of the disease, though reduced caloric intake early in the disease has also been reported (Shatenstein et al., 2007).

6.5.2 AP142-expressing flies are nutritionally deprived and lived longer when supplemented with EAA

Lowering food intake extends lifespan across evolutionary distant organisms (Fontana et al., 2010; Piper and Bartke, 2008). Lowering food intake in the context of impaired proteostasis would allow the organism to reduce the rate of protein translation, thereby reducing the cellular burden of toxic proteins, and to increase the degradation of damaged proteins and organelles through autophagy (Bjedov and Partridge, 2011; Taylor and Dillin, 2011). Lowering the activity of the IIS or mTOR pathways have been shown to improve several phenotypes in AD models (Cohen et al., 2009; Killick et al., 2009; Majumder et al., 2011; Spilman et al., 2010). This is complementary to the reports showing that DR improves Af31_42 pathology (Patel et al., 2005; Qin et al., 2006). On the other hand, increased nutrient intake, which limits lifespan, is associated with increased risk of developing AD (Fontana et al., 2010; Luchsinger et al., 2002). Therefore my results showing that EAA supplementation extended the lifespan of flies expressing A131_42 was somehow unexpected. However, there is a limit to the degree of DR beyond which malnourishment is established (Fontana et al., 2010; Piper and Bartke, 2008). I consider that the reduced feeding behaviour and secondary nutrient deprivation have tipped the AD flies to the malnourishment side of the DR tent (shift to the left; see Chapter 1). As nutrients are restored lifespan is extended. Thus, my results support that flies expressing Af31_42 are nutritionally deprived and this compromises their survival.

6.5.3 Ap_{142}-induced anorexia altered the response to orally-delivered toxins: implications for drug studies

A secondary consequence of the reduced feeding behaviour observed in flies expressing A131_42 was that toxins delivered through the fly media were less bioavailable to the flies. This translated in inconsistent results when flies were assessed for their response to the pro-oxidant PQ (sometimes showing sensitivity, but most commonly resistance to PQ). Similarly, flies expressing A131_42 were resistant to H202 when delivered through the fly medium. When PQ was administered to the flies as a single intrathoracic injection, they showed an age-dependent sensitivity. Thus, Af31_42 increases vulnerability to oxidative stressors and this phenotype is also present before the onset of locomotor alterations.

These results also have significant implications for using this model to test potential therapeutic compounds that are delivered in the fly medium. Higher

concentrations of drugs may be required to gain therapeutic concentrations and potentially beneficial compounds may have been previously missed as a result of their poor oral uptake in Af31_42-expressing flies.

6.5.4 $Ap_{1–42}$ induced starvation resistance through alterations in lipid metabolism

A significant result observed in this study was that flies expressing Af31_42 were extremely resistant to starvation (doubling the lifespan of control flies). This was unexpected given that they barely eat and therefore would be expected to be very sensitive to starvation conditions. However, flies are equipped to sense low nutrient availability and switch on a programme to store energy, primarily in the form of triglycerides. I showed here that although indeed the Alzheimer's flies seem to be starving, they do have higher levels of triglycerides, the main energy store in *Drosophila.* Since they have higher energy stores, they are able to cope for longer when completely deprived of nutrients (Ballard et al., 2008). I established that this effect is likely dependent on protein/EAA. Flies that were induced to express Af31_42 in medium without yeast (but containing sucrose) were sensitive to starvation. As the yeast content increased to our standard medium levels the flies gradually developed the resistance to starvation phenotype. This effect was restored to control fly levels by adding 50% more yeast to the medium. Similarly, adding EAA to the medium blunted the starvation resistance phenotype induced by Af31_42 and restored the lower levels of triglycerides typical for wild-type flies. Such a switch has not been identified in humans, though lower meal frequency has been associated with higher circulating lipid levels (Farshchi et al., 2005). Higher circulating lipid levels have been associated with increased Af31_42 deposition load in AD patients (Reed et al., 2013). Moreover, elevated circulating levels of triglycerides have also been reported in two mouse models of AD. In these models the increase in triglycerides preceded A131-42 deposition (Burgess et al., 2006). Though the latter effect is likely mediated by high levels of circulating amyloid, which could interfere with enzyme(s) important in lipid metabolism, these results highlight the fact that Af31_42 pathology can directly and indirectly impair lipid metabolism.

6.5.5 What underlies the behavioural and metabolic alterations induced by AP1-42?

Food intake is controlled by a sophisticated set of behaviours regulated by a number of specialised genes and is aimed at maintaining nutritional homeostasis (Itskov and Ribeiro, 2013). I further explored the microarray data set used throughout the results for potential transcriptional changes that could suggest a mechanism for altered feeding (Favrin et al., 2013). To my surprise I found that at least two neuropeptides involved in the regulation of feeding were altered. Though I have not confirmed these results, I would like to speculate about their potential relevance. Little is known about their involvement in the adult fly, but larval studies are very informative. Over-expression of *hugin* suppresses feeding in larvae. Interestingly, reduction of *hugin* in adult flies translates into early initiation of feeding behaviour (Melcher and Pankratz, 2005). The transcriptional data set by Favrin et al., (2013) suggested that Af31_42 increases the expression of hugin. *Hugin* is the fly ortholog of Neuromedin U (NMU) in mammals. NMU has limited expression in the brain, but high levels are present in the hypothalamus, anterior pituitary, nucleus accumbens, globus pallidus, septum, amygdala and medulla oblongata (Budhiraja and Chugh, 2009). Its limited distribution in the brain highlights its role in the regulation of energy balance. When NMU is administered intracerebroventricularly (i.c.v.) reduces food intake in several species including rats and chicks. Contrary, when anti-NMU antisera is delivered i.c.v. it increases feeding in rats (Budhiraja and Chugh, 2009; Mitchell et al., 2009). Elegant genetic mouse models have shown that NMU knockout animals are hyperphagic and obese, while its over-expression reduces food intake and makes animals leaner (Hanada et al., 2004; Kowalski et al., 2005). NMU is therefore an anorexigenic neuropeptide and is gaining renewed interest as it has potential as an anti-obesity target (Mitchell et al., 2009).

Another neuropeptide seemingly regulated by A131_42 is neuropeptide F (NPF), the ortholog of neuropeptide Y (NPY) in mammals (Itskov and Ribeiro, 2013; Wu et al., 2003). NPF seems to have similar function as NPY in promoting feeding behaviour (Wu et al., 2003, 2005). NPY GABAergic neurons control feeding behaviour by inhibiting the inhibitory function of pro-opiomelanocortin and are therefore anorexigenic. Centrally delivered NPY (i.c.v.) promotes a robust feeding behaviour in different mammalian models (Beck, 2006; Chee and Colmers, 2008). Of importance to my results is the fact that adult-only ablation of NPY-producing neurons reduces feeding and promotes weight loss in mice (Luquet et al., 2005). The microarray data set suggested that the anorexigenic

signal from *hugin* was increased while the orexigenic (feeding promoting) effect was reduced in Af31_42-expressing flies (Favrin et al., 2013).

The findings suggested by the microarray data are suggestive, yet I need to corroborate them at the mRNA and/or protein level. However, they would potentially point towards a particular sensitivity that these neurons would have to the expression of A131-42. They might even suggest that expressing A01_42 only in these neurons could be sufficient to produce relevant phenotypes in *Drosophila*. I will be looking at these neurons with particular interest in the next coming months.

Chapter 7

Final thoughts: drugs in ageing and age-related diseases

"To him who devotes his life to science, nothing can give more happiness than increasing the number of discoveries, but his cup of joy is full when the results of his studies immediately find practical applications."
Louis Pasteur

7.1 General conclusions: treating ageing

Ageing is undeniably an intriguing area of study in biology. The challenges are two tiers. First, the quest for the preservation of the youthful state has inspired scientists and conquerors to speculate not only of the existence of a magic remedy to preserve it, or to reverse to it once lost, but also as to how science can provide answers. Secondly, ageing remains to be an enigmatic area in evolutionary biology. Why has evolution maintained a process that leads to an unfit state? Evolutionary biologists have wondered hard and long about this. The jury is still out there. We are still far from understanding the processes that have led to the existence of the ageing process. While some have considered it a misfortunate trait that allows fitness early on in life, others have proposed that mutations accumulate leading to an increase burden of molecular damage that the organisms' repair mechanisms are unable to cope with it.

Furthermore, ageing has become an attractive area of study in the biomedical field because of its implication to public health. Ageing is the major risk factor for the most prevalent killer diseases of modern times. Estimations have made evident that the contribution of curing these diseases (dementia, cardiovascular and cancer), individually or collective, to life expectancy are minimal in comparison to eradicating the ageing process. Life expectancy is not expected to cap at any time soon, in spite of the twin epidemic of obesity and diabetes. As our populations live longer, age-related diseases become more prevalent. This is in spite of the compression of morbidity that is allowing the rise in life expectancy. So how is research into ageing going to contribute to alleviate this problem? Over the last 30 years, biogerontology has really taken off as an important part of biomedicine. The first observations that ageing could be retarded by genetic manipulations came from studies in the nematode *C. elegans* and soon after these same interventions

were shown to be evolutionary conserved in fruit flies and mice. This period in biogerontology has often been termed as the "new science of ageing" given that it showed the revolutionary principle that ageing is plastic and amenable to genetic manipulations. It is not a surprise that ever since the field has exploded. Nowadays, manipulations to single genes are very often reported to affect lifespan and healthspan in model organisms. The challenge now has become to find ways to translate these fmdings from comforts of basic research into the clinic to have a real impact in public health. The most obvious way is to synthesise drugs that can act on the pathways already identified by genetics. However, this approach has many pitfalls. First, drug development is a very lengthy process that often requires over a decade (in most cases longer) to reach pharmacies shelves. This applies to the fortunate case in which a drug passes the complicated steps of Phase 1 and Phase 2 clinical trials. Second, ageing is not a disease, therefore agencies regulating the safety and efficacy of drugs would not allow the compound to be tested for the required experimentation for human use. Indeed, anti-ageing compounds are regularly found in shops specialised in nutraceuticals and supplements, as they are not licensed for disease treatment. An alternative is to test the pro-longevity effect of compounds already licensed for human use and to repurpose them. This is a strategy that has been gaining popularity in the pharmaceutical industry as measure of optimising the huge investments they often make to take compounds to market. Finding a new application for a drug that is already approved accelerates the process required for the drug to be approved for an alternative disease as it is very likely that the drug has already complied with the earlier phases of clinical experimentation.

Rapamycin and metformin have now become the pillars of this new wave of drug repurposing. Rapamycin is licensed as an immunosuppressant and chemotherapeutic agent, while metformin in anti-diabetic drug. Both these drugs can extend lifespan, and most importantly healthspan in evolutionary distant organisms, which makes them ideal drugs to be considered for the treatment of ageing. Rapamycin is often dismissed as a real anti-ageing compound given its side effects. For example, rapamycin leads to glucose and lipid abnormalities which could negatively impact human health in the long run. Additionally as rapamycin is licensed as an immunosuppressant there is concern that its use could lead to deadly infections, particularly at older ages, when the immune system is not at its fullest capacity and infections like pneumonia are particularly dangerous.

Metformin is one of the most prescribed drugs in the world and its potential to improve healthspan is greater, in spite of its side effects often confined to the

gastrointestinal system. However, the only report to have shown that metformin extends lifespan in mammals showed that the effect was rather small, which begs the question as to whether metformin acts to modify longevity or to improve health by acting on diseases. Recently David Gems made the argument that even compounds acting on specific diseases rather than on the ageing process should be considered anti-ageing as they are likely to contribute to some aspects of the ageing process. According to Gems anti-ageing medications could be of three classes:

1. Drugs that act on the ageing process by promoting lifespan extension without necessarily increasing healthspan.
2. Drugs that promote healthspan and lifespan by acting on the ageing process.
3. Drugs that act on disease, which could be viewed as a minor branch of the ageing process.

Drugs acting by any of these mechanisms are likely to contribute to public health, but drugs that act both in healthspan and lifespan are the most likely to show broad spectrum benefits for disease and thus impact public health.

7.2 Lithium and GSK-3 in ageing and neurodegeneration: an integrative perspective

My results showed that lithium is a pro-longevity drug that can extend lifespan independent of sex and genetic background. Importantly, the lifespan extension conferred by lithium did not reduce fecundity and improved metabolism by lowering triglyceride levels. Reduced fecundity and altered metabolism (increases levels of carbohydrate and lipid metabolites) are among the features of DR, IIS down-regulation and rapamycin treatment. Lithium seems to be different to these interventions. The lipid-lowering ability of lithium proved to be very effective at blocking the hypertriglyceridemia produced by rapamycin treatment. Moreover, the combination of lithium plus rapamycin proved to be more effective than either treatment on its own for lifespan extension. I discussed in a previous Chapter that these could be relevant for the establishment of a polypill for ageing. These fmdings make lithium an exciting and interesting drug for future work.

In Chapter 6 I described how flies expressing A131_42 show altered metabolism secondary to poor food intake. We have previously shown that lithium is able to improve

the locomotor capacity and lifespan of these flies (Sofola et al., 2010; Adesakin-Sofola et al., 2014). However, in light of the new phenotypes described here it would be relevant to assess the potential added benefits of supplementing flies expressing $Af3_{1\_42}$ with lithium. Expression of Af31_42 leads to reduced feeding, starvation resistance and high lipid content. Given that lithium reduces triglyceride levels, this could potentially be tackled by supplementing these flies with lithium. Another potential added benefit would be stress-resistance. I showed in Chapter 4 that lithium increases stress resistance by allowing a transcriptional response capable of mounting detoxification and protection against oxidative stress. Moreover, I showed in Chapter 6 that flies expressing A131_42 show altered stress response and potentially this is because of poor regulation of CncC. Therefore, lithium could be beneficial at regulating the response to stress in flies expressing $Af3_{1\_42}$. A confounding effect of lithium treatment in flies is that it reduced the synthesis of Af31_42 as it impacts on translation initiation (Adesakin-Sofola et al., 2014). Reducing translation would lower the level of the peptide being induced before it causes damage, this intervention would be as effective as lowering (or even eliminating) the inducer from the medium. However, if the treatment was applied after Af31_42 has already accumulated in neurons I should be able to see rescue of the effects in CncC and stress resistance. I have preliminary data to suggest that this could be overcome by supplementing lithium only after the production of AP_{1_42} has been stopped. Rogers and colleagues showed that even when the induction of AP1_42 is stopped, the peptide remains in the fly head until it dies, and only brief periods of induction are enough to elicit the survival and locomotor phenotypes (Rogers et al., 2012). I have expanded this to the new phenotypes I described here. Additionally my preliminary data suggest that lithium could impact on all phenotypes even when AP1_42 is not being lowered. In a way this is not surprising given that lithium can improve many features in control flies.

What would be the next step for lithium? First a mechanism of action would need to be consolidated in *Drosophila.* Second, evolutionary conservation will need to be proved. In Chapter 3 I discussed some strategies as to when and how to evaluate the effects of lithium treatment in rodents. These considerations might be useful in moving the drug forward. Third, as with rapamycin, once clear mechanisms of action are established, testing for more selective compounds would be appropriate. Lithium is a very dirty drug; perhaps this is its best feature at the same time that it has been my worst nightmare. Though it would be incredibly satisfying to find one mechanism of action to explain all of the positive features of lithium, I think this is rather unrealistic. This is not only because

lithium is known to interact with many biological pathways, but also because it is very complicated to dissect complex organismal traits with one drug. Assuming that all cells will react in the same way to a particular stimulus would be denying the beauty and complexity of multicellularity. As muticellular organisms respond in concert rather than as isolated cells, cell-specific responses can be easily masked by the overall organismal response. Moreover, because some cells and tissues are to some extent more vital than others, it is likely that they will be spared for longer at the expense of 'less' vital tissues. All of these responses need to be considered when evaluating complex traits like ageing, stress-response and metabolism. My data showing that *sgg* expression has tissue specific effects clearly highlight that different cell types respond differently to stressors. Understanding the contribution of specific cells to the overall organismal struggle for survival should be amongst the concerted efforts of the biomedical community as a whole.

7.3 Are drugs the way forward in the ageing field?

DR is the most studied intervention to promote healthy ageing. DR has been shown to increase lifespan, to promote health, and to ameliorate a host of age-related diseases in almost all organisms tested. It is very unlikely that DR will become the cure for ageing. The benefits of DR have long been known and nothing has changed so far. The ageing filed does require the aid of pharmacology to drive the field forward. As discussed before, I considered that it is unlikely we will find the one drug that will cure ageing. Instead we will need to find cocktails of drugs. My data on the combination of lithium and rapamycin is encouraging, but if it was up to me I would immediately add aspirin, a statin and metformin to the mix. Studying the interaction of drugs will be an essential requirement for the new era of interventional biogerontology.

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Appendix

Appendix 1

Castillo-Quan it Parkin' control: regulation of PGC-1 a through PARIS in Parkinson's disease. Dis Model Mech 2011; 4: 427-429.

Appendix 5 PCR primers

Gene	Sequence
Sgg	TCACAGAGCTTCAGCACAGC
pUAS	AACCAGCAACCAAGTAAATCAA
dFOXO (forward)	TTGGTAGTGCCTATGATCCAG
dFOXO (reverse)	AAGGTAGTGCCTAGATCCAG
Chico (forward)	AGTTAATTCAAACCCCACGG
Chico (reverse)	AAACGGCGATTGATGTTGAAG

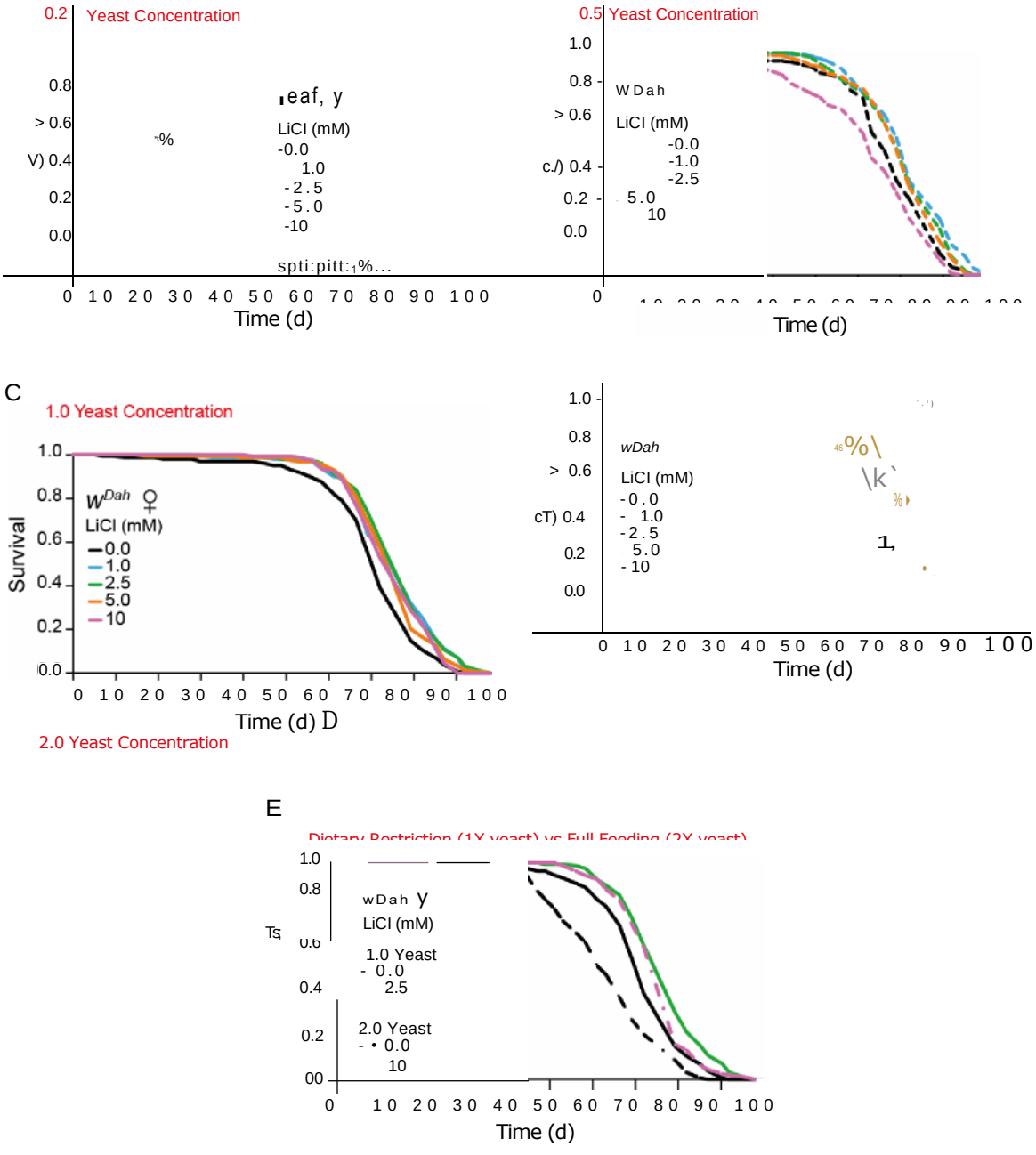
Appendix 6 qPCR primers

Gene	Sequence
Scylla (forward)	GGATCAAACAAAAATAAAGATTGG
Scylla (reverse)	TCTTGGCCTTCTTGGACATC
Charibdys (forward)	ATCCGTCGGTATGACCACTC
Charibdys (reverse)	CAGCACGTTGGTCATGTAGG
Fz3 (forward)	GCTTTTTGCTCTGCTCGTCT
Fz3 (reverse)	TCCATACACTCGCCCCTAAC
Naked (forward)	CGACTGGAGGAATTCACCTG
Naked (reverse)	AACTGCAGCGGCTGCGATGA
dInR (forward)	GCGGGATACGGCGATTTCAC
dInR (reverse)	AACGATCAGGAACGCTAGGC
Chico (forward)	AGTTAATTCAAACCCCACCGG
Chico (reverse)	AAACGGCGATTGATGTTG
4E-BP (forward)	CACTCCTGGAGGCACCA
4E-BP (reverse)	GAGTTCCCCTCAGCAAGCAA
Actin (forward)	GAGCGCGGTTACTCTTTCAC
Actin (reverse)	GCCATCTCCTGCTCAAAGTC
RP49 (forward)	ATGACCATCCGCCCAGCATCAGG
RP49 (reverse)	ATCTCGCCGCAGTAAACG

Appendix 7 Recipe for SY preparation for DR tent (for 1 L)

Ingredient	0.0 SY	0.2SY	0.5 SY	1.0 SY	1.5 SY	2.0 SY
ddH2O (mL)	700	700	700	700	700	700
Agar (g)	15	15	15	15	15	15
Sucrose (g)	50	50	50	50	50	50
Yeast (g)	0	20	50	100	150	200
Additional ddH2O (mL)	223	212	196	170	144	118
Nipagin (mL)	30	30	30	30	30	30
Propionic acid (mL)	3	3	3	3	3	3

1. Agar was added to the initial volume of water (ddH2O) just before heating. Stirring was continuous thought the preparation process to prevent any of the ingredients sticking to the saucepan.
2. Just after the initial boiling point sucrose was added and brought to boil.
3. The yeast was added and brought to boil again. The saucepan was removed from the heating source and the additional ddH2O was added to cool down the medium.
4. Only after the medium was below 60°C (preferentially between 50 and 58°C) nipagin and propionic acid were added.
5. Medium was ready for dispensing.



Appendix 8. Survival curves from DR tents showed in Figure 3.6. N= 160 females per condition.

Appendix 9 Recipe for EAA solution preparation for 1 L of 1SY medium

Ingredients	Quantity*	Catalogue Number§
Arginine	0.425	A5131
Histidine	0.210	118000
Isoleucine	0.340	12752
Leucine	0.475	L8912
Lysine	0.515	L5626
Methionine	0.100	M9625
Phenylalanine	0.260	P2126
Threonine	0.365	T8625
Tryptophan	0.090	T0254
Valine	0.400	V0500

* to add to a final volume of 50 mL of ddH2O.

§ all from Sigma.

Appendix 10 Ingredients for 1 L of holidic medium

Before autoclaving

Ingredient	Ammount	Notes
Agar	20 g	From Difco (214530).
Isoleucine	0.58 g	From Sigma 12752.
Leucine	0.82 g	From Sigma L8912.
Tyrosine	0.42 g	From Sigma T3754.
Sucrose	17.12 g	From Sigma S1888.
Cholesterol	15 mL	20 mg/mL in 100% ethanol. From Sigma C8667.
Buffer 100 mL	For 1 L mix (in ddH2O) 30 mL of acetic acid (Fisher, A/0400/PB15), 30 g of KH_2PO_4 (Sigma, P9791), 10 g of NaHCO3 (Sigma, S8875).	
CaC12	1 mL	250 g/L. From Sigma C7902.
MgSO4	1 mL	250 g/L. From Sigma M7506.
CuSO4	1 mL	2.5 g/L. From Sigma C7631.
FeSO4	1 mL	25 g/L. From Sigma F7002.
MnC12	1 mL	1 g/L. From Sigma M3634.
ZnSO4	1 mL	25 g/L. From Sigma Z0251.
TOTAL 883.7 mL		

After autoclaving

Ingredient	Ammount	Notes
Nucleic acid / Lipid solution	8 mL	For 1 L mix the following in ddH2O: 6.25 g of choline chloride (Sigma, C1879), 0.63 g of myo-inositol (Sigma, 17508), 8.13 g of inosine (Sigma, 14125), and 7.5 g of uridine (Sigma, U3750).
EAA solution	30.255 mL	See Appendix 9.
Non EAA solution	30.255 mL	See Appendix 9.
Glutamate	9.11 mL	100 g/mL From Sigma G5889.

Cysteine	2.64 mL	50 g/mL in HC1. From Sigma C 1276.
Vitamin solution	14 mL	For 1 L mix (in ddH2O) the following: 0.1 g of thiamine aneurin (Sigma, T4625), 0.05 g of riboflavin (Sigma, R4500), 0.6 g of nicotinic acid (Sigma, N4126), 0.775 g of Ca pentothenate (Sigma P2250), 0.125 g of pyridoxine (Sigma, P9755), and 0.01 g of biotin (Sigma, B4501).
Folic acid	1 mL	0.5 g/L (ddH2O). From Sigma F7876.
Propionic acid	16 mL	From Sigma P5561.
Nipagin	15 mL	100 g/L methyl 4-hydroxybenzoate in 95% ethanol. From Clariant Nipagin M.

Appendix 11 Ingredients for EAA and non EAA solutions for holidic medium

Essential amino acid (EAA) stock solution (in 400 mL of ddH2O)

Ingredient	Ammount	Notes
Arginine	9.4 g	From Sigma A5131.
Histidine	4.48 g	From Sigma 118000.
Lysine	11.48 g	From Sigma L5626.
Methionine	2.25 g	From Sigma M9625.
Phenylalalnine	6.06 g	From Sigma P2126.
Threonine	8.56 g	From Sigma T8625.
Tryptophan	2.91 g	From Sigma T0254.
Valine	8.85 g	From Sigma V0500.

Non EAA stock solution (in 400 mL of ddH2O)

Ingredient	Ammount	Notes
Alanine	10.5 g	From Sigma A7627.
Asparagine	5.55 g	From Sigma A0884.
Aspartic acid	5.55 g	From Sigma A6383.
Glycine	7.15 g	From Sigma G7126.
Proline	3.73 g	From Sigma P0380.
Serine	5.03 g	From Sigma S4500.