UNIVERSITY OF LIVERPOOL

Rilmenidine is a Calorie Restriction Mimetic that Improves Survival and Indicators of Healthy Ageing in *C. elegans* via a Novel IRAS Ortholog, *f13e9.1*

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By

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Abstract

Ageing is currently the inevitable consequence of human existence. The ageing process is a disease that internally imposes limitations and reductions in liberty through enfeeblement, frailty and pathology, leading to dramatic physiological and psychological suffering. Therefore, it is paramount we identify immediate strategies which defer the progression of age-associated diseases and compress morbidity.

At present, calorie restriction (CR) offers the most feasible and robust intervention to decelerate ageing. However, despite the attractive pro-longevity effects of CR, several concerns surrounding the applicability, tolerability and safety have precluded its widespread adoption. Using the Connectivity Map to identify drugs with a similar transcriptome to CR, I sought to repurpose a clinically approved drug that demonstrated broad tolerability and safety whilst representing an easily translatable, potential calorie restriction mimetic.

I identified rilmenidine: a widely-prescribed, centrally-acting antihypertensive that mimicked gene expression changes previously observed in CR. Furthermore, rilmenidine significantly opposed the transcriptional direction of ageing such that it rescued age-associated overexpression of genes enriched for immune-regulation, inflammation and ECM degradation.

I validated the potential geroprotective properties of rilmenidine in the model organism *C. elegans*. Rilmenidine administration extended lifespan in WT worms, when commenced from early adulthood or only once the worm had aged. Furthermore, it decelerated the development of decrepitude, without altering developmental periods. Lifespan extension was not possible in genetic models of CR *(eat-2),* suggesting a longevity effect was induced through CR mimicry. Moreover, this was supported by rilmenidine-induced lifespan effects being dependent on 3 key CR nexuses: *DAF-16*, AMPK and TOR.

In *C. elegans,* Rilmenidine elicited increases in ERK activity, typical of *in-vitro* imidazoline agonist exposure, which was abrogated following blockade of an imidazoline binding site. This effect was mimicked following the knockout of *f13e9.1*, which was characterised herein as the nematode ortholog of the human imidazoline type 1 receptor (IRAS). Indeed, rilmenidine also increased nematode thermotolerance as well as autophagy, both dependent on imidazoline binding, and demonstrated a capacity to attenuate the accumulation of polyQ aggregates. Autophagy was essential to the longevity effect of rilmenidine and was increased independent of the previously hypothesised cAMP pathway.

Thus, this research presents a strong novel case for rilmenidine to be a CR mimetic that warrants substantial clinical investigation as to the auxiliary benefits of rilmenidine which may belie its overt purpose as an anti-hypertensive, such that it be preferentially prescribed to hypertensives which provides a safe, tolerable method of CR mimicry to over 25% of the population.

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Abbreviations

4EBP	eIF4E Binding Protein
βhB	β-hydroxybutyrate
aa	amino acid
ACE	angiotensin-converting-enzyme
AD	Alzheimer's disease
ADMET	absorption, distribution, metabolism, and excretion
ADP	adenosine diphosphate
ALM	anterior lateral microtubule
ALS	amyotrophic lateral sclerosis
AMP(K)	adenosine monophosphate (activated protein kinase)
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BMI	body mass index
bp	base pair
BP	blood pressure
cAMP	cyclic adenosine monophosphate
CC	coiled coil
CCB	calcium channel blockers

CEO	Chief Executive Officer
CFP	Cyan Fluorescence Protein
CGC	Caenorhabditis Genetics Center
CHMP	chromatin-modifying protein/charged multivesicular body protein
CHO	Chinese Hamster Ovary
cMAP	connectivity map
CPRD	Clinical Practice Research Datalink
CR(M)	calorie restriction (mimetic)
CRON	calorie restriction with optimal nutrition
CV	coefficient of variation
CV(D)	cardiovascular (disease)
DAPI	4',6-diamidino-2-phenylindole
DDE	2',5'-Dideoxyadenosine
DDR	DNA damage response
DEG	differentially expressed gene
DMSO	dimethyl sulfoxide
DR	dietary restriction
DSB	double strand break
ECM	extracellular matrix

EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
eIF4E	eukaryotic translation initiation factor 4E
EMS	ethyl methanesulfonate
ERK	extracellular signal-regulated kinase
FC	fold change
FDA	Food and Drug Administration
FDR	false discovery rate
FEV1	forced expiratory volume in one-second
FGFR	fibroblast growth factor receptor
FOXO	Forkhead box protein O
FRET	fluorescence resonance energy transfer
FUDR	floxuridine
FVC	forced vital capacity
GABA	gamma-Aminobutyric acid
GEO	Gene Expression Omnibus
GH(A)	growth hormone (receptor antagonist)
GHRKO	growth hormone receptor knockout

GI	gastrointestinal
GLUT4	glucose transporter type 4
GO	gene ontology
GOF	gain of function
GPBAR1	G-protein coupled bile acid receptor
GSK	GlaxoSmithKline
HF	high-fidelity
HFD	high-fat diet
HIF	hypoxia-inducible factor
HSP	heat shock protein
HSR	heat shock response
I1R(A)	imidazoline type 1 receptor (agonist)
IBS	imidazoline binding site
ICL	interstrand crosslink
IGF(R)	insulin growth factor receptor
IIS	insulin/IGF-1 signalling
IQR	interquartile range
IR	insulin receptor
IRAS	imidazoline receptor antisera-selected

IRR	insulin related receptor
IRS	insulin receptor substrate
ISS	insulin substrate signalling
ITP	Intervention Testing Program
LB	lysogeny broth
LINCS	Library of Integrated Network-based Cellular Signatures
LKB1	liver kinase B1
LOF	loss of function
LRR	leucine rich repeat
LVH	left-ventricular hypertrophy
MOR	Mu opioid receptor
mTOR	mammalian target of rapamycin
MVBs	multivesicular bodies
NAC	N-acetylcysteine
NCBI	National Center for Biotechnology Information
NGM	nematode growth media
NGS	next generation sequencing
NICE	National Institute for Health and Care Excellence
NIH	National Institute of Health

PAGE	parametric analysis of gene-set enrichment
PAK1	P21 activated kinase 1
PCA	principal component analysis
PCNA	proliferating cell nuclear antigen
PC-PLC	phosphatidylcholine-hydrolyzing phospholipase C
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PI3K	phosphoinositide 3-kinase
PI3P	phosphatidylinositol 3-phosphate
PKC	protein kinase C
PLM	posterior lateral microtubule
PP2A	protein phosphatase 2A
PR	protein restriction
PTEN	Phosphatase and tensin homolog
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
ROS	reactive oxygen species
RSK	ribosomal protein S6 kinase
RT	reverse transcriptase

RVLM	rostral ventral lateral medulla
S6K1	p70S6 Kinase 1
SASP	senescence-associated secretory phenotype
SA-β-gal	senescence-associated β-galactosidase
SEM	standard error of the mean
SERPINs	serine protease inhibitors
SHBG	sex hormone binding globulin
SNA	sympathetic nerve activity
SNP	single nucleotide polymorphism
SNS	sympathetic nervous system
SOD	superoxide dismutase
SREBP	sterol responsive element binding protein
SRS	Silver–Russell syndrome
SSB	single strand break
T2DM	Type 2 Diabetes mellitus
TAE	Tris-acetate-EDTA
TBST	tris buffered saline with Tween
TCA	the citric acid cycle
TORC1	target of rapamycin complex 1

TSC	tuberous sclerosis complex
UK	United Kingdom
ULK-1	Unc-51 Like Autophagy Activating Kinase 1
US	United States
UV	ultraviolet
WGS	whole genome sequencing
WHO	World Health Organization
WIF	WNT inhibitory factor
WT	wild type
YFP	yellow fluorescent protein

1. Introduction

1.1. A Meditation on The Purpose of Ageing Research And The Acceptance of Death As a Postponable But Necessary Inevitability

1.1.1. Immortality Quests To Buffer Mortality Salience

The inevitability of death fascinates, haunts and compels human existence. Indeed, life itself must be defined by limitation to exist; the immutability of transience is inextricably woven into the fabric of reality. Hence, a sense of moribund impermanence has been ingrained into the human psyche and thus, not unsurprisingly, man has sought to soothe his mortal soul in the contemplation of immortality.

Humanity's oldest recorded tale, the Sumerian epic of "Gilgamesh", written during the latter half of the third millennium, vicariously confronts transcendent immortality with all its desperation, greed and wisdom (Dalley, 2000). Gilgamesh was a demi-god king who became tortured by the prospect of his own mortality. Initially told that he would be given rule over the city of Urak in lieu of immortality, he accepts his fate and seeks to become The Great Ruler, escribing his legacy into history to remain long after his physical departure. However, this *joie-de-vivre* soon evaporates, when his rampaging and narcissistic adventures, are punished by the Gods through the death of his good friend Enkidu. Petrified by cold reality of his impending fate, Gilgamesh revolts against death, denouncing it as an evil crime against the ordered and good universe (Mason, 1970.). He begs the Gods to let him live forever and is promised immortality upon the completion of two tasks: firstly, to remain awake for 6 days and 7 nights, and secondly to retrieve the flowers of life, from a river bed. Alas, encumbered by his earthly limitations, Gilgamesh falls woefully short and eventually resigns himself to his fate, proclaiming, "Only the gods live forever . . . As for mankind, numbered are their days; Whatever they achieve is but the wind!" (Starr, 2010).

The themes of this tale have been echoed throughout the ages, universally in all cultures: an imprudent quest for longevity, driven by an egocentric fear of death, fruitless in its conclusion. The desire for immortality, routinely presented in literature and mythology, is thought to tame and buffer our sense of vulnerability and impermanence. The human mind is remarkably complex in its self-awareness and thus the comprehension that life must end, when juxtaposed with the Darwinian predisposition for the continuation of life, provides Mankind with an unprecedented level of existential anxiety (Landau et al., 2007). As asserted in the Terror Management Theory, in order to avoid this continuous confrontation, society has constructed cultural worldviews that give meaning to life and enduring value and significance

unto themselves (Pyszczynski et al., 2015). Through the adoption of social roles and the consequent satisfaction of standards of value, one's social and cultural worth is increased leading to a sense of self-worth and esteem (Pyszczynski et al., 2004). In this instance, the contemplation of death is so costly, that the creation of literary, mythical and religious worlds is a necessity to provide a context of unceasing heroism. Likewise, participation in cultural values enables people to feel part of something larger, more significant, and more eternal than their mortal self, thereby imbuing the finitude of life with meaning (Pyszczynski et al., 1997). In turn their contribution and legacy may live on and offer symbolic immortality, as attempted by Gilgamesh in early parts of his epic. Belief systems, religions and stories that denied the finality of death may have evolved and grown in part due to their all-encompassing and compelling offer of literal immortality (Yetzer, Pyszczynski & Greenberg, 2018). Whilst such offers of immortality have been a mainstay in mythology and literature for most of humanity, the development of radical lifespan extension as a rational and diligently pursued narrative to off-set the final confrontation, has been prominent since the age of enlightenment (Cave, 2012). Such endeavours constitute the Elixir story of Cave's four main death-denial mechanisms; the others comprising of resurrection, continuation of the soul and symbolic eternity in your legacy (Cave, 2012). Whilst most anti-aging interventions were literary and symbolic rather than plausible, as early as 1550, the paradigm had shifted to more conservative and achievable perceptions of lifespan extension: Luigi Comaro's Art of Living Long, espoused sage advice on the importance of moderation in the maintenance of vitality into old age. Considered by some to be the first book of dieting, its popularity has had a profound influence on the modification of lifespan and only the Bible and the Koran have had more re-printings (Hayflick, 2006).

Throughout the following centuries, concepts of fighting the ageing process matured, the prospects of entirely defeating the laws of entropy eroded, and the preservation of functionality in old age became paramount (Stambler, 2014). However, to dismiss all early meditations on radical longevity as charlatan whims, would serve to be blind to the wealth of biomedical contributions that the quest for lifespan extension has offered. Indeed, whilst the motivation was to find the elixir of youth, along the way pre 20th century life-extension attempts have produced the modern hormone replacement therapy or therapeutic endocrinology at large, the benefits of probiotic diets, and adjuvant-driven immunotherapy (Stambler, 2014).

During the early 20th century, rejuvenative anti-aging science became a well-established scientific field with hundreds of publications on the subject in the years preceding the 2nd World War (Stambler, 2014). But such research remained speculative and primarily academic: real changes in average lifespan were occurring through the direct treatment of disease. Often considered the greatest human accomplishment

of the last century, from the beginning of the 20th century, and the 110 years that followed, life expectancy in the Western world nearly doubled (Oeppen and Vaupel, 2002). This remarkable and indirect increase in lifespan expectancy came primarily through the reduction of childhood mortality mediated by improvements in basic public healthcare. In the first half of the 20th century, average life expectancy from birth, increased by nearly 20 years owing to improved sanitation and working conditions, better nutrition and housing, organized sewage disposal, improved animal and pest control, water chlorination, national vaccination programs, development of antibiotics and better medical practice (Fontana, 2009). Thus, the infectious disease model emerged for increasing lifespan. Quicker and more lucrative lifespan returns were possible via a whack-a-mole style treatment of emergent societal diseases. The epidemiological transition of life expectancy and an improved economic situation led to the replacement of infectious diseases with the chronic sequalae of non-communicable diseases (Omran, 2005). In this vein, the latter half of the 20th century saw more modest reductions in mid-life mortality through the curtailment of the emanating heart disease epidemic which was treated by advances in pharmaceutical cholesterol and hypertension control (Clarke et al., 2009). Subsequently, this resulted in an increase in UK life expectancy of approximately 10 years between 1950 and 2000 (Office for National Statistics, 2019). In the 21st century, earnest battles against cancer, Alzheimer's Disease (AD) and other age-associated disease have been continuing with ongoing progress in reducing mortality rates. Life expectancy has grown linearly for nearly 100 years now, but the maximum achieved lifespan has entirely plateaued since the 1990s and the length of a healthy life span has remained unchanged (Vaupel, 2010; Robine and Cubaynes, 2017; Lakdawalla et al., 2004). A distribution shift has occurred and death and morbidity have been concentrated at older ages: the failure of success (Gruenberg, 1977). Children born in this decade have an 85% chance of dying after 65 years from aged-related diseases far more complex, intertwined and chronic than previous models (Olshansky, 2015). Diminishing returns are inevitable as life expectancy approaches maximum lifespan and thus an emergent Faustian bargain has been proposed (Olshansky, 2017).

Faust, the eponymous character of Goethe's magnum opus, was a fictitious scholar, much loved by God. His virtuous quest for erudition attracted the attention of Mephistopheles, the Germanic folklore demon. Becoming increasingly unsatisfied and despairing at the vanity of scientific, humanitarian and religious learning, Faust turned to magic for the showering of infinite knowledge. Mephistopheles tempts Faust into unlimited knowledge and pleasure for a limited time in exchange for his soul, which he duly agrees. Comparatively, human desire for immortality appears insatiable, and such rapid and profitable returns in lifespan have of course been met with a Faustian-like price in the burden of disease, increased financial costs of care and pension schemes met with an aged and infirm population (Olshansky, 2017).

As discussed, previous gains in life expectancy were mostly due to reductions in mortality and as such the Cave's elixir of life has remained elusive. Disease and death are still inescapable, merely in abeyance, in echo to Goethe's tale. However, policy makers today continue to pursue the current medical model of disease treatment offering incrementally diminishing growth in life expectancy. The Department of Health and Social Care policy paper intends to improve healthy life expectancy by an additional 5 years by 2035, through preventative healthcare (Department of Health and Social Care, 2019). This is a noticeably conservative outlook formulated on a remodeling of cardiovascular disease prevention of the 70's targeted to contemporary problems of obesity, alcohol consumption and sleep deprivation (Cheung, 2018; Department of Health, 2016). Whilst this strategy has been excellent at improving quality of life for those at risk of premature disease, it offers little distraction from the salience of mortality and the fear of impending suffering and frailty which is a narrative not everyone is willing to follow (Fontana et al., 2014).

As early as 1956, the concept of replacing the single disease model with an anti-aging model was being formalised as evidenced by McCay et al. concluding that treating ageing itself would be, "retarding the onset of certain chronic terminal illnesses" (McCay et al., 1956). Ageing represents a major risk factor for a cluster of diseases with a conjoined causal process and delaying it could offset the co-morbidities of frailty (Niccoli and Partridge, 2012). Those willing to pursue this hypothesis over the last 25 years have been able to improve the health and lifespan of model organisms, from worms and flies, to rodents and fish, in turn increasing the length of disease-free life and postponing age-related disease (reviewed in: Bulterijs et al., 2015). But such remarkable results were met with cynicism within the gerontological community. The interspecies and even intraspecies ageing phenotype is complex and variable, with many considering ageing merely an unavoidable culmination of stochastic damage (Martin, 2002; Finch and Kirkwood, 2000; Gems, 2015). As such, ageing is rendered intractable. Amelioration of the impact of one type of ageing-related damage would leave the majority unaffected and would therefore have little impact on overall ageing-related decline (Partridge, 2010). This sentiment is echoed to this day in approaches to geriatric medicine. Although care may be tailored towards old-age, geriatricians still focus their efforts on the treatment of individual ailments within the elderly with little holistic direction from gerontological research. This is at odds to other specialties such as oncology that routinely draw on basic translatable guidelines established from sizeable and well-funded research communities (Hayflick, 2004; Nikolich-Žugich et al., 2016; Witham et al., 2019).

Furthermore, a dichotomous perception of pro-longevity research has existed in the public. Bolstered by societal desires for immortality, research into the field of ageing can appear seductive and intriguing, whilst simultaneously, transgressive, suspicious, and even dangerous (Fishman et al., 2008) . If the public

were to contemplate such a field of research, it could spark anxieties and existential threats of inescapable death and fallibility (Martens et al., 2005). This, in line, with the continuously evolving complexities of ageing and an historical perception of transgressions and immorality has led geroscience into a form of "forbidden knowledge" (Kempner et al., 2005). As such the public have, until recently, been largely unaware or unable to digest the benefits of prolongevity research and, in fact, misinterpretation and fear of radical societal change, through improper distillation of legitimate research has been a considerable challenge to gerontology (Sethe and de Magalhães, 2013). The truthful non-specialist communication of advances in geroscience is in constant conflict with mainstream demonization of old-age and bashful boundary science (van Wijngaarden et al., 2018; Cole and Cole, 2014). As, succinctly expressed by Baars, society is experiencing an increasingly prevalent sense of "premature cultural senescing", driven by an acceleration of information and compression of time (Baars, 2012). The dominant preference throughout modernity for the new or most advanced item has in late modernity come to include "young" (Baars, 2012). Such perception denigrates and undermines the utility and wisdom of ageing whilst wrongly glorifying the necessity of youth. Old age has become something to fear and avoid at all costs, a concept lapped up by a $42.51 billion anti-ageing industry (Orbis Research, 2018). On a background of paradoxically increasing chronic morbidity and decreasing mortality, the sufferance of ageing has led to widespread public revulsion of growing old, and its association with loneliness, isolation, powerlessness and uselessness (Gilleard, 2018). Thus, it is that unsurprising the public often consider lifespan extension a Tithonian Error.

1.1.2. The Tithonian Error: Healthspan As A Vital Component of Geroprotection

In the Pio Monte della Misericordi, a Neapolitan church, hangs a baroque painting by De Mura of the blonde female deity Eos, celestially poised and surrounded by love-putti, little angels, and maidens scattering flowers. Below, shielding his eyes is a withered and incongruous Tithonius, wearisomely slumped on a rock, for there he has been laid in solitude, burdening them both by his incapacity. Eos, had previously seduced the once young and vivacious son of Troy, Tithonius, and for years they had lived rapturously, but as the first grey hairs began to ripple from his comely head and noble chin, Eos was reminded of their disparate fates (Mordini, 2015). Despondent about Tithonus's inevitable demise, Eos naively begged her father, Zeus, to accord Tithonius with immortality. However, perfidious Zeus displeased with his daughter's infatuation with a mortal, deceivingly granted Tithonius eternality but not eternal youth. Thus, as the Homeric hymn goes, "*when loathsome old age pressed full upon him, and he could not move nor lift his limbs, this seemed to her in her heart the best counsel: she laid him in a room and put to the shining doors. There he babbles endlessly, and no more has strength at all, such as once he had in his supple limbs"* (Hine, 1970).

Tithonius' folly represents the publicly perceived threat of lifespan extension: an ever-increasing lifespan, but at the cost of progressive enfeeblement, ill health and dementia. Whether such beliefs may be merely irrational responses to mortality salience or a media-manipulated perspective of valueless infirmity in old-age, public ambivalence on the benefits of lifespan extension are apparent. A survey by the Pew Research Centre found a majority (57%) of individuals would refuse medical intervention to live to 120, with only 9% of participants wishing to live beyond 100 at all with current medical capabilities (Pew Research Centre, 2013). Freudian scepticism would argue this is more fear of infirmity than an honest wish for limited finitude: Freud viewed the discussion of death as lip-service and in reality, "our unconscious does not believe in its own death; it behaves as if immortal. At the bottom no one believes in his own death" (Furer and Walker, 2008). Against the former, research is repeatedly showing that lifespan extension offers more than just years of life, but life in years. Built into increases in lifespan can be a "compression of morbidity": a phrase coined by James Fries in 1980 (Fries, 1980). The truth in this dichotomy is no better exemplified than in humans. Approximately 90% of centenarians are functionally independent at the average age of 92 years, requiring no mobility support or daily intervention (Perls, 2006). But not only those lucky enough to live naturally to 100 can enjoy the freedom of health in later life: a growing body of interventions can elicit even more spectacular pro-longevity morbidity compressions in model organisms.

Using the term "healthspan", scientists have used murine models to demonstrate how frequently prescribed pharmaceuticals can be used to extend the life of the animal in conjunction with increased disease-free survival and improved functionality into old age. Specifically, the administration of metformin, a first line treatment for diabetes, has the capacity to increase mean lifespan in female outbred mice by ~40%, whilst also increasing healthspan by ~30% (Anisimov et al., 2008; Allard et al., 2016). This is reinforced by retrospective evidence in humans that type-2 diabetics treated with metformin had survival rates similar to (and, among those age > 70, even better than) their matched non-diabetic control group (Bannister et al., 2014). As follow-up, preliminary trials in healthy human beings to reduce morbidity and extend healthspan/lifespan using metformin are in process (Hall, 2015).

Moreover, additional murine work has demonstrated that rapamycin, an immunosuppressive therapy for kidney transplant, has the capacity to increase median lifespan by up to ~30%, further thrusting the concept of pharmaceutical intervention for increased lifespan and disease compression into the limelight (Johnson et al., 2014). However, in this instance, the healthspan benefits have been less clear-cut. Whilst several laboratories have shown rapamycin to slow numerous physiologic metrics ofageing, including liver degeneration, myocardial abnormalities, endometrial hyperplasia and non-lethal adrenal tumours,

disturbingly, other labs have shown the adverse effect of increasing cataract formation and testicular degeneration in male mice (Wilkinson et al., 2012). Therein lies the first problem of directing interventions at ageing itself: the effects of targeting an upstream process as far-reaching as ageing, given its reconditeness and myriad nature, may lead to malapropos ancillary effects beyond mere survival. This is well-exemplified when considering the delicate balance of fecundity and lifespan. An established inverse correlation between the two exists, whereby, in nature, longer lifespans are associated with decreased fecundity (Read and Harvey, 1989; Partridge et al., 2005; Holmes et al., 2001). Furthermore, anti-ageing interventions, including rapamycin, that increase lifespan, tend to accentuate this trade-off (Reznick et al., 2000; Bjedov et al., 2010). Thus, when considering their applicability, in the knowledge of potential limitations, there exists a continued sense of circumspection and suspicion. However, it is important to note that as research continues into the underlying mechanisms of ageing, and relationships between other capacities are established, the accuracy of interventions are likely to improve. Yet, the relatively long lifespan of humans makes for unprecedented longitudinal study of efficacy and off-site target that would bring into question the justification of research costs and ethical conditions.

Additionally, many of the most striking and unexpected interventions have been genetic, some through the manipulation of single genes (Kenyon et al., 1993). Of course, this raises clear, multi-disciplinary concerns surrounding the ethical pillars of medical research, and of course, such basic genetic manipulation is currently limited to animal models. With that said, the genetic tractability and malleability of the animal ageing process has spearheaded research over the last 30 years; genetic interventions have increased healthy lifespan across taxa including mammalian models. In particular, single mutations in nematodes have offered the most robust lifespan extension that has been recorded in a laboratory, with deletion of *daf-2* doubling *C. elegans* lifespan, and homozygous nonsense mutations in the *age-1* gene providing a near 10-fold increase (Kenyon et al., 1993; Ayyadevara et al., 2008). This is perhaps the greatest achievement of the gerontological field to date, and arguably the driving force behind the surge in popularity of ageing research. Kenyon excellently summarises this sense of hope: “which genes can extend lifespan? Can we augment their activities and live even longer? After centuries of wistful poetry and wild imagination, we are now getting answers, often unexpected ones, to these fundamental questions.” (Kenyon, 2010).

1.1.3. Expediting The Cure For Ageing Through Bio-Tech Investment

The devilish tangibility of truly modifying the ageing process, the end of mortality, has proved too tempting for the capitalist fist, and as such Mephistopheles has come to rear his head again. Some of the largest companies in the world, Google and Facebook, with limitless capital have set up shop firmly at the

forefront of biogerontology with profit in mind (Scott and DeFrancesco, 2015). Investment in longevity is by no means new; The National Institute of Ageing has existed since the 70's: but the level of public and corporate interest is unprecedented, and the numbers are astonishing. In 2018, 145 anti-aging clinical trials were initiated and over $800 billion of private investment was pumped in longevity start-ups. In August 2018, Samumed raised $438 million in equity funding, a stunning investment for a company that, while promising, still has no approved drugs (CBS Insights, 2018.). Furthermore, Unity Biotech has received more than $300 million in funding with key investment from Amazon founder Jeff Bezos and PayPal co-founder Peter Thiel; they too have no clinically approved products (CBS Insights, 2018). It is undoubtable that recent advances in ageing research have accelerated the slurry of investment, but something more visceral must be at play as logic does not favour this sector. If history is anything to go by, longevity research is a terrible bet; there are no success stories. It could be argued there exists little difference from the alchemists of the middle ages, or the gland grafters of the early twentieth century, or the hormone vendors now peddling their wares, to the modern practitioners of anti-aging medicine whom endeavour to convince the public the fountain of youth is near (Olshansky, 2017). No where is this more obvious than the collapse of Sirtis, a start-up company selling a resveratrol analogue which was purchased by GSK for $750 million, only to be shut down after clinical trials showed it caused unexpected safety concerns (Popat et al., 2013). Furthermore, Faustian-like prices are being paid for unproven cryonic preservation, as individuals pay over £100,000 for the distant possibility that in the future, science may have solved the currently unassailable task of bringing a frozen human back to life (Jones, 2013).

But for Silicon Valley, the epicentre of death denial, no price is too high and furthermore, understanding isn't a precondition for successful intervention. Whilst the desire for immortality or rather agelessness has long existed, a viable vehicle to realistically carry that desire is only recent; Ponce de Leon was merely too early. Death denial is no longer an abstract condition, but a mission statement for the resolution of a purely technological challenge which requires minor reprogramming of the "ageing bug". Tech-prophets of Silicon Valley with their doctrines of immortality have inevitably appealed to a Western American society losing faith in religious notions of heavenly immortality, believing instead in the power of technology and science to save humanity. The San Francisco Bay Area is among the least religious areas of the US, according to the Pew Research Center, with only 42% of adults saying they believe in God with absolute certainty (Pew Research Center, 2018). Psychological research supports this idea: individuals with low religiosity exposed to indefinite lifespan extension propaganda measurably reduce their belief of an afterlife in favour of lifespan extension (Lifshin et al., 2018).

The denizens of Silicon Valley are no longer satisfied by the power of their legacy, their symbolic immortality; they secured symbolic immortality when their ideas became household names which revolutionised the Western World as we knew it. And seduced by the hubris of their success, some believe these technocrats take themselves seriously enough to consider their immortality or delayed death in humanity's best interests (Begley, 2018). After all, the desire for immortality could be considered the ultimate expression of narcissism or perhaps for Silicon Valley to conquer death would be the ultimate humanitarian achievement.

In the Western world, food is omnipresent, water systems are clean, vaccinations prevent disease and yet the problem of death remains unresolved. It is ironic therefore, whilst many third world countries still lack the basic necessities for civilised life, that Silicon Valley should pursue the luxurious concept of immortality: the final inequality.

1.1.4. The Expedience of Anti-Aging Medicine As a Moral and Economic Obligation

Importantly though, and despite commitment and appeal from leading scientists, any field looking to gain significant institutional and charitable momentum, must offer more than a narcissistic escape from the salience of mortality (Bulterijs et al., 2015). Thus, the reclassification of ageing as a disease that needs offsetting and which maintains focus on fundamental ageing mechanisms, rather than on "age-related disease" serves to resolve one's scepticism on the medical utility of studying ageing. This dilemma has been fiercely debated in literature (Gladyshev and Gladyshev, 2016). Individuals, still dissatisfied with geroscience's purpose, would argue that a disease must require pathology, and a deviation from the norm (Faragher, 2015). Given that ageing is inevitable in every individual, like puberty, maturation and fertilisation, it should be considered a normal and natural process and therefore not a disease that requires treatment (Caplan, 2005). If ageing is not a disease then anti-ageing is merely human enhancement and a selfish transgression from nature (Gems, 2011; Juengst et al., 2003; Rantanen, 2014). However, the universality of ageing does not negate the consideration of ageing as a disease, rather it exaggerates it as a special type of disease (Gems, 2011a). The logic of medicine is predicated on the belief that disease is a state in which the limits of what is normal have been transgressed resulting in any abnormality of bodily structure or function, other than those arising directly from physical injury (Bulterijs et al., 2015; Caplan, 2005). Ageing is a process characterised by accumulative transgressions from the norm and broad spectrum of pathology which will inevitably lead to death, the ultimate pathology. Surely, ageing must have a *prima facie* claim to being counted as a disease? Moreover, the failure to define ageing as a disease is a dangerous one as it creates an intellectual barrier and disincentivises research (Faragher, 2015). Yet, some would argue that any attempt to counter the concept of ageing

would be fruitless and a waste of money. Specifically Gladyshev and Gladeyshev, fear a misallocation of economic and social resources to a futile cause, where the only result might be to prolong periods of illness and/or pain (Gladyshev and Gladyshev, 2016). Furthermore, normality and universality have an inescapable sense of benignancy, a naturalistic fallacy based on the consideration that all which is natural must be "good". Ageing roots out the weak and makes way for the young and therefore it is in our best interest to grow old for the furtherance of mankind. Faragher equates this fallacious logic to the erroneous belief that avalanches, volcanoes and tsunamis must be good (because they are natural) whilst books, houses and clothing are bad (because they are not found in nature) (Faragher, 2015). Normality and universality does not mean harmlessness. Malignant neoplasms, viral infections, gingivitis, acne and hypertension are all ubiquitous phenomena, yet medicine does not reject these as untreatable. Even if one continues to rationalise these as separate entities to ageing, natural processes such as pregnancy, although not a "disease" per se, remain an accepted target for prevention in the form of contraception (Boorse, 1975). Lastly, the recategorization of ageing as a disease would at least offer some FDA-approved regulation to prevent the swindling of anti-ageing products to the vulnerable elderly (Mehlman et al., 2004) .

So, it follows that if ageing should be considered a disease which routinely imparts dramatic physiological and psychological suffering, is not the moral obligation of the medical community to do their utmost to reduce such consequences? It is strange that biogerontology has been forced to justify the ethics behind its endeavours when the ageing phenotype seamlessly moves between AD, cancer and macular degeneration, all areas of research which have never remotely required justification of their pursuit. The purpose of medicine must follow the universal moral principle that good is to be done and evil avoided (Andorno and Baffone, 2014). Suffering is an intrinsic evil and thus there is a moral duty to prevent human suffering. As stated by Karl Popper: "human suffering makes a direct moral appeal, namely, the appeal for help, while there is no similar call to increase the happiness of a man who is doing well anyway" (Popper, 2003). Thus, provided biogerontological research continues to extend lifespan for the purpose of offsetting or compressing the physical and psychological suffering associated with senescence, and not for vain enhancement, there should be little moral objection.

However, as expressed earlier in this chapter, deceleration of ageing that provides protection against the full spectrum of diseases of ageing thereby assuring late-life health extension does unfortunately come with an incidental corollary of increased lifespan, which as we have touched on, has thrown up some objection. This is not lifespan extension in the realms of medical immortality but a likely average lifespan of 112 years, and a maximum lifespan of 140 (Miller, 2002). Nonetheless, are these extra years simply a

postponement of the inevitable? Will people still die of the same diseases only a few decades later? Of course, they will. It would be foolish to assume that the deceleration of ageing would somehow provide a complete escape of age-associated disease. There is no such thing as life time protection against pathology, only deferral of pathology: a concept championed by Gems and Blagosklonny (Gems, 2015; Blagosklonny, 2006). But what is the alternative: the willful acceptance of premature suffering and death? Mankind is Inherently and genetically programmed to fear of death and will always choose life free from disease over death (de Magalhaes, 2018). I suspect, many would choose AD tomorrow rather than today. However, notably, the deceleration of ageing comes with no cure. Researchers are thus morally obliged to "continue on indefinitely, seeking ever greater decelerations of ageing, ever later postponements of illness and greater extensions of lifespan" (Gems, 2011a). But under the current model of disease and medicine this is the only prescription (Callahan, 2018). Ultimately, the success of any medical treatment is presently gauged in terms of the degree to which it extends a healthy lifespan (Gems, 2011b). To that end, finding a treatment that decelerates ageing is a path that must be followed.

So if we now have a morally and medically advisable target to control the disease of ageing which if ameliorated can lead to moderate lifespan and healthspan extension and a reduction in suffering, how does one approach the funding and investigation of the subject? On the face of it, ageing should be considered the leading cause of death worldwide: 100,000 people globally die each day from age-related causes— a disease with a 100% mortality rate (De Grey and Rae, 2008). Once ageing is considered a disease, would it dwarf all competing claims for biomedical research funding because it affects every last one of us (Farrelly, 2008)? This is doubtful - the over-hyped propaganda of some gerontologists is unlikely to receive equal enthusiasm from investors and policy makers who quite frankly believe such research to be utopian and unrealistic (Farrelly, 2008). Furthermore, the research of ageing would be considered a luxury to those who live in suffering. To divert funds away from research into poverty, infection and premature death associated diseases would be an insult to the 50% of all the individuals on this planet who died before they even got the chance to experience agedness (Ritchie, 2018). The equality-driven prioritarian viewpoint stipulates that investment imperative should always be weighted towards worse off individuals. To this end, the first-world elite would rarely qualify for funding (Clayton and Williams, 2000). However, the field should be wary not to undersell the potential gains any successful intervention on ageing would have on humanity. Some have projected that the effect of delayed ageing resulting in 2.2 years additional life expectancy would yield US$7 trillion in savings over 50 years (de Magalhães et al., 2017), whilst others claim that a modest retardation of human ageing by just seven years could confer benefits greater than those that could be achieved by the elimination of cancer (Farrelly, 2010). It is also important that we are honest about the current limits to our understanding of the ageing process. Whilst,

as previously mentioned, understanding is not a prerequisite to treatment, blindly piling money in prospective fringe areas of the field would be inappropriate. Additionally, to request significant funding on false hopes and overstated presentiment, that fail to deliver, would also be greatly inappropriate. I an ideal world we need to find the middle ground between promising too much and promising too little (Farrelly, 2008). Significant costs into anti-ageing research would emerge upon the commencement of human clinical trials. It has been suggested that human trials into anti-ageing would cost at least $1 billion (Nikolich-Žugich et al., 2016; Paul et al., 2010) and that the reasonable amount which the NIH should be willing to commit to research has been estimated at $3 billion (Farrelly, 2010). Thus, ageing research, for now, should prioritise basic science with a translational, easily employable, clinical element, using resources already available to the scientist. For example, the utilisation of freely accessible, large, cross-field "omics" databases and the repurposement of currently available drugs offers a cost-effective strategy to find implementable interventions for the ageing process without the need of an extensive drug development and testing pipeline (de Magalhães et al., 2017).

Thus, this research thesis sets out to repurpose a drug for the deceleration of ageing and compression of morbidity mediated through currently studied pathways of ageing, enabling individuals to live a longer healthier life. It should serve to slow the manifestation of age-associated disease through the holistic retardation of the underlying causes of ageing leading to the reduction in geriatric suffering and emancipation of the ageing population. Freedom lies in the human capacity for self-determination. The ageing process internally imposes limitations and reductions in liberty through enfeeblement, frailty and pathology and thus, a failure to advocate and fund scientific research into ageing violates one's right to health (Farrelly, 2019). Part and parcel of one's physiological loss of liberty and capacity in old age is the inevitable psychological suffering that ensues, hastened by a societal revulsion of old age and a cultural narrative that prejudices the old. And whilst emancipatory efforts are made to reduce the suffering of ageing through the promotional ideal of "healthy ageing", It leaves us with a sense of a widening gap between the fit and the frail. It is this frail, darker, lonelier side of ageing which society most fears driven by the physiological imprisonment of getting old.

The provision of an intervention which can curb the progression of age-associated disease and compress morbidity so that it enables ageing individuals to continue their roles within society may help to remove the prejudices, segregation and isolation imposed upon the elderly. Such prejudice exists primarily because elderly people personify our inevitable demise (Martens et al., 2005). Furthermore, one's animality is revealed in old age: a divulsion of physical vulnerability and basicness as systems fail and mind no longer controls body. This lack of evolutionary fitness is unattractive to those in polite society

unwilling to accept their own physical, animal nature (Martens et al., 2005). Lastly, the tribal mentality within human society demands a contribution from everyone and the incapacities of the aged has increased the tendency to regard them as non-contributing burdens, resulting in resentment and marginalisation (Wisdom et al., 2014). Whilst, intrinsically, such ageism will always exist towards those oldest old, when disease becomes an inevitability; such age-decelerating interventions should help to compress the period in which old age may feel like a personal and societal burden and help individuals continue to manifest their own symbolic immortality and continue to be fit and valued members of their community.

In conclusion, this research, henceforth, does not search for the elixir of life, or the snake-oil of eternity: it is a reasonable, affordable, and rational approach to reduce the suffering of old-age and improve one's wellbeing in later life, representing the moral obligation of the medical and scientific community. Beyond the individual, this research hopes that by slowing down the processes of ageing—even by a moderate amount— it could yield dramatic financial benefits to society and remove prejudices against old age, reducing fear of mortality and providing an increased sense of happiness and wellbeing. "For age is opportunity no less than youth itself, though in another dress. And as the evening twilight fades away the sky is filled with stars, invisible by day" (Longfellow, 1825).

1.2. Biology of Ageing

1.2.1. Ageing As A Susceptibility to Death Which Encumbers Intervention Testing

For ageing to be a viable target for intervention, it is advisable to present a clear understanding of its phenotype such that its outcomes may be monitored and measured appropriately. However, despite its universality, and clear perceptibility, it remains the most elusive and least well-understood aspect of human biology (Kirkwood, 2005). Simply put, humans, like all things on earth are subject to the inexorable forces of entropy where deterioration will always prevail over synthesis. Such deterioration, characterized by a progressive loss of physiological integrity, leading to impaired function, renders human beings progressively more likely to die (López-Otín et al., 2013; Medawar, 1952; Pedro De Magalhães, 2004) Thus, ageing is merely a time-dependent increase in disease susceptibility and mortality. But what does that look like and can interventions fundamentally defer susceptibility?

First, mathematically, that answer is relatively homogenous and derived from the acceleration of mortality risk with age. This has culminated in various attempts to map rates of ageing with the Gompertz law perhaps being the most recognised (Finch and Pike, 1996). Gompertz's actuarial work focused on quantifying the rate of ageing within a population but suggested that a law of individual ageing was

revealed through the net pattern of human mortality data (Milne, 2009). Therein he proposed that mortality rates increase exponentially with age, doubling approximately every eight years of age until there is death in an entire population or individual within a decade or two past the age of 100 (Kirkwood, 2015; Fries, 2005). Almost all ageing animals and populations are subject to this model and as such, the ability to modulate this "rate of ageing" is a *sine qua non* of any anti-ageing intervention. Specifically researchers use the mortality rate doubling time, the amount of time it takes for the mortality rate to double for a given cohort, to define the decelerative success of any intervention (de Magalhães et al., 2005). Whilst, this method has its limitations, it serves as a practical metric of physical deterioration and health. Additionally, and used throughout this thesis, mean and maximum lifespan represents a broad and accessible metric to interpret the efficacy of an intervention and will be greatly referred to throughout this thesis.

As introduced, there is a clear and supported relationship between population mortality and intrinsic biological senescence at the individual level (Gurven and Fenelon, 2009). However, what does disease susceptibility look like, and what physical deterioration is apparent? Expectedly, the physiological manifestations of ageing are vast and heterogenous. The Digital Ageing Atlas, for example, a "one stop shop" for age-related changes lists over 300 physiological changes, which include cellular, hormonal and changes at various scales (including organs and the whole organism); and psychological or cognitive changes (Craig et al., 2015). However, James Fries, reinforced by other researchers, posits that these changes are best considered within a collective decline in maximal function of the vital organs (Atamna et al., 2018; Fries, 2005; Khan et al., 2017; Strehler, 1960; Strehler and Mildvan, 1960). The human body, and, specifically individual organs, strive to maintain homeostasis and express the capacity to endure recurrently stressful conditions. The young individual can withstand up to 7 to 11 times more physiological stress than the average quotidian demand (Manhapra, 2004). However, this fortitude and the subsequent ability to restore normal homeostatic balance and function in a relatively short recovery time becomes compromised in old age (Atamna et al., 2018). Such functionality, termed "organ reserve", progressively declines at a rate of 0.5% to 1.4% per year from the early twenties onwards and may further accelerate from the fifth decade (Bortz and Bortz, 1996; Sehl and Yates, 2001). Even in the absence of disease, one's capacity will continue to erode, until ultimately, the most routine of daily perturbations, become unmanageable and culminate in mortality (Fries, 2005). Thus, "Death occurs when the rate at which an organism does work to restore the original state is less than that demanded to overcome the effects of a given challenge", whether that challenge be acute disease, infection or trauma (Neustadt and Pieczenik, 2008). To that end, the actuarial observations of Gompertz are indeed correlative to the decreased capacity of an organism (Fries, 2005).

The indicators of decline in reserve and homeostatic elasticity are organ specific. Furthermore, it is essential to remember that rates of organ or organismal decline are different for everyone, rarely linear, and may be hastened, modified or slowed by disease burden, lifestyle and nutritional status. That said, general patterns of age-change are common in certain tissue and moreover, such changes have been elucidated in most organ systems and serve as the source of many age-associated chronic diseases. Fries, again, considers these diseases, universal and inevitable manifestations of age that accelerate and can become symptomatic in line with functional decline, further compromising one's stress resistance and vulnerability to death (Fries, 2005). The ability to compress the period of overt, symptomatic age-associated disease, and maintain "healthy ageing" is one of the central tenants of anti-ageing research and a key target for pharmaceutical companies (Stefánsson, 2005).

However, despite continued understanding of the associated pathologies of ageing, translating such "academic" knowledge into "anti-ageing" clinical trials with useful "hard" outcomes such as burden of chronic diseases, functional dependence, and/or mortality is poor (reference). Firstly, the complexity of human ageing that spans multiple physiological domains, cannot be accounted for *in-vitro*. Whilst organ function can be in part reconstituted *in-vitro*, the interconnected influence of human or animal systems on disease and age-related phenotypes often results in observations being invalid after mammalian follow-up (Artal-Sanz et al., 2006).

Indeed, whilst there are movements towards using humans for longevity research, such clinical trials are extremely expensive and rare (Nikolich-Žugich et al., 2016; Paul et al., 2010). Specifically, the current NIH annual budget would cover no more than a couple of human clinical trials (Farrelly, 2010). Even if human clinical research applications were to overcome the significant ethical hurdles, human life spans are long, and chronic diseases of aging tend to manifest in the later decades of life. Thus, antiaging intervention trials targeting such chronic disease will require decades of follow-up before such "hard" outcomes could be measured. Indeed, these studies could be accelerated if reliable markers of the human ageing rate could quantify pretreatment baseline, during-treatment change, and posttreatment outcome of participants in randomized clinical trials of rejuvenation therapies, but these are yet to be sufficiently validated (Moffitt et al., 2017).

Furthermore, human anti-ageing intervention trials face significant operational challenges which reduce the robustness of generated data. These include protocol adherence by study participants, drop-outs leading to incomplete and diminished data; result interpretation of the "intention to treat" analysis, where everyone in the treatment group is analysed together regardless of their adherence to the study intervention and the heterogeneity of participants (reviewed in: Newman et al., 2016).

Therefore, the *de facto* method to assess the effects of potential anti-ageing interventions is the utilisation of model organisms. Ideally researchers wish to use organisms that share similar physiology and functionality to humans and as such, mice and other rodents have dominated the anti-ageing intervention research field (Ingram and de Cabo, 2017; West et al., 2000). Unfortunately, however, mice often live more than 2 and half years. Despite a richness in phenotype scoring, they require continued housing and care, hence making rodent ageing study very laborious, expensive and time consuming (Maglioni et al., 2016). These models are therefore commonly reserved for "end of pipeline" testing prior to preclinical study in humans. For example, from the discovery of rapamycin's mechanism of action in 1995 a further 14 years of study continued before it was successfully tested for complete lifespan effects in mice (Johnson et al., 2013).

1.2.2. *C. elegans* as a model organism for anti-ageing intervention research

However, in the early 1960s, ponderings by an academic called Sydney Brenner provided an alternate solution (Artal-Sanz et al., 2006; Nigon and Félix, 2017). Brenner utilised *Caenorhabditis elegans* as a model organism for evolutionary genetics study, legitimising its use by stating, "one would need the simplest differentiated organism, small enough to be handled in large numbers, easy to cultivate, with a short life cycle and amenable to genetic analysis" (Artal-Sanz et al., 2006). This, of course, has significant cross-over for ageing research.

Indeed, it is simple and small: a ~1mm transparent body comprising 959 somatic cells in hermaphrodites (1031 for males) that form multiple distinct tissues observable by light and fluorescent microscopy (Mack et al., 2018). Worms have differentiated muscle cells, hypodermis, intestine, gonads, glands, an excretory system and nervous system. It was the fully mapped connectome of 302 neurons that so enticed Brenner, as it provided a unique balance of complexity and manageability (Mack et al., 2018). The *C. elegans* intestine also provides an excellent platform for the analysis of drug absorption, distribution, metabolism, excretion and toxicity (ADMET), comparable to human gastroenterology and performing functions akin to mammalian stomach, bowel, liver, pancreas and adipose tissue (Artal-Sanz et al., 2006; Mcghee, 2007).

The cultivation and maintenance of *C. elegans* is also relatively straightforward and inexpensive. They can be cultured on both solid and liquid media set in Petri dishes, tubes, or 6-, 12-, 24-, 96-, or 384-well plates (Leung et al., 2008). In laboratory conditions, *C. elegans* routinely subsist solely on a bacterial lawn of *E.coli* spread over NGM agar supplemented with cholesterol. Owing to their ability to enter a survival statsis known as Dauer, they can survive indefinitely in axenic conditions, deprived of *E.coli*, and are the only known multicellular organism than can be routinely cryo-preserved: this of course

permits the generation of archival libraries (Ma et al., 2018). Additionally, their hermaphroditism enables self-fertilisation and hence invariant progeny with a stable strain lineage which can be stored indefinitely. Furthermore, self-fertilisation results in large-scale production of progeny (300 in just 3 days) in the expected Mendelian ratios, which can be easily cultivated on single plates for high-throughput screening. Such progeny is also primarily hermaphrodite although males can arise through spontaneous X-chromosome non-disjunction during meiosis (∼0.2%) (Mack et al., 2018). Thus, hermaphrodites are almost exclusively used for large-scale lifespan assays owing to their isogenic invariance. On the other hand, males allow the creation of new allele combinations through cross-fertilisation, further facilitating the genetic amenability of *C. elegans* (Mack et al., 2018).

As such, it is no surprise that *C. elegans* was the first animal to have its genome completely sequenced and it is likely to be the first animal with a library of loss-of-function mutations in every gene (Lai et al., 2000; McDiarmid et al., 2018; The *C. elegans* Sequencing Consortium, 1998). From this, it has been estimated that, despite being 1/30 the size of a human genome, the number of protein coding genes between the two species is similar, and ∼40–80% (depending on similarity thresholds) of *C. elegans* proteins have been estimated to have orthologs in humans. Moreover, it is thought that 40% of genes known to be associated with human diseases have clear orthologs in the *C. elegans* genome (Mack et al., 2018) .

Researchers use the wealth of genetic information surrounding the *C. elegans* genome to optimise both forward and reverse genetic approaches. In forward screens, worms with specific phenotypes are randomly mutagenized, often by EMS, to identify candidate genes which exacerbate or alleviate the trait of interest. This process has been greatly expediated in recent years following the economisation of NGS and also deep sequencing (Sin et al., 2014). In reverse genetic approaches, the functionality of specific genes can be rapidly and easily explored. Not only does a phenomenally large library of cheap mutant strains already exist, but the worm community is extremely sharing of their own endeavours, making accessibility to various knockout models unprecedented. Failing that, the knockout or knockdown of genes in *C. elegans* is quickly and cheaply accomplished. This is principally established through either the permanent deletion of a gene or RNAi knockdown of gene function without altering the organism's DNA (Ahringer, 2006). First, in the creation of knockout mutants, a similar EMS mutagenesis library is created as in a forward genetic approach. However, this is followed by screening PCR to identify animals carrying lesions in the gene of interest and subsequent outcrossing to clean-up mutated DNA. In more recent years, since the implementation of CRISPR-based genome engineering, targeted deletion or editing of any gene of interest in <1 month, is now possible, with far less offsite

mutation. Second, knockdown by RNAi in *C. elegans* has been available for over 15 years and is enacted principally through the feeding of bacteria carrying dsRNA-encoding plamids purchased from one of two libraries (Ahringer or Vidal) that collectively cover >90% of the worm genome. Importantly, this technique permits post-developmental gene knockdown which is particularly useful in separating developmental phenotypes from ageing phenotypes. Beyond the downregulation of specific gene function, the *C. elegans* genome is equally amenable to the fluorescent transgenesis of genes of interest as well as the expression of human genes to induce a disease-related phenotype in *C. elegans*.

However, as briefly highlighted, perhaps the most relevant phenotype of *C. elegans* that lends itself to ageing research is its short life-cycle and lifespan (average approximately 17 days at 20 °C) which still permits the statistical identification of modest lifespan effects. As such, its efficacy as a pharmacological tool has been demonstrated by the recent documentation of small molecules with pro-longevity effects, representing possible strategies to delay ageing and the onset of age-associated diseases in mammals.

1.2.3. Cellular Manifestations of Ageing Influencing Universal Organismal Ageing

As described, ageing is defined as an increasing risk of mortality caused by progressive deterioration of organ and tissue integrity (Fries, 1980). Whilst, the appearance of such deterioration, is for the moment unstoppable, its hinderance allows for the repression of chronic disease borne out of acceleration of the ageing phenotype. However, as shown, organs degenerate in their own manner and at their own rates, permitting or denying the ability for some tissue to manifest in disease. It would perhaps be more beneficial to consider “upstream” targets.

At a cellular level, the inexorability of entropy is equally manifest as in the organism itself. Whilst the purpose is still questioned, damage accrued through endogenous fallibilities in metabolism and repair, or through exogenous inputs of environment and lifestyle are ultimately liable for ones ageing. It would be amiss to define the hallmarks of ageing through a lens other than that devised by Lopez-Otin et al. Their nine hallmarks of ageing can be clustered into 3 groups described by Aunan et al. as the primary hallmarks that cause damage to cellular functions; antagonistic hallmarks in the response to such damage; and, finally, integrative hallmarks that are the result of clinical phenotype, which ultimately contribute to the clinical effects of ageing as seen in physiological loss of reserve, organ decline and reduced function (Aunan et al., 2016; López-Otín et al., 2013) . For the purpose of this thesis, I have sought to categorise these hallmarks into three distinct aberrations: genomic related, proteomic and signalling. Whilst, other hallmarks are valid in their own right, their detailed discussion does not pertain to the changes in cellular ageing which this thesis serves to address.

1.2.3.1. Genomic, Epigenetic and Telomeric Aberrations

Genome damage accrued through endogenous and exogenous sources is thought to precipitate much of the aged cell phenotype (Vijg and Montagna, 2017). Whilst mutagens are encountered throughout one's lifespan, dysregulation of the mechanisms used to excise and repair such damage also accumulates which greatly accentuates the problem, leading to a viscous cycle. Furthermore, regulatory mechanisms to control the expression of genes at an epigenetic, telomeric and transcriptional level become aberrant, promoting cell cycle malfunction, carcinogenesis and senescence (Hoeijmakers, 2009).

One well-established theory of ageing denotes that direct damage to DNA is the formative and casual process of ageing itself (Alexander, 1967; Gensler and Bernstein, 1981). The genome is regularly exposed to insults which form various lesions along the DNA; it is proposed that as many as 100,000 lesions are thought to occur daily in each somatic cell (Hoeijmakers, 2009). Such lesions comprise of base modifications, single-strand breaks (SSBs), double strand breaks (DSBs), and interstrand cross-links (ICLs), which can manifest in point mutations, gene deletions and translocations alongside chromosomal changes and telomere shortening (Maynard et al., 2015). Research has elucidated a correlation between age and damage levels or mutation frequency, and consequent longevity (Blokzijl et al., 2016; Pan et al., 2016). Whilst, accumulation of mutations is unavoidable with age, their frequency can be hastened through age-related decline in repair capacity (Vijg and Montagna, 2017). In juvenescence, an elaborate plethora of repair mechanisms exist to excise and replace damaged DNA, however, there is sufficient evidence that all pathways of DNA repair, mismatch, excision and double strand break repair, become less efficient with age, leading to accumulation of mutations and subsequent loss of genome stability (Gorbunova et al., 2007). Indeed, human diseases caused by inherited defects in DNA repair genes can closely recapitulate a premature ageing phenotype. Werner Syndrome patients, for example, defined by mutations in the DNA repair gene, WRN, exhibit hallmark symptoms of ageing including: premature greying of the hair and baldness, skin and muscular atrophy, hypogonadism, poor wound healing, atherosclerosis, osteoporosis, soft-tissue calcification, juvenile cataracts, a tendency towards diabetes, and an elevated cancer frequency (reviewed in Muftuoglu et al., 2008). Importantly, deficient DNA repair in Werner's Syndrome culminate in significant chromosomal abnormalities, increased frequency of deleterious mutations and accumulation of DNA double-strand breaks (Ariyoshi et al., 2007; Freitas and de Magalhães, 2011). Conversely, however, long-lived animals appear to show upregulation of DNA repair. Specifically, the bowhead whales demonstrates duplications in genes linked to DNA damage repair and ageing, such as PCNA (proliferating cell nuclear antigen) (Keane et al., 2015).

Additionally, long-lived species of bat and rodents appear to show significant upregulation of DNA repair genes in comparison to their short lived counterparts (Conde-Pérezprina et al., 2012; Fang et al., 2014). With that said, it is important to note that, whilst direct DNA damage alone may contribute to ageing in its ability to self-disrupt transcription and its triggering of apoptosis or senescence, it is generally considered, the mutations generated from such damage, are the important factors driving genome instability and ageing (Vijg and Montagna, 2017). This is exemplified by the curved development of the ageing phenotype rather than a cliff, demonstrating a dominant role for stochastic events like somatic DNA damage causing ageing and the forming the mutational theory of ageing (Kenyon, 2010).

Genomic instability refers to an increased tendency of alterations in the genome during the life cycle of cells driven by the accumulation of mutation (Vijg and Suh, 2013). For example, research estimates that through ageing, up to 10% of cells exhibit aneuploidy, and changes such as this are sufficient to drive age-related change through two primary mechanisms: induction of senescence and exhaustion of regenerative capacity (Macedo et al., 2018; Mukherjee and Thomas, 1997; Thomas and Fenech, 2008; Westra et al., 2008).

Mack et al. (2018) recently published a review detailing changes in *C. elegans* ageing and evaluated their comparability to human hallmarks of ageing. Indeed *C. elegans* possess similar DNA repair machinery to that of humans, such that human segmental premature ageing syndromes, e.g. Werner, Bloom, and Cockayne syndromes may be elicited in worms (Babu et al., 2014; Lee et al., 2004; Wicky et al., 2004). Cellular responses to DNA damage appear conserved as well: transcriptional blocking DNA lesions provoke activation of a FOXO transcription factor in *C. elegans* and notably, *age-1* and other long-lived mutants of the nematode *C. elegans* show increased DNA repair capacity (Mueller et al., 2014). However, the direct role of DNA damage towards *C. elegans* ageing is controversial and it is uncertain whether this animal is suitable for studying the contribution of spontaneously induced DNA damage to ageing (Lans et al., 2013). Whilst genotoxic levels of UV shorten *C. elegans* lifespan, genetic mutants that induce endogenous mutations do not consistently shorten lifespan and furthermore, exogenous DNA damage may extend lifespan in long-lived mutants potentially primed to tolerate such stress (Hyun et al., 2008). Importantly, *C. elegans* exist in a post-mitotic state with a definite number of steady post-mitotic cells and do not technically undergo cell senescence and lifespan or ageing appears unrelated to telomere length in worms (Lidzbarsky et al., 2018) . Nonetheless, *C. elegans* exhibit an increase in single-strand DNA breaks, 5-methylcytosine , and protein carbonyls accumulation with age (Back et al., 2012). Likewise, DR restriction appears to enhance SOD and catalase activity and oxidative stress resistance (Houthoofd et al., 2002a; Van

Raamsdonk and Hekimi, 2010). This apparent tolerance is not entirely mediated through IGF-1 signalling in response to DR; specifically *sod* knockout has little or no effect on *daf-2* strain longevity and in some cases, it even further increases lifespan of *daf-2* (Zhou et al., 2011). That said, improved stress resistance to genotoxic agents forms one of the key assays used by biogerontologists to assess the ability of an intervention to affect ageing (Senchuk et al., 2017).

1.2.3.2. Proteomic Aberrations

Cellular ageing encompasses a labyrinth of processe which, whilst not uniformly manifested causes the, so far, ineluctable amassment of cellular damage leading to time dependent loss of cellular fitness. Loss of proteostasis is one such of those manifestation. Under normal and burgeoning conditions, the proteome is preserved via the stabilisation of folded proteins, management of efficient protein trafficking and controlled degradation, necessary to control the functional levels of proteins in their native state and minimize non-productive or harmful off-pathway reactions (reviewed in Hipp et al., 2019).

First, deleterious genomic instability in ageing promotes the production of misfolded proteins (Kikis et al., 2010). Furthermore, inherent polymorphisms, which occur at an incidence as high as two per coding sequence, can predispose individuals to misfolding: and the fallibility of translation machinery can lead to mutation in up to 18% of expressed proteins (Drummond and Wilke, 2008). Although no quantification of protein error levels in any ageing system exists, estimates in animals such as chick and rat have identified an age-related increase in protein error (Hipkiss, 2006; Holliday, 1996). Extrinsically, this is compounded by exposure to ROS and other stressors, which further imparts proteotoxic stress and post-translational modifications which can seemingly lock misfolded proteins in their position, thus preventing their degradation (reviewed in Krisko and Radman, 2019). Failure to rescue protein misfolding can result in aberrant aggregate formation, whose accumulation can eventually lead to characteristic age-related neuronal plaque formation and, in some instances, precipitate disease phenotypes (reviewed in Kikis et al., 2010).

Thus it is essential that organisms possess sufficient mechanisms to remove and clear potentially toxic or aggregative proteins from the cell (Komatsu et al., 2006). This can be performed through three distinct processes: chaperone-mediated refolding and/or transport of proteins into non-toxic entities or intracellular inclusions, degradation by the ubiquitin protease system, or finally degraded via autophagy (each mechanism reviewed in Klaips et al., 2018). It is the latter of these processes that is most pertinent to this thesis and thus will receive a more detailed review. Furthermore, its modulation is one of the key targets of gerontological research and a pathway regularly affected by geroprotective drugs.

The discovery that middle aged mice fed rapamycin, an autophagic induction drug, displayed increased lifespan triggered vast research into the role upregulating autophagy may have in decelerating the ageing process (Kroemer and Levine, 2008). Furthermore, mitochondrial dysfunction, an additional hallmark of ageing, is characterised by loss of membrane potential and profligate leakage of respiratory electrons, toxic apoptotic mediators and ROS: autophagic processes are able to selectively remove such cellular contamination (Murrow and Debnath, 2013). In a similar pathway to toxic protein removal, cellular contaminants are quarantined within a double membraned vesicle known as an autophagosome and degraded via the autophagosome's fusion with a lysosome (Ravikumar et al., 2010). This is particularly important for post-mitotic cells such as cardiomyocytes and neural cells that must rely on homeostatic "cleansing" methods such as autophagy to maintain cell viability (L. Wang et al., 2015).

Additionally, during inimical periods such as nutrient deprivation, growth factor withdrawal and oxidative stress, autophagy becomes an essential pro-survival pathway through its reproduction of amino acids and metabolic intermediates such as glutmaine (Altman and Rathmell, 2012; T. C. Lin et al., 2012). Furthermore, autophagy serves a mitigating role to degrade cellular pathogens, defective organelles, and more pernicious compounds such as aggregate-prone intracytoplasmic proteins associated with many neurodegenerative diseases (Fujikake et al., 2018). Thus it would appear logical that any malfunction or cessation in a cell's autophagic machinery would have deleterious consequences: indeed, autophagic inhibition of otherwise homeostatic mice precipitated the aggregation of morbific protein aggregates (Komatsu et al., 2006). Pertinently, autophagic malfunction, in a generalist view, would result in an accelerated accumulation of aberrant macromolecules and organelles mimicking a senescent phenotype, that compromises cellular and tissue integrity (Barbosa et al., 2019). Correlatively, literature demonstrates a reduced expression of autophagic proteins in aged tissues, whilst nematode autophagy mutants display reduced life span (Chang et al., 2017; Chapin et al., 2015).

There appears to be a plethora of factors, morphologies and processes which contribute to the aged phenotype, many of which autophagy cannot avert. However, it is undeniable that the vast majority of cellular ageing interventions currently available not only rely on proficient autophagy, but require its upregulation (Hansen et al., 2018; Lapierre and Hansen, 2012).The prototypical and most robust example of longevity is often considered to be the manipulation of the highly conserved primordial insulin-like growth factor pathway (Junnila et al., 2013). Countless genetic, pharmacological and behavioural studies have elucidated that the downregulation of this pathway causes increased longevity in many animal and human models (Gelino and Hansen, 2012). In *C. elegans*, IGF-1 receptor (*daf-2*) mutants display a dramatically increased longevity, only when autophagic machinery is intact (Hansen et al., 2008). Equally,

loss of function mutations in key autophagic genes or RNAi attentuated the longevity effects of dietary restriction, rapamycin, spermidine and increased FOXO expression in nematodes, drosophilae and mice (Morselli et al., 2010). Similarly, *Sirt-1* is able to prolong lifespan upon its transgenic overexpression, and reports suggest that this overexpression induces increased autophagy, whilst autophagy knockout abrogates its effect (Morselli et al., 2010).

It would appear that controlled upregulation of autophagy delays the development of cellular ageing (Madeo et al., 2015). This is supported by studies in Drosophila, in which the upregulation of Atg8, an autophagy gene, increased longevity (Simonsen et al., 2008) Similarly, improving protein homeostasis through autophagy in *C. elegans* through expression of heat shock factor is sufficient to promote longevity (Kumsta et al., 2017). It is thus important to study any compounds which appear to be increasing autophagy and assess whether this attribute is transferrable to life extension. Conversely, any putative compounds that improve organismal longevity should be studied to assess whether this attribute is due to autophagy upregulation.

Numerous reports have demonstrated a global reduction in *C. elegans* autophagy during ageing. Whole body extracts of worms show a reduction in autophagic activity, and an age-associated increase in uncleared autophagosomes can be observed in four major tissues (intestine, body-wall muscle, pharyngeal muscle and neurons), presumably caused by impaired autophagic activity or reductions in lysosomal protease activity (Chang et al., 2017; Sarkis et al., 1988; Wilhelm et al., 2017). Indeed, age-related protein aggregation and misfolding is evident in *C. elegans* and precipitates a pronounced, widespread decline in proteostasis, which some argue is a programmed event (Labbadia and Morimoto, 2014). Recent research has demonstrated that defective autophagy in ageing *C. elegans* affects lifespan and healthspan, and inhibition of autophagic machinery results in a significant lifespan increase and enhanced neural integrity (Medina and Richly, 2018; Wilhelm et al., 2017). As concluded by the authors, “Hence, by avoiding this detrimental autophagic deadlock in its early stages the toxic effects of dysfunctional autophagy are alleviated.”

One important hallmark of neurodegenerative diseases is the accumulation of protein aggregates, which should typically be degraded via autophagy. As such, researchers have been continually using models of neurodegenerative diseases in *C. elegans* to elucidate their molecular mechanisms . Chronic exposure of misfolded proteins could interfere, block or over-utilise autophagic machinery, disturbing normal protein homeostasis (proteostasis) causing cellular dysfunction and death in neurodegenerative diseases. Indeed, expression of aggregation-prone proteins in various model systems was shown to interfere with cellular proteostasis which coincided with in age-dependent collapse of proteostasis

(Brignull et al., 2006; Shemesh et al., 2017). In this particular model, poly-Q expansions were expressed throughout the nervous system of the worm, mimicking a number a neurodegenerative diseases characterised by expansion of glutamine (Q, encoded by CAG) repeats within otherwise unrelated genes to cause misfolding, oligomerization, and aggregation. A model of poly-q disorders was first developed in *1999* in the laboratory of Anne Hart. However, subsequent developments by the Morimoto group were able to capitalise on the transparency of *C. elegans* to fuse Yellow Fluorescent Protein (YFP) to the polq locus, permitting the real-time *in-vivo* visualisation of aggregation akin to that observed in Huntington disease patients (Morley et al., 2002). This provides ease and convenience which has now been expanded to various promoters corresponding to neuronal or muscular expression. Similar models exist for other neurodegenerative diseases through the expression of human aggregative proteins in a number of different tissues fluorescence and phenotype induction read-outs. A popular AD model of the latter utilises the expression of the N-terminally truncated Aβ1–42 peptide in *C. elegans* body wall muscle cells which causes paralysis when induced through temperature upshift (McColl et al., 2012). These models can be widely used for genetic or pharmocological screens to discover enhancers and/or suppressors of polyglutamine proteotoxicity and other neurodegenerative diseases (Sin et al., 2014). The inexorable tie between proteostatic collapse and ageing means many interventions that extend lifespan in worms also alleviate phenotypes of aggregative transgenic worms including poly-q expansion models (Devagi et al., 2018; Keowkase et al., 2018; Kim et al., 2019; Marcellino et al., 2018; Negi et al., 2017; Zhang et al., 2012). Moreover, researchers often demonstrate that potential pro-longevity compounds upregulate autophagy alongside a conferred amelioration of aggregation phenotypes in worms, whilst also increasing lifespan in wild-type worms (Boasquívis et al., 2018; Denzel et al., 2014).

1.2.3.3. Nutrient-Signalling Aberrations

A fundamental challenge in the ageing field is to isolate the source of heterogeneity in the ageing process. As highlighted in Lopez-Otin et al., (2013) the plethora of effects mediated through loss of genetic and protein homeostasis can often be consummately ameliorated or advanced by alterations in central signalling axes. Indeed, the loss of signalling regulation is arguably the most conserved and robustly mediated facet of ageing. Specifically, the method in which cells and organisms sense nutrients and signal such interpretation can dramatically influence organismal lifespan and ageing. In mammals, it is the somatotropic axis which influences three main signalling pathways (AMPK, mTOR and IGF-1) that has been the focus of much ageing research over the last few decades.

Inclusive of unicellular organisms, the ability to sense intra and extracellular nutrient availability and scarcity is a primordially conserved attribute across all taxa (Efeyan et al., 2015). Transmembrane chemoreceptors expressed by *Escherichia coli* (*E.coli)*, sense local aspartate and malate availability and thus signal rotary motors to propel the cell via peritrichous flagella action closer to the energy source (Parkinson et al., 2015). Likewise, odour detection within terrestrial mammals senses the desirability of certain food quality, triggering positive feedback within the limbic area and promoting food consumption (Hoover, 2010).

The dramatic divergence between prokaryotic and eukaryote evolution is thought to be driven by the engulfment of oxidative bacteria, the predecessors of mitochondria (Gray, 2012). This provided an abundant source of ATP which could support the genomic capacity and regulation of hundreds of thousands-fold more genes (Lane and Martin, 2010). Thus, the conservation of ATP levels became paramount in the maintenance of cellular viability and moreover, the availability of ATP developed into a vital barometer of energy status within the cell. The hydrolysis of ATP to ADP and phosphate (or AMP and pyrophosphate) underpins the catalysis of enzymatic reactions and anabolic processes essential to cells, and therefore, the ratio between these three molecules can accurately determine and regulate the relative energy availability of a cell. This is governed through a heterotrimeric enzymatic complex called AMPK, considered the master regulator of metabolism within eukaryotes (Yuan et al., 2013). Both ADP and ATP willingly phosphorylate AMPK at residues T172 of the α and γ subunit respectively, whilst additionally promoting supplementary phosphorylation by liver kinase B1 (LKB1) allosterically activating AMPK 2-10 fold (Gowans et al., 2013). Conversely, ATP competitively inhibits the binding of both AMP and ADP to the γ-subunit during energy repletion (Jeon, 2016). The cellular effects of AMPK activation facilitate the catabolism of energy storage whilst inhibiting proliferative and anabolic capacities of the cell. AMPK promotes lipolysis of triglycerides whilst promoting the uptake of circulating free fatty acids and inhibiting the *de novo* synthesis of fatty acids or the storage of these molecules as triglycerides (Daval et al., 2005). This pattern is similarly mimicked in relation to glucose. AMPK actively inhibits glycogenosis and gluconeogenesis, whilst increasing the expression of GLUT transporters to increase glucose uptake into the cell for subsequent glycolysis (Jeon, 2016).

In worms, *aak-1* mutants have a shortened life and are unable to regulate metabolism effectively, whereas overexpression of AMPK units increases lifespan (Apfeld et al., 2004; Narbonne and Roy, 2009). Additionally, the beneficial effects of metformin require the activity *AAK-2* and its upstream kinase LKB1/PAR-4 (Onken and Driscoll, 2010). More recently, oxidative stress in *C. elegans* led to a constant activation of AMPK and consequent hyperactivation of autophagy (Kosztelnik et al., 2019). It is

also well established in worms, that AMPK activation, like in mammals leads to activation of key longevity transcription factors such as *skn-1* and *daf-16* (Templeman and Murphy, 2018). In fact, *daf-2* mutants display enhanced autophagy that is AMPK dependent and which contributes significantly to its effects on lifespan extension (Egan et al., 2011).

Activated AMPK minimises energy expenditure through the inhibition of non-essential protein synthesis instead promoting the repurposement of proteins for energy substrates via autophagy (Kim et al., 2011; Wilson et al., 2015). This is regulated by AMPK inhibiting the activity of the anabolic mammalian target of rapamycin (mTOR), a signalling protein which upregulates protein synthesis and cell growth. Unsurprisingly, mTOR is an equally vital signalling molecule and among other processes, the mTOR pathway is central in amino-acid nutrient sensing. mTOR denotes a catalytic subunit of two distinct protein complexes, known as mTOR Complex 1 (mTORC1) and 2 (mTORC2), which are individually comprised of the following components: mTOR, mLST8, DEPTOR and either Raptor for the mTOR1 complex or Rictor for the mTOR2 complex (Saxton and Sabatini, 2017).

Mammalian lysosomes, orthologous to the storage vacuoles of yeast, in part function as amino acid pools in cells (Li and Kane, 2009). The observation that loading of lysosomes with amino-acids was sufficient to activate mTOR *in-vitro*, forged the way in the identification of SLC38A9, a lysosomal transmembrane protein with homology to amino acid transporters, as a positive regulator of the mTORC1 pathway (Zoncu et al., 2011). Further research pinpointed leucine and arginine levels as the specific amino acids that activate or inhibit mTOR (S. Wang et al., 2015; Wolfson et al., 2016). Recent research, reviewed in Wolfson and Sabatini (2017), highlight the novel discoveries of three amino acid sensors upstream of mTORC1 (SLC38A9, Sestrin1/2, and CASTOR1) which inhibit mTOR during shortages of leucine or arginine but in amino acid sufficiency activate mTOR consequently driving anabolic activities. Furthermore, mTOR can be activated by signalling from numerous growth factor receptors such as the insulin receptor or the epidermal growth factor (EGF) receptor which activate tyrosine kinase adaptor molecules at the cell membrane (Tanaka et al., 2011). This leads to the recruitment of the class I family of PI3K to the receptor complex, stimulating a phosphorylative cascade involving Akt and the successive activation of mTOR through the inhibition of its negative regulator TSC. Thus, in burgeoning conditions of high amino acid pools and circulating growth factors, mTOR will drive a number of anabolic activities, however, during homeostasis under non-proliferating conditions in vivo, most cells do not show active mTORC1 or mTORC2 signalling (Weichhart, 2018).

Downstream of mTOR, key transcription factors are activated which increase protein synthesis, increase metabolism substrates and prohibit the catalysis of protein through autophagic or UPS systems.

mTORC1 promotes protein synthesis largely through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4EBP). Activation of S6K1 causes binding to S6 ribosomal protein to support increased translation following the release of eukaryotic translation initiation factor 4E (eIF4E) from translation inhibitors eIF4E-binding protein 1 (4E-BP1) and 4E-BP2 by mTOR1. *De novo* lipid synthesis is increased by mTOR through the upregulation of sterol responsive element binding protein (SREBP) transcription factors which activates lipogenic gene expression (Porstmann et al., 2008). In carbohydrate metabolism, activated mTORC1 signalling stimulates glucose uptake and consequent glycolysis to provide carbon backbones for biosynthetic processes (Düvel et al., 2010). As previously discussed, AMPK stimulates autophagy in nutrient depleted environments. This is mediated through ULK-1 activation which phosphorylates a key autophagy protein beclin-1 (Russell et al., 2013). mTOR importantly, when activated, blocks the activation of ULK-1 by AMPK, whilst also inhibiting the expression of lysosomal biogenesis and the autophagy machinery (Kim et al., 2011; Saxton and Sabatini, 2017).

In similar concert to mammalian models, *C. elegans* AMPK appears to regulate the longevity pathways of mTOR. TOR in worms is coded for by *let-363*, and its deletion causes dauer-like larval arrest which limits its gerontological research utility (Jia et al., 2004). As such, downregulation of the TOR pathway and subsequent longevity, in *C. elegans* can be achieved through downstream *rsks-1*/S6K mutants: α/*AAK-2* is required for this effect (Selman et al., 2009). Notably, the majority TORC1 components are conserved in *C. elegans*, although the TSC seems to be absent (Zhang and Mair, 2017).

The TOR pathway in worms also controls growth and reproduction in response to the availability of amino acids and growth factors (Lapierre and Hansen, 2012). Key downstream factors of TOR: S6K, eIF2 and eIF4G, all increase lifespan in *C. elegans* when supressed (Hansen et al., 2007). The cellular mechanisms thought to be induced through TOR downregulation in *C. elegans* is: increased lipolysis, autophagy upregulation and overlapping transcriptional activation of DR induced transcripts (Lapierre and Hansen, 2012). Lastly, the beneficial effects of rapamycin on *C. elegans* lifespan appear to be mediated through the TOR pathway (Robida-Stubbs et al., 2012).

Upstream of mTOR and affecting AMPK activity is a complex interplay of three growth factor receptors and their associative signalling pathways. Three different mammalian insulin/IGF tyrosine kinase receptors have been identified: the insulin receptor (IR), IGF-1 receptor (IGF-1R), and the orphan IR related receptor (IRR), which all interact with three different ligands: insulin, IGF-1 and IGF-2. Upon increased endocrine signalling during anabolic and trophic states, increased growth hormone binding to insulin/IGF tyrosine kinase receptors converges onto insulin receptor substrate (IRS) which can initiate

signal transduction via two major branches: the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the Ras/MAPK pathway (Templeman and Murphy, 2018). The PI-3K- PKB/AKT pathway has been shown to regulate most of the metabolic effects of insulin/IGF-1 signalling, whereas the Ras-MAPK pathway had been shown to regulate most of the mitogenic effects of insulin/IGF-1 signalling (Taniguchi et al., 2006; van Heemst, 2010). The activation of Akt phosphorylates many targets, including the Forkhead box O (FOXO) transcription factor family and importantly TSC2, a regulator of mTOR activity (Saxton and Sabatini, 2017). Specifically, FOXO transcription factors are inhibited, through their exclusion from the nucleus by the insulin/insulin-like growth factor (IGF) signalling pathway (Kenyon, 2010). There exists four different members of the mammalian FoxO family (FOXO1A, FOXO3A, FOXO4 and FOXO6), which regulate an impressive array of gene expression targets related to cell cycle, stress resistance, and longevity (van der Horst and Burgering, 2007). Whilst direct targets of FOXO have been identified through CHIP-seq, precisely how FOXOs perform cell type-specific functions remains unknown (Webb et al., 2016). Nonetheless, FOXOs are often considered "master-switches" in the response to food shortage. When uninhibited by IGF signalling during low insulin/growth hormone drive, important transcriptional shifts initiated by FOXO promote cell survival through, improved nutrient uptake, lipid oxidation, cell cycle arrest and quiescence (van Heemst, 2010). Conversely, during Insulin/GH-IRS-induced Akt activation, FOXOs become inhibited whilst mTOR becomes activated. This is thought to be mediated primarily through the phosphorylation of TSC and PRAS40. Akt directly phosphorylates TSC2 at two sites (S939 and T1462 on the full length of the human protein) and possibly at two or three more sites (S981 and S1130/S1132), leading to inhibition of mTOR's negative regulation (Yoon, 2017). PRAS40 has been also shown to interact with mTORC1, which negatively regulates mTORC1 signalling (Sancak et al., 2007). Phosphorylated PRAS40 binds to 14-3-3 proteins, which have been proposed to sequester PRAS40 away from mTORC1 (Yoon, 2017). Furthermore, to compliment the anabolic state induced by growth hormone signalling, key cell survival starvation mechanism initiated by AMPK become inhibited. Again, the activation of AKT phosphorylates the AMPK α1-subunit (S487 in humans) but does not phosphorylate an equivalent site in the AMPK α2-subunit (S491), thus leading to inhibition of phosphorylation by upstream kinases at the activating site of AMPK, Thr172 (Hawley et al., 2014).

To add a further layer of complexity to nutrient signalling within mammals, the mitogenic Ras-MAPK, activated by IRS signalling further regulates the PI3K/AKT pathway. Singularly, downstream targets of activated Ras-MAPK include the p90 ribosomal protein S6 kinase (RSK) and the transcription factor ELK1, which influence cell growth, proliferation, differentiation, and survival (Templeman and Murphy, 2018). However, evidence suggests multiple levels of cross-talk between both Ras and AKT signalling (Aksamitiene et al., 2012).

The age-related decline in metabolic homeostasis of these pathways is likely an important contributing factor to general organismal ageing and has been demonstrated to potentiate the manifestation of aged phenotypes. Explicitly, sarcopenia, a central component of frailty in old age is thought to be associated with a decline in GH and IGF-1 levels. Functional loss of hypothalamic–pituitary axis presents with GH production reduction of 14% per decade after the age of 30 years, with a parallel decline in IGF-1 secretion (Veldhuis et al., 1995). Importantly, IGF-1 is the primary mediator of muscle repair and growth by stimulating satellite cell proliferation and muscle protein synthesis, inhibiting proteolysis and counteracting inflammation and fibrosis (Vitale et al., 2016). Thus it is well represented in a number of cohort studies that reduced serum IGF-1 independently increases the risk of sarcopenia, whilst also reducing gait speed and muscle mass (Gielen et al., 2015; Tay et al., 2015; Volpato et al., 2014).

It has been hypothesised that reduced IGF/GH signalling may serve a protective function aimed at minimizing cell growth and metabolism which would help to limit cancer development and also entropic damage during ageing (Junnila et al., 2013; López-Otín et al., 2013). However, paradoxically, senescent cells, another hallmark of the ageing phenotype, display constitutively activated mTOR. Senescent cells are highly metabolically active, and are often identified by their pro-growth phenotypes such as an enlarged size, increased organelle content (including mitochondria and lysosomes), increased metabolism and the potent SASP (Carroll and Korolchuk, 2018). It has been shown that malapropic mTOR activation drives the transcription of SASP immune mediators (Laberge et al., 2015), whilst additionally increasing intracellular ROS upregulating a senescent-inducing DDR (Correia-Melo et al., 2016; Laberge et al., 2015). Likewise, AMPK activity is reduced in senescence, further permitting exaggerated metabolic and anabolic processes in senescent cells (Salminen et al., 2016). This pattern is represented across aged tissue: hyperactive mTOR is present in ovarian tissue and sarcopenic muscle of aged donors, and also in the adipose tissue of aged mice (Baar et al., 2016; Bajwa et al., 2016; Joseph et al., 2019). Additionally, increased mTORC1 signalling is associated with numerous age-related diseases and pathologies, including AD (An et al., 2003), diabetes (Inoki et al., 2011; Volkers et al., 2014), and cancer (Bar-Peled et al., 2013; Grabiner et al., 2014).

However, importantly, research demonstrates that changes in mTOR signalling are sex and tissue specific, and a more complex dysregulation in mTOR is apparent (Baar et al., 2016). Whilst, levels of mTOR activity do fluctuate between sex and tissues, the pattern of repression observed in some tissue, may still represent an inappropriately high level, and research has indicated that baseline fasted levels of mTOR activity are abnormally high in the liver of old mice (O. V Leontieva et al., 2014). However, as demonstrated above, age-changes in IGF/Insulin signalling upstream of mTOR show equivocal changes.

Moreover, an additional regulator of mTOR activity, AMPK, demonstrates a loss in signalling capacity with age to cope with both inherent and environmental stresses, highlighting a general loss of anabolic and metabolic homeostasis within the cell which could differentially affect mTOR signalling (Salminen et al., 2016).

1.3. Calorie Restriction

1.3.1. Calorie Restriction As a Means To Slow the Hallmarks of Ageing

The field of age-related metabolic dysregulation has been largely driven by observations that reduced growth signalling confers improved longevity and healthspan. As early as the 1910s, it was shown that blunting the growth of female rats by decreasing their food intake led to life extension and the ability to reproduce at older ages than the rats that did not undergo a period of slow growth (Masoro, 2010; Osborne et al., 1917).

Today, over a 100 years of research, across a multitude of model organism, has firmly established calorie restriction as the most robust and reliable geroprotective intervention (Liang et al., 2018). Two ground-breaking and ongoing studies have managed to provide complete lifespan data for CR in Rhesus monkeys with 93% genetic homology to humans (Colman et al., 2009; Mattison et al., 2012). Unfortunately, whilst both promotive of CR, their conclusions are different: Colman et al. report healthspan and survival extension, whilst Mattison et al. report only healthspan extension. Subsequent comparison and review has failed to ascertain which of the two studies is right, but instead, served to highlight firstly, the intricacies of defining the composite requirements for optimum calorie restriction, and secondly the wavering conservation of CR in line with evolution complexity (Mattison et al., 2017; Vaughan et al., 2017). Significant calorie restriction induced lifespan extension is relatively straightforward to elicit in lower order species. At an invertebrate level, mere glucose depletion, the most common practice to mimic calorie restriction in yeast cultures, progressively increases the mean and maximum life span when glucose concentration drops from 2% up to 0.01% (Lee et al., 1999). Similarly, many studies have shown single amino acid depletion or the restriction of nitrogen availability is sufficient to increase lifespan in yeast (reviewed in Taormina and Mirisola, 2014). Again, in *C. elegans*, a non-parasitic nematode worm, unregulated and generic calorie restriction through feeding-defective mutants confers significant improvements in survival (Avery, 1993), whereas, in flies, diluted yet freely available diets with a fixed concentration of sugar can increase lifespan by 50% or more (Tatar, 2006). Whilst invertebrate models offer relative malleability in lifespan modulation by calorie restriction, they have also provided a vital insight into the importance of dietary ratios and nutrient content. Dietary restriction (DR), encompassing the depletion of specific nutrients, rather than the reduction of the overall energy intake,

with focus on protein-restriction, has been argued to be responsible for the increased longevity observed in fruit flies (Mair et al., 2005). Moreover, evidence continues to highlight the ratio between protein and carbohydrate as their most important regulator of lifespan (Carvalho et al., 2005; Fanson and Taylor, 2012). During the Rhesus monkey trial that elicited lifespan extension, the animals were fed mainly once a day whilst twice in the other trial, which may have contributed to the 1.8 lower mortality compared to controls in the former. Thus, despite significant unravelling of the various modalities of DR, the molecular mechanisms coordinating this pro-longevity response remain obscure.

There are numerous ways to induce DR/CR in the bacterivorous nematode (Zhang and Mair, 2017). Researchers discussing CR in worms are reluctant to use the term “calorie restriction”, instead preferring DR, given the variable mechanisms of food intake restriction which are not strictly defined by their calories (Zhang and Mair, 2017). DR was shown to increase lifespan in *C. elegans* are far back as 1977 (Klass, 1977). In this first iteration, worms were grown in liquid S-medium with a limited bacterial food source determined by using a Petroff-Hauser counter and adjusted by serial dilution. This method was adopted by early investigators and was amenable to high-throughput study, permitting easy quantifiable definitions of food intake. Researchers have noted lifespan extension across ranges of dilution with correlative changes in fecundity (Panowski et al., 2007; Zhang and Mair, 2017). However, despite its early adoption, concerns regarding continuous swimming for worms and the consequent effects on metabolism and stress has led to other ways to induce DR using the standard agar plate-based methods. The Brunet lab developed solid DR method by feeding animals with *E.coli* at different concentrations, but they were unable to elicit such empathic levels of lifespan extension as compared to liquid media, perhaps due to the DR initiation after development which was not the case in liquid media (Jianfeng et al., 2015). Indeed this also comes with its limitations, particularly in the standardisation of NGM media and peptone content, alongside the unregimented movement and bacterial consumption by *C. elegans* on the plate leading to great variability of DR effects between labs. As such, to bypass such variability axenic medium assays have been developed which confer lifespan extension, but unfortunately equally elicit a starvation response which is genetically and phenotypically separate to that of a DR response.

Thus researchers have sought to utilise the vast mutant library to induce DR. The most popular of these is the *eat-2* mutant, which lacks the encoded nicotinic acetyl-choline receptor and renders the pharyngeal muscle unresponsive. Animals are unable to efficiently grind the bacteria, inducing DR on regular media plates. These animals live around 30% longer than their WT counter parts in part due to downregulated TOR signalling, reduced phosphorylation of S6K and increased autophagy (Lakowski

and Hekimi, 1998). Importantly however, these animals are subject to DR during development, confounding longevity variables, and furthermore, due to the lethality of null mutants, the utilized alleles are typically hypomorphic mutations (Kapahi et al., 2017). In other papers, attempts to instead limit the uptake of nutrients in the gut have been made to induce DR. Fei et al. used RNAi to knockdown sodium-dicarboxylate cotransporters (NAC-2 – NAC-3), a key TCA intermediate transport protein in the gut, to reduce nutrient availability, and conferred significant lifespan extension (Fei et al., 2003).

Whenever testing the efficacy of any DR model in worm, researchers tend to establish its connectivity and integration to ISS signalling. As described in other model organisms, downregulating the conserved Ins/IGF-1 pathway also significantly extends lifespan in *C. elegans*. In fact, genes acting on this pathway were the first genes to be directly linked to ageing and some of the genes in this pathway, including the FOXO transcription factor *DAF-16* have been linked to DR (Kenyon, 2010). The IIS pathway is regulated by insulin-like peptide ligands that bind to the insulin/IGF-1 transmembrane receptor (IGFR) ortholog *DAF-2*. Activated *DAF-2* results in recruitment and activation of the phosphoinositide 3-kinase AGE-1/PI3K. In turn, the serine/threonine kinases PDK-1, AKT-1, and AKT-2 are activated, resulting in phosphorylation of the *DAF-16*/FoxO, and translocation to the nucleus and transcription of key longevity genes. Mutations in either *age-1* or *daf-2* almost double lifespan compared to wild-type worms and this prolonged lifespan phenotype is dependent on the FOXO-family transcription factor *daf-16* (Kenyon, 2010; Kenyon et al., 1993). Importantly, downregulation of the ISS pathway elicits significant improvements to healthspan in *C. elegans* relevant to human ageing including sustained neuromuscular junction activity, learning and memory, supressed neurodegenerative protein aggregation, and pertinent to the limitations of DR: an improved immune system (Cohen et al., 2006; Evans et al., 2008; Hsu et al., 2009; Kaletsky et al., 2016; Kauffman et al., 2010). The necessity of various components of the ISS pathway for geroprotection in *C. elegans* is varied depending on the dietary regime, highlighting not only the difficulty in homogenising DR regimes in worms, but also demonstrating that a single CR "master switch" is unlikely to exist. Specifically, *daf-16* is only indispensable for lifespan extension by dietary restriction on solid media, and notably this is mediated by AMPK, or in longevity mediated by CR in middle-aged organisms (Greer et al., 2007). Bacterial dietary restriction (bDR), liquid DR (lDR), modified solid DR (msDR) and anexic medium all extend lifespan independent of *daf-16* (Jianfeng et al., 2015). Furthermore, dietary restriction by bDR, axenic mediums and chronic bacterial deprivation all extend the lifespan of *daf-2* hypomorphic mutants (Zhang and Mair, 2017). *Eat-2* mutants also seem to extend lifespan independent of *daf-2*: *eat-2;daf-2* double mutant lived 20% longer than *daf-2* alone and *daf-2* on axenic medium live 274% longer than WT (Taormina and Mirisola, 2014). This non-linear complexity is furthered by the fact only 14 out of 17

eat mutants have increased mean and maximal lifespan, and therein they differ in their mechanisms of action: *eat-2*(ad1113) and *eat-2*(ad465) alleles require *sir-2.1* to promote longevity whereas the *eat-2*(ad1116) allele does not (Gao et al., 2018; Kapahi et al., 2017). Nonetheless, *eat-2* mutants remain a favoured and versatile model of DR in worms, particularly given its amenability to bacterial RNAi.

Supportively, rodent studies have demonstrated that CR without malnutrition, or DR through sole methionine restriction, suppresses circulating IGF-1 and insulin levels in proportion to the level of restriction (Vitale *et al.*, 2019; Sanchez-Roman and Barja, 2013). This correlates with the observation that serum IGF-1 concentration is inversely correlated with median lifespan in 31, genetically-diverse, inbred mouse strains (Yuan et al., 2009). Thus, the downregulation of IGF-1/GH/Insulin signalling has been suggested to mediate some of the lifespan extension properties of CR.

In humans, whilst IGF-1 levels have been correlated to lifespan, the data is inconclusive. Some studies report reduced IGF-1 in centenarians (Arai et al., 2001) and long-lived individuals (Bonafè et al., 2003), whilst other research demonstrates an opposite relationship (Paolisso et al., 1997). Furthermore, in humans, calorie restriction does not reduce serum IGF-1 concentration, however a recent meta-analysis has challenged this (Lettieri-Barbato et al., 2016). Additionally, most recently, the CALORIE study group has offered the first adequately powered research into the clinically controlled and reported effects of long term calorie restriction (Fontana et al., 2016). They designed a multicentre randomized controlled trial of calorie restriction lasting 2 years. Results again corroborate findings from shorter term (6 month-1 year) studies, demonstrating an inability of CR to reduce IGF-1. Researchers did nevertheless note a significant and persistent increase in serum IGFBP-1, an inhibitory binding protein which should confer a reduction in IGF-1 potency.

As highlighted earlier, the two most prominent downstream targets of IGF signalling is mTOR and AMPK, however inputs from other sources effect the activity of these enzymes. mTORC1 has for many years been considered a key nutrient sensor, fuelling speculation that the lifespan-enhancing effects of CR are at least partly mediated by decreased mTORC1 signalling. A role for reduced mTOR activity in ageing was first demonstrated in worms deficient in their mTOR ortholog *let-363* which more than doubled its natural lifespan (Vellai et al., 2003). In flies, repression of mTOR activity through inhibition of upstream TSC complexes conferred a significant increase in lifespan (Kapahi et al., 2004). Given the essentiality of mTOR in mammalian growth and development, knockout is lethal in mice and presumably in higher order species: mTOR mutant murine embryos lack forebrain development and body axis rotation, resulting in death at midgestation (12.5 days post) coitum (Hentges et al., 2001). That said, several mouse models of decreased mTOR signalling exist, with single allelic copy mTOR+/- mice demonstrating

a 14.4% increase in mean life span relative to wild type (Lamming et al., 2012). Likewise in flies, downstream of mTOR, caloric restriction up-regulates the Drosophila equivalent of 4E-BP (d4E-BP), and augmenting d4E-BP is sufficient to extend lifespan in this organism (Zid et al., 2009). Furthermore, the most robust pharmaceutical intervention that extends lifespan is rapamycin, an inhibitor of the mTOR that can improve longevity in yeast, worms, flies, and even mice (Ehninger et al., 2014).

Some researchers believe the anti-aging benefits of CR are solely and directly mediated by mTOR. Blagosklonny (2010), has stated that, "nutrients activate TOR, which is involved in ageing. By deactivating TOR, CR slows ageing. There is no aim, no selective advantage, no purpose. Simply: food activates TOR and TOR drives ageing." As discussed, stress resistance and homeostatic flexibility play a central role in preventing mortality with age. Research in both kidney and liver has showed that, DR can improve resistance to multifactorial acute stress of ischaemia and reperfusion; however this protection is dependent on mTOR signalling (Harputlugil et al., 2014; Robertson et al., 2015). Further hepatic evidence suggest specific DR through amino acid restriction reduces mTOR phosphorylation and activity. Isocaloric restriction of protein markedly inhibits prostate and breast cancer growth in human xenograft animal models of neoplasia in part via a down-regulation of intratumor mTOR activity (Fontana et al., 2013). Given the amino acid specific signalling of mTOR, this research provides evidence that the proportion of lifespan extension of CR mediated by protein restriction may be elicited through reduced mTOR signalling. Whilst CR-like regimens do not addtively lengthen the lifespan of yeast or worms already exhibiting downregulated mTOR, this is not entirely replicated in higher order species or indeed other worm models (Hansen et al., 2007; Kaeberlein et al., 2005). In flies, rapamycin fed to CR flies was able to additively increase maximal lifespan, suggesting CR may be more complex and affecting survival through pathways supplementary to mTOR. Furthermore, that RNAi knockdown of key downstream mTOR targets, S6K and translation initiation factors, yield an additive lifespan extension when combined with dietary restriction in *C. elegans* (Hansen et al., 2007). In mammalian models, the research is even more inconclusive as data suggests that mTOR signalling may only be reduced in specific tissues . Furthermore, rapamycin-induced inhibition of mTOR activity elicits significantly distinct and unique transcriptional and metabolomic profiles to that of CR (Wilson C Fok et al., 2014). However, in combination, DR mice given Rapa showed a significant change in many transcripts and metabolites, which were not changed significantly by DR or Rapa alone (Wilson C Fok et al., 2014). As the authors state, "These data suggest that feeding DR mice Rapa would increase lifespan over that observed in DR mice." Recent evidence has shed some light on the possible reason behind the diversity of results, in that mTOR may not be constitutively downregulated at all times by CR/DR. In a very recent paper, Tulsian et al. demonstrate that the effect of CR on mTOR activity is entirely time-dependent (Tulsian et al., 2018).

CR induced mTORC1 activity at one time, reduced at two times and has no effect during other times, suggesting that the unification of feeding times across experiments may further research into the ambiguity of mTOR signalling and CR. Whilst a direct linear relationship between CR and mTOR activity may not hold true, owing in part to complex organ function, non-unified feeding regimes, and inconsistent quantification of mTOR signalling, there is broad consensus that reduced mTORC1 signalling probably contributes to longevity and health benefits as a result of dietary restriction, whether that be through energy depletion per se or isocaloric protein restriction (Johnson et al., 2013).

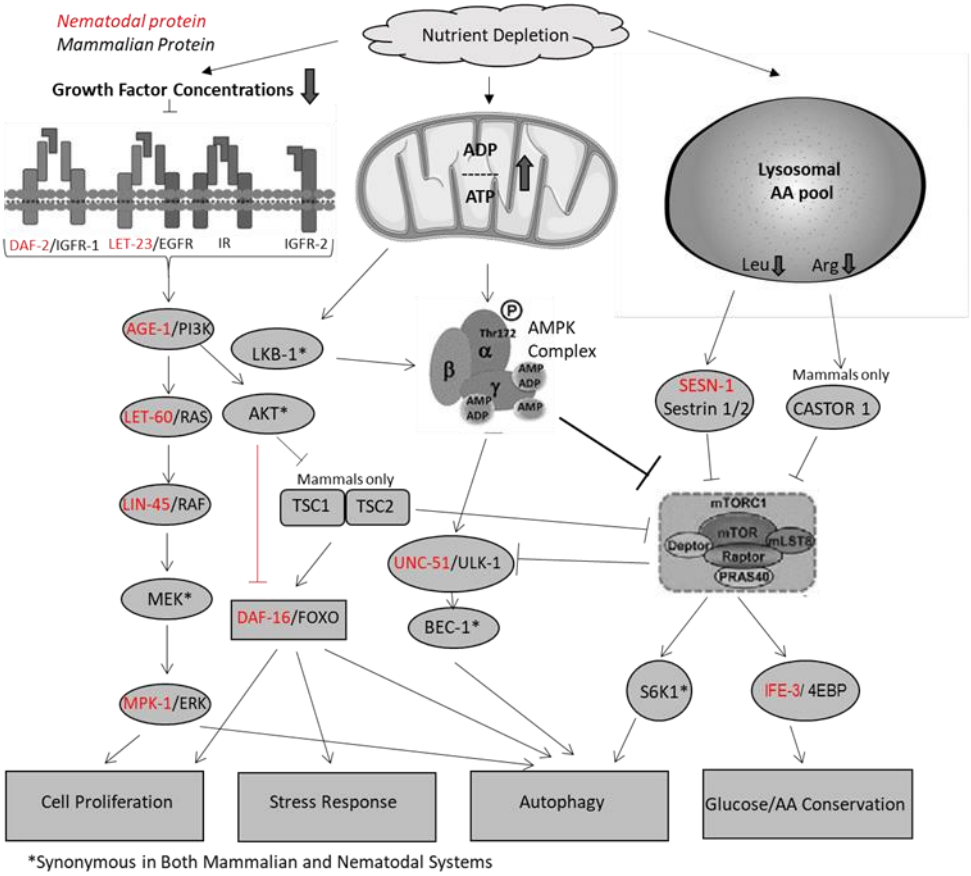


Figure 1. Nutrient Sensing Pathways Associated with Ageing And The Geroprotective Effects of Calorie Restriction. *Schematic illustration of three evolutionary conserved pathways which interlink to regulate the response to nutrient availability in multicellular eukaryotes. Decreased nutrient availability triggers a reduction in extracellular growth factors and intracellular ATP and amino acid levels. Downstream activation of AMPK and concomitant downregulation of ERK, AKT and TOR signalling in both mammalian and nematodal systems results in metabolic conservation and attenuation of anabolic activity. C.elegans orthologs are represented in red and proteins evolutionary and synonymously conserved in both mammalian and nemotodal systems are represented by way of an asterisks.*

Of course, AMPK activation is the best-described intracellular trigger for mTOR inhibition, and unsurprisingly, CR is continually associated with AMPK activity. However, unfortunately the relationship is also complex. At a most basic level, glucose depletion in yeast causes an upregulation of AMPK activity and nuclear localisation (Turcotte et al., 2010). A collection of research has demonstrated that the sole overexpression of *aak-2*, a *C. elegans* AMPK ortholog, in worms can increase lifespan whilst its deletion reduces lifespan (reviewed in Cantó and Auwerx, 2011). Furthermore, *aak-2* is increased during glucose restriction and calorie restriction demands the functional presence of *aak-2*. Similarly, in fruit flies, RNAi knockdown of the gamma subunit (AMPKγ) extended lifespan and abrogated any additive lifespan extension through starvation (Johnson et al., 2010). However, much like mTOR signalling, a linear relationship is not sufficient when approaching mammalian models: increased AMPK activity is not the sole reason for the benefits seen in calorie restriction. Indeed, some research suggests chronic AMPK activation may shorten lifespan (in yeast) and incur myopathies in mammals (Burwinkel et al., 2005). With that said, it is clear that AMPK relates to CR. Perhaps a more plausible explanation may be that AMPK may help coordinate some adaptions to CR through temporal and spatial changes in phosphorylation, rather than being the activated nexus of a single pathway.

Ageing is the most universal contributors to the etiologies of metabolic decline and disease and given the role AMPK has in nutrient sensing, it is highly likely any relationship between AMPK and CR would exist through an improved sensitivity to insulin and response (Barzilai et al., 2012). Ageing commonly presents with insulin resistance, manifesting in unrestrained hepatic gluconeogenesis, adipose lipogenesis, and defective glycogen synthesis, glucose uptake in skeletal muscle and potential type-2 diabetes. This is progression is in part precipitated by increased age-associated adiposity which proliferates inflammatory signalling and free-fatty acid release, all of which can contribute to the pathogenesis the age-associated chronic condition. The CALERIE studies have clearly demonstrated that CR ameliorates adiposity and improves insulin sensitivity and reduced fasting insulin and lipid profiles (Larson-Meyer et al., 2006). The aforementioned CR Rhesus monkey trials both reported age-related divergence in fasting glucose levels, with increases more marked in control monkeys (Mattison et al., 2017). Additionally, CR animals had lower incidence of glucoregulatory dysfunction than controls in both studies. Again, adiponectin is central to ageing with it not only being increased in young and CR individuals high circulating levels are found in several long-lived mouse mutants and human centenarians (Fontana et al., 2010). Unsurprisingly, adiponectin is an insulin sensitizer with anti-inflammatory properties and a potent activator of AMP-activated protein kinase (AMPK). A further connection between the physiological effects of CR and AMPK is mitochondrial dysfunction. In brief, AMPK through ULK1 can increase mitophagy and can phosphorylate mitochondrial fission factor and promote mitochondrial fission upon energetic stress,

ensuring AMPK acts as a signal integration platform to maintain mitochondrial health (Herzig and Shaw, 2018).

However, the nexus of AMPK and indeed mTOR is not limited to an amelioration of insulin resistance and oxidative stress. CR is routinely cited as the most robust physiological inducer of autophagy and nutrient depletion is the gold-standard for autophagic induction in culture (Kroemer et al., 2010). In vivo, autophagic response to CR is oscillatory: autophagy is rapidly upregulated as early as 30 min after access to nutrients has been limited, before shortly being downregulated only to surge again after 10 hours. It is suggested that this serves to prevent pathologically excessive autophagy and maintain proteostatsis (Zhang and Cuervo, 2008). Thus, autophagy is a vital component of CR-induced longevity. Multiple models have demonstrated that the benefits of dietary restriction are abrogated following the knockdown of autophagic machinery (Rubinsztein et al., 2011). Regulation of this machinery is tightly controlled, as previously discussed, by mTOR and AMPK. Long-Term Calorie Restriction in humans not only increases the expression of AMPK, but also SIRT family transcripts, ULK1, ATG101, APG12, GAPRAP/GATE-6, beclin-1, autophagin-1, and LC3 gene expression, as well as protein expression of FOXOs, PGC1α, beclin-1, and LC3 (Yang et al., 2016). Murine work has explicitly shown CR to upregulate neuronal autophagy, limitingly in young mice, as effect of CR in older mice is less present (Alirezaei et al., 2010). Thus, in application, an AD mouse model fasted for three months caused significant autophagic induction however was unable to significantly clear aggregates (Chen et al., 2015) . This is at odds to other research using specific autophagic activators such as rapamycin that attentuate cognitive deficits and reduces amyloid-beta level (Spilman et al., 2010). With that said, a plethora of studies have highlighted the effects of CR on hepatic and sarcoplasmic autophagy which can be reviewed in Bagherniya et al., 2018. Of most interest is the observation that mild CR could attenuate the age-related sarcopenic impairment of autophagy in rodent skeletal muscle, which might be one of the mechanisms by which CR attenuates age-related cellular damage and cell death in skeletal muscle *in vivo* (Wohlgemuth et al., 2010).

In summary, CR should serve to attenuate the anabolic signal of insulin and overnutrition which send positive growth and proliferative signals, pushing cellular balance toward growth and cell division. “Increased metabolism will produce ROS from mitochondria as well as shortening the time in which cells repair or replace old or damaged molecules. Although enough energy is present to produce daughter cells, these rapid rates of cell division may be harmful. Therefore, CR may promote increased lifespan by decreasing rates of cell division and favouring repair and maintenance” (Gillespie et al., 2016).

1.3.2. Concerns and Pitfalls of Calorie Restriction

Despite the seemingly beneficial profile of CR, there has of course been significant concerns and pitfalls highlighted by critics. On a purely practical level, defining CR is difficult, and no unified composition and intake has been established. In terms of total caloric intake, it is apparent that severe rather than mild/moderate CR in humans results in the same metabolic and molecular adaptations typical of long-lived CR animals, whilst improving overall health and reducing multiple metabolic factors implicated in the pathogenesis of the most common chronic diseases typical of Western countries (Most et al., 2017). For many though, severe CR with adequate micronutrition is not an option. Primarily, intentional CR in elderly populations would contribute to significantly higher risk of functional impairment and incident disability (Coker and Wolfe, 2018). In an exhaustive review of dietary interventions in the elderly it was concluded that caloric restriction or "dietary alone"–induced weight loss contributed to the development of sarcopenia and bone loss, and likely precipitated the genesis of frailty in vulnerable individuals (Batsis et al., 2015). These concerns are mirrored in a recent review by Ingram and de Cabo, who describe the limited utility of CR in aged rodents, and the potential implications for humans (Ingram and de Cabo, 2017). Both mice and rats initiated on CR in late-life often results in negligible effects or indeed reductions in longevity (Forster et al., 2003; Lipman et al., 1998, 1995; Ross, 2009). Research into the latest beneficial initiation point is ongoing, however Dhahbi et al. initiated CR (~40%) in 19-mo old male C3B6F1 mice and reported significant increases in lifespan accompanied by reduced tumour rates as well as a global gene transcriptional profile that resembled life-long CR (Dhahbi et al., 2004).

However, responsiveness to CR is not a ubiquitous phenomenon, and its ineffectiveness is not limited to the elderly. This is at odds to the attractive paradigm of longevity prolongation by CR being universal among animals. If the applicability of CR to human longevity is not upheld through evolutionary conservation, it is critical to the rationale of its implementation. This is particularly concerning given the apparent unresponsiveness of Colman's Rhesus monkeys, who despite demonstrating reduced morbidity, failed to live any longer (Colman et al., 2009). Subsequent discussion has indeed highlighted the possibility that CR is genotype sensitive, and optimum food intake is genotype and sex-specific, explaining the disparity of responsiveness between the two rhesus monkey trials (Mattison et al., 2017). This idea has been explored further in rodents. Research at two separate sites showed that the effects of 40% CR were very dissimilar in different ILSXISS recombinant inbred strains of mice, with some strains exhibited an adverse response (Liao et al., 2010; Rikke et al., 2010). This has been reciprocated in short lived DBA/2J mice that exhibit a genotype-specific variability in CR responsiveness ranging from a slight reduction to a greater than 60 % increase in mean lifespan (Ingram and de Cabo, 2017). Researchers suspect that stringent CR regimes of ~40% may be too aggressive for certain strains, which would result

in undernutrition. Thus, some strains would maybe exhibit increased lifespan if they were tested at a lower level of dietary restriction. Furthermore, it may be that longevity-extending effect of CR is only evident when animals have been housed in laboratory conditions for several generations, evidenced by an inability to extend lifespan in wild caught mice (Harper et al., 2006).

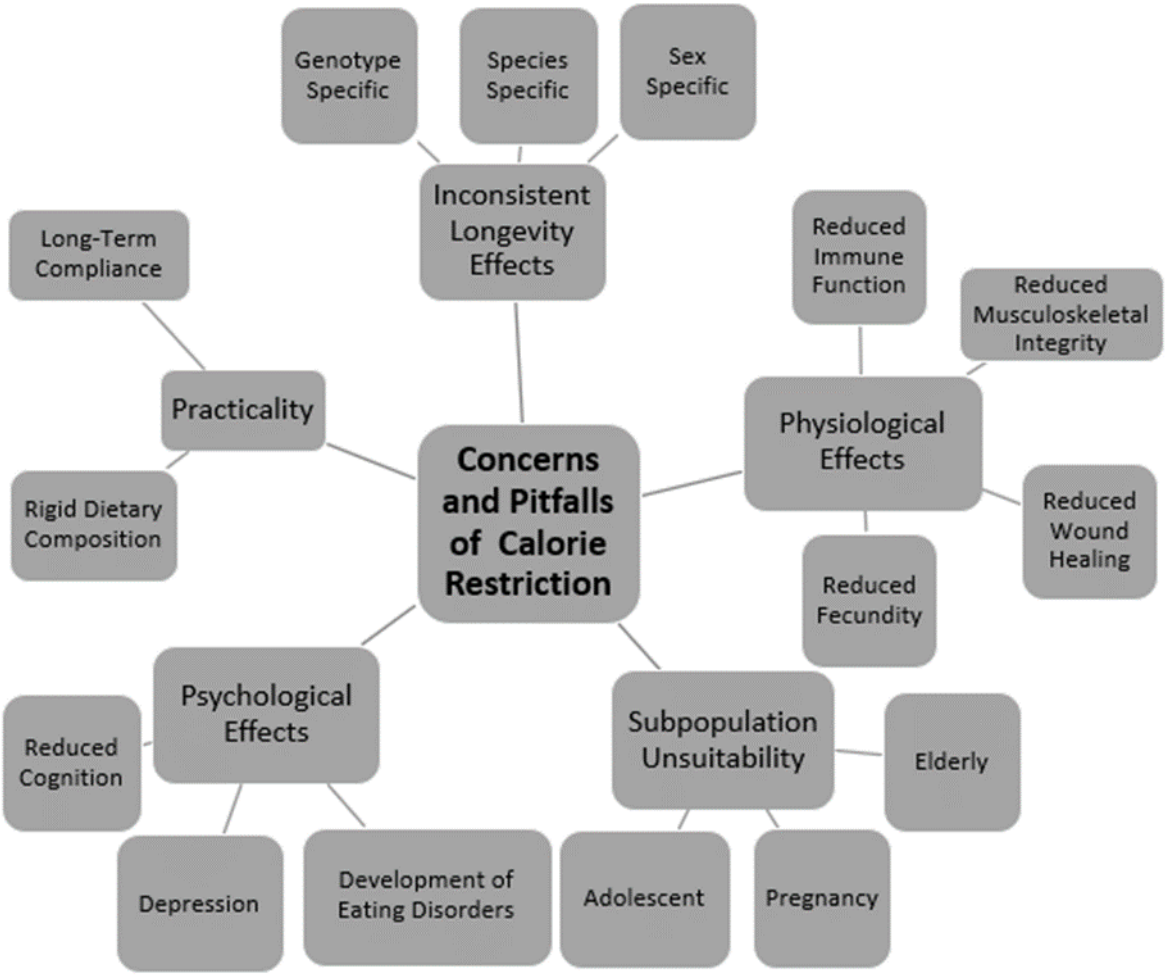


Figure 2 Concerns and pitfalls regarding the clinical applicability of calorie restriction for geroprotection in humans.

The modulation of dietary intake to test optimal dosages is achievable in rodent models due to the administration of a fixed and regulated simple diet. This is extremely difficult and is succinctly expressed in a recent review, "Randomized controlled trials for single micronutrients or bioactive ingredients are much easier to accomplish than macronutrient, whole food, or dietary pattern studies. The more complex the design of menus; the procurement, storage, and transfer of the intervention to the participant; and the participant's ability to handle receipt of the intervention, including storage, preparation, and protocol compliance, can be daunting" (Weaver and Miller, 2017) . One of the largest obstacles to CR in human populations is undoubtedly compliance and this can be greatly improved if the regime is tolerable. However, research has continually raised the psychological problems induced by CR, mostly pertaining to mental challenge of reduced food intake. Active participants have reported an increased desire to eat and a decreased satisfaction of appetite (Anton et al., 2009). This has been tied to pressing concerns following the development of eating disorders such as binge eating, anorexia and bulimia in CR trials (Redman and Ravussin, 2011). Reassuringly, however, well-controlled studies such as CALORIE have reported no adverse effects of CR on mood, cognitive performance, memory, attention and health-related quality of life after 2 years of CR (Martin et al., 2016; Velthuis-te Wierik et al., 1994). The effects of decades of CR are still unknown and furthermore, the effects of long-term CR on cognition and neurodegeneration in humans will remain unknown for years to come. Little research on cognition has emerged from the rhesus monkey studies. That said, a pertinent study in non-human primates using lemurs noted that, whilst cognitive and behavioural performances were not modulated by CR, a significant acceleration atrophy of grey matter occurred following CR (Pifferi et al., 2018). This reinforces potential concerns regarding the long-term administration of CR on brain integrity in human populations.

Concerns have also been raised regarding the immunological function of animals under CR. Gardner et al. (2005; 2011) in a series of papers first provided experimental evidence that CR mice exhibited CR impaired responses to the influenza virus resulting in a much higher risk of mortality. These concerns were compounded by increased colonisation by various nematode infections following inoculation in CR mouse strains, which additionally proliferated an aberrant t-cell response (Kristan, 2007; Shi et al., 1998). Furthermore, infection with *Helicobacter hepaticus* induced greater colitis and resultant mortality in *CR* SMAD3−/−mice vs control SMAD3−/−mice (McCaskey et al., 2012). Conversely, many papers have associated CR with improved immune function. This stems from research suggesting mice injected with influenza faired significantly better than their controls (Effros et al., 1991). Kristen has since reviewed the relationship between CR and infection and concluded that despite an enhanced immune function, "CR hosts are more susceptible to infection by intact pathogens than their fully fed counterparts"(Kristan, 2008). It is likely, that the metabolic demands required to mount an appropriate immune response may

not be met in CR rodents. This reflects similar concerns of slower wound healing in CR rodents which may translate to reduced reserve function in humans. For some decades, a continuous flow of research has amounted. As early as 1988, studies reported that mice, and then later, that rats on CR had slower rate of wound healing measured by in vivo assays (Harrison and Archer, 1987; Hunt et al., 2012; Reed et al., 1996). This is supported by mechanistic studies elucidating that CR mice have affected collagen production, extracellular matrix deposition and neovascularisation (Otranto et al., 2009; Spanheimer et al., 1991). It is thought that CR does not diminish the ability to heal wounds but rather preserves one's proliferative capacity, and upon the reinstating of sufficient nutrient reserve, wound healing is actually enhanced (Dirks and Leeuwenburgh, 2006). This theory is bolstered by murine work showing that upon *ad libitum* refeeding of CR mice 4 weeks prior to injury, resulted in improved wound healing compared to controls (Reed et al., 1996). In humans, results again lack long term inference. Mild leukopenia has been observed in the CALORIE-2 CR population but there is a negligible impact on immunity (Contreras et al., 2018). In non-human primates and mice, the number of naive t-cells in CR populations is higher and unchanged in human populations undergoing over a decade of CR (Messaoudi et al., 2006; Meydani et al., 2016; Tomiyama et al., 2017). Thus CR must be approached with caution in those who are immune-deficient or indeed vulnerable to infection, and more work must be done to establish CR dosages that do not significantly impinge on immune function.

Critics also highlight the danger of reduced musculoskeletal integrity associated with CR (Larson-Meyer et al., 2010; Velthuis-te Wierik et al., 1994). Additionally, reduced muscle mass and energy reserve, as a result, have been shown to reduce physical performance in humans and model organisms. Short-term CR studies in humans demonstrate a shortened time to exhaustion and reduced strength and aerobic fitness (Larson-Meyer et al., 2010; Velthuis-te Wierik et al., 1994). Undoubtedly, human trials have demonstrated that CR produces favourable body compositions. CR participants experience reductions in fat mass with a higher lean skeletal muscle mass-to-fat mass ratio (Das et al., 2017; Mercken et al., 2013). There is of course a concomitant reduction in free-fat mass and obviously muscle mass (Das et al., 2017). This was initially concerning; however, it is now apparent that upon nutritional equilibrium to muscle mass, skeletal muscle declines plateau (Dirks and Leeuwenburgh, 2006). From thereon, research suggest that muscle strength actually increases relative to body mass in CR participants (Racette et al., 2017). However, whilst participants remained relatively strong, the objective sarcopenia must be tempered with thermogenesis and protective concerns. Individuals with less muscle mass may have a reduced ability to generate heat, manifesting in increased cold sensitivity in participants (Dirks and Leeuwenburgh, 2006). This of course endangers individuals with comprised thermoregulation or indeed reduced stress resistance. Importantly, reduced body weight, insulation and muscle mass may lead to

increased exposure of potentially weakened muscles to trauma. Long term calorie restriction has been shown to significantly decrease bone mass and mineralisation in both mice and humans (Ingram and de Cabo, 2017; Villareal et al., 2016). Concerningly in humans, this bone loss was found to be at clinically important sites of osteoporotic fractures such as the hip and femoral neck and the lumbar spine (Villareal et al., 2011). This is compounded by mouse studies showing that mid-life CR resulted in detrimental effects to bone architecture and strength (Banu et al., 2001; Colman et al., 2007). Clinical trials in humans have challenged the effects of reduced bone mass. Indeed, members of the CR society actually show no change in the rate of bone turnover (Villareal et al., 2011). Furthermore, trabecular bone architecture, (a major determinant of bone strength and a good predictor of the risk of developing fragility fracture) was not significantly different between the CR and control groups, despite the CR-mediated low bone mineral density (Most et al., 2017; Villareal et al., 2011). It has been suggested that although bone density may decrease, bone quality and strength is improved through a reduction of bone turnover and a prevention of secondary hyperparathyroidism (Most et al., 2017; Villareal et al., 2011). As suspected, CR does have a proclivity to affect bone quality and mass, however these appear to be dose and site-dependent. In dosage tested in mice, only 30-40% CR induced negative effects on bone architecture (Berrigan et al., 2002). This reinforces concerns regarding the appropriate composition and dosage of CR for humans to maintain appropriate body composition and musculoskeletal integrity. Moreover, the significant reduction in fat mass seen from CR, can lead to sexual dysfunction, infertility and menstrual irregularities in women (Dirks and Leeuwenburgh, 2006). Imperatively, women with low BMI are at greater risk for pre-term delivery and birth of low birth-weight infants (Allen et al., 1994). These associations detail more broader concerns of CR on reproduction in animals and humans.

Fecundity concerns of CR centre around the disposable soma theory: the reallocation of resources away from reproduction to somatic maintenance (and thus survival) to increase the chance of surviving the period of resource limitation. Thus, calorie restriction should, in theory, result in reduced reproductive capacity. Simply, reproductive output is negatively associated with lifespan in humans (Tabatabaie et al., 2011). However direct evidence in humans is limited. In mice, long lived species tend to have a markedly reduced reproductive capacity and CR indeed supresses fecundity (Partridge et al., 2005; Soultoukis and Partridge, 2016). Mice on CR, additionally, produce offspring with low birth weight and multiple metabolic defects, including early life adiposity, altered pancreatic function and progressive glucose intolerance (Ingram and de Cabo, 2017). A recent meta-analysis looking at the effect of reproduction and CR in 26 studies on over 20 different species, based on 1096 control and 1132 treatment subjects drew three conclusions (Moatt et al., 2016). First and foremost, and in agreement with other research by Nakagawa et al., CR does lead to a reduction in reproduction but, in line with longevity (Nakagawa et al., 2012).

Second, reproduction declines linearly with increasing CR, at odds with both current evolutionary theories of CR. However, they were uncertain of the reliability of this pattern given the paucity of data at the tails of distribution. Lastly, they noted a sexually dimorphic effect of CR, with a greater effect in females. The authors primary concern regarding all three conclusions hinged on a selection bias towards routinely used model organisms. Indeed they highlighted the citation bias towards 3 studies using drosophila and rats that may be shaping false assertions between fecundity and CR. The meta-analyses found the rate of decline of reproduction with increasing CR was steeper in model than non-model species. This could be due to two contributing factors. Frist, laboratory animals have been in nutrient rich environments for many generations, putting selective pressure towards fecundity rather than longevity. Thus, CR model organisms respond more plastically to food availability. Second, it may be that after decades of research, the dosages required to elicit efficacious CR in these model organisms has been well established compared to other animals, such that effect of CR on fecundity is further unmasked.

In monkeys however, the effects of moderate CR on reproductive function is less apparent. Female rhesus monkeys undergoing 30% CR displayed no adverse effects (Sitzmann et al., 2008). In males, CR appears to have little impact on testicular function or sperm parameters, however ejaculate osmolality and pH were benignly changed in response to nutritional status (Sitzmann et al., 2010).

In conclusion, CR is clearly not appropriate for certain sub-populations, such as women of child-bearing age, developing children and adolescents, and elderly or infirmed populations. Furthermore, until an appropriate dosage of CR can be established the risks posed by inappropriate levels of CR remain a large obstacle. Even with appropriate dosage and composition, the window of opportunity to prescribe CR is small and may be ineffective. Taking females for example, their childhood and adolescence would prohibit CR. Then during their twenties there may be a small window to incorporate CR. Then throughout child-bearing ages of 30, CR again becomes inappropriate. Then, provided they remain free from illness, which is unlikely, there may be a period to implement CR from 40-60. From 60 onwards, particularly in menopausal woman, concerns over musculoskeletal integrity may prohibit CR. Would this be a big enough period of implementation?

1.3.3. Calorie Restriction Mimetics

There is a clear moral and economic obligation to rapidly provide an intervention to decelerate the ageing process. At present, calorie restriction offers the most feasible and robust intervention. However, as highlighted, several concerns surrounding the applicability, tolerability and safety of calorie restriction currently precludes its adoption. Thus, the search for a viable alternative that could recapitulate the phenotype of calorie restriction without dietary implementation has greatly accelerated

in the last decade. In 1998, the discovery that feeding 2--Deoxy-d-glucose to rats mimicked the physiological effects of calorie restriction, led to the coining of the term “calorie restriction mimetic” (CRM) (Lane et al., 1998). Since then, the number of citations associated with the term has grown from 21 citations in 2006, to 75 citations in 2015 (Ingram and Roth, 2015).

A number of recent reviews on CRM state the main limiting factor which justifies the use of CRMs is a poor compliance to the demands of a CR lifestyle, likely explaining some failures in observational clinical studies (Madeo et al., 2019; Picca et al., 2017; Redman et al., 2018; Shintani et al., 2018). Indeed, the overarching benefit of CRMs lies in the fact one may have their cake and eat it. In fact, according to Ingram and Roth, CRMs by definition, must not exert their benefit through a reduction in food intake and any doing so would constitute an induction of CR rather than a *de facto* mimicry (Ingram and Roth, 2015). Key here is the ease of implementation. CRMs, manifest normally as pharmacological intervention, and offer users the hormonal, metabolic or physiological effects of CR without a substantial change in diet or caloric intake. Thus, the compliance to such a regime is far more likely to persist for the requisite duration that CR demands. Furthermore, given the vital caveats surrounding the daily implementation of CR in terms of dietary composition and timing, CRMs should fare better. A single CRM would require tailoring only in its dosage and timing. This expediates trials in model organisms as researchers need to account for less factors. Consequently, an optimum dose and regime should be found sooner, and if translatable to humans, offers a routine and followable prescription.

The oft-cited unsuitability of CR in many subpopulations is in part driven by the pleiotropic nature of CR that induces a catalogue of physiological effects. The numerous and largely undefined pathways which mediate the ageing process, do of course, also involve themselves in the regulation of immunity, tissue repair, thermoregulation and reproduction (Le Couteur et al., 2012). Ideally CRMs would bypass the pleiotropy of CR and provide a more targeted approach. Indeed, this distinction prefaces the duality of CRM research: those that search to provide upstream substrates that fully recapitulates CR and those that wish to elicit certain cellular responses of CR through downstream activation of key pathways.

Some researchers propose that the effects of CR can only result from upstream processes as rather than being driven through a single or a few vital genetic pathways, ageing is manifested through the actions of multiple pathways. Thus a screen for identifying candidate CRM should focus on direct manipulation of metabolic and energy sensing systems (Ingram et al., 2006). As briefly mentioned, 2--Deoxy-d-glucose was the first example of an upstream CRM and exerts its prolongevity effect through the suppression of glycolytic activity (Roth et al., 2005). Consequently, metabolism is shifted to mitochondrially-based

oxidation of fat and an increased formation of ROS. This is represented by a reduction in metabolic rate, manifested as a reduction in locomotory activity, heart rate, and blood pressure similar to CR (Wan et al., 2003). Elegant *C. elegans* study demonstrated that the likely longevity benefit of 2-deoxy-d-glucose was derived from "mito-hormesis": a mild stress response from low dose free radical toxicity that encourages cell survival and preservation (Schulz et al., 2007). It follows on from the more general concept of "hormesis" which stipulates that low-dose stress on an organism can improve healthspan and lifespan. A similar compound, d-Glucosamine, also demonstrates glycolytic inhibition which upregulates the mitohormetic response and improves longevity in model organisms (Weimer et al., 2014). Subsequent work shed some light on the mechanisms of this metabolic shift, showing that glucosamine upregulated autophagy in an mTOR independent manner, and this upregulation was essential for increased lifespan (Shintani et al., 2010). Many compounds, including amino acids and metabolic intermediates like βHB and α-ketoglutarate are upstream CRMs (Chin et al., 2014; Kim et al., 2018a). Notably, pharmacologically induced glucose restriction mediated by a number of compounds, such as 2-Deoxy-d-glucose, glucosamine and d-allulose extends healthy lifespan in worms (Schulz et al., 2007; Shintani et al., 2017).

Another technique that has been employed to directly inhibit the utilization of ingested calories is through the blockade or retardation of fat digestion or absorption. The two most well-studied candidates are chitosan and orlistat (Ingram and Roth, 2015). Chitoson is a supplement purported to bind to or "trap" dietary fat, preventing fat absorption and causing fat excretion. These reports are based on rodent assessment, however human analysis has revealed little change in faecal fat excretion (Gades and Stern, 2005). Orlistat, a commonly prescribed anti-obesity medication, similarly works by slowing fat breakdown through the inhibition of pancreatic lipases (Hollywood and Ogden, 2011; Zhi et al., 1995). Undoubtedly, these compounds have elicited significant physiological changes suggestive of CR such as weight loss and favourable changes in lipid profile and glycaemic control in overweight individuals (Jacob et al., 2009; Jull et al., 2008; Rucker et al., 2007). However, there have been no studies demonstrating any effect of these compounds on lifespan or indeed healthspan and it is unlikely efforts have not previously been made. Thus, at present glycolytic inhibition appears the most recapitulative upstream mechanism to mimic CR longevity. Importantly though the main limitation to upstream energy targeting lies in the precise holistic mimicry of CR. Whilst their activation of anti-aging pathways may be undifferentiated, they will of course also lack orthogonality, which CRMs were intended to epitomise.

As such, researchers have looked to modulate specific downstream pathways of CR in a hope it might provide a more precise and directed evocation of the beneficial aspects of CR without any adverse

associations. Indeed, the most successful interventions have focussed on the 3 main age-associated pathways: mTOR, AMPK and IGF-1. When assessing the validity of a compound as a CRM beyond its inability to alter food intake, researchers propose that they must also activate stress response pathways, provide protection against stressors and lead to a preservation of healthspan (Ingram et al., 2006). Two major drugs have emerged as leaders of the race to define the first *bone-fide* prescriptible CRM.

Firstly, pharmacological interventions aimed at activating the AMPK nexus have been developed or repurposed to mimic CR downstream. These substances include some FDA-approved drugs including biguanides, resveratrol, thiazolidinediones, salicylates and glucagon-like peptide-1 receptor agonists (Vaiserman et al., 2016). Biguanides represent a class of compounds that include phenformin, buformin, and the modern-day metformin and have been used since the 1950s as major anti-diabetic treatments due to their robust ability to reduce hyperglycaemia, insulin, gluconeogenesis, intestinal glucose absorption, serum lipids and somatomedin (Ingram and Roth, 2015). However, in response to safety concerns over lactic acidosis development in many patients on forerunner biguanides (phenformin and buformin), metformin has since become the most widely used biguanide drug to treat type 2 diabetes mellitus (T2DM) and metabolic syndrome with over 120 million prescriptions worldwide (Anisimov, 2003; Lushchak et al., 2017).

The ubiquitous and long-term clinical use of metformin has provided data for the thousands of publications and precipitated over 2000 metformin clinical trials (Balasubramanian et al., 2017). Moreover, this has provided a platform for key epidemiological study and meta-analyses linking metformin usage to improved survival outcomes and ageing in general. Early studies by Scarpello (2003) and also Eurich et al., (2005) noted increased survival from all-cause mortality in diabetic and heart-failure patients. At a similar time, concordant research observed a reduction in cardiovascular risk in individuals with diabetes treated with metformin (Johnson et al., 2005). This has since been supported by recent work demonstrating a 6% reduction in CV risk and also carotid artery intima-media thickness (a surrogate marker of CVD) in metformin treated diabetics (Petrie et al., 2017; Wang et al., 2017). Meta-analyses have revealed conflicting evidence with the first suggesting reported no significant over-all mortality benefit of metformin whilst the more recent, and arguably more fastidious, revealing that diabetics taking the drug had lower all-cause mortality and age-associated diseases than non-diabetics (Campbell et al., 2017). Nonetheless, a multitude of research has indicated that metformin seems to be beneficial against a number of other age-related diseases, including cancer, cardiovascular disorders and chronic kidney disease (reviewed in Valencia et al., 2017).

In light of these compelling findings, research on the geroprotective properties of metformin has been rapidly developing in model organisms to determine dose effectiveness and safety, physiological/metabolic effects, and mechanisms. 2008 was the first time metformin, and moreover, any pharmacological agent, had been proven to extend lifespan in a rodent model (Anisimov et al., 2008). Admittedly, this model, the outbred Swiss-derived female mice, is rarely used outside Russia, however it led the way for further, somewhat conflicting publications. Numerous lifespan studies, including the former, have now assessed metformin in different genetic backgrounds. The most cited paper (586 citations as of April 2019), by de Cabo's lab, tested two doses of metformin (0.1% and 1%) in regular C57BL/6 mice initiated at 12 months of age. A clear and contrasting dose response was observed with the 0.1% w/w male group showing increased the mean life span (6%), whilst lifespan was significantly reduced at the higher dose (Martin-Montalvo et al., 2013). However, the 0.1% dosage was subsequently tested by ITP using their UM-HET3 mouse model: they found that when initiated at 9 months of age, metformin did not significantly increase the life span of either male or female mice (Strong et al., 2016). Despite the somewhat indeterminacies of these studies on lifespan, metformin has repeatedly demonstrated a proclivity to improve healthspan in rodent models. In the de Cabo lab experiments, the lower dose increased healthspan as indicated by a number of parameters measured at older ages, including improved glucose tolerance, increased treadmill endurance, better rotarod performance, higher levels of locomotor activity, and reduced incidence of cataracts (Martin-Montalvo et al., 2013). Furthermore, other reports have noted improved mitochondrial function, reduced inflammation, cognitive performance and estrous function (Allard et al., 2016; Anisimov et al., 2011).

Similar difficulties in eliciting lifespan extension has been observed in drosophilae. Administration of metformin to flies did reduce adiposity in line with rodent models, but ultimately failed to prolong lifespan; higher does were actually toxic (Slack et al., 2012). Interestingly however, metformin does robustly activate AMPK, the kinase which is thought to mediate the majority of metabolic actions by metformin (Viollet et al., 2012). Indeed AMPK activity is significantly upregulated in mice and *C. elegans* treated with metformin (Martin-Montalvo et al., 2013; Onken and Driscoll, 2010). It has been proposed that the changes in cell signalling from the activation of AMPK, occur from an AMP:ATP shift precipitated by the inhibition of electron transport chain complex I (Foretz et al., 2014; Owen et al., 2000). Activated AMPK can then drive the catabolic and insulin sensitising effects associated with improved physiological integrity such as cellular uptake of glucose, β-oxidation of fatty acids, increased glucose transporter 4 (GLUT4) expression, and mitochondrial biogenesis (Solon-Biet et al., 2015). However, some of the physiological benefits of metformin cannot be accounted for by AMPK activation alone. For example, reduced gluconeogenesis, a process central to the anti-diabetic

response to metformin, occurs through non-competitively inhibiting the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase to produce an altered hepatocellular redox state that reduces the conversion of lactate and glycerol to glucose (Madiraju et al., 2014). Furthermore, evidence also exists that the prolongevity effects of metformin may occur independently of AMPK through target of rapamycin complex 1 (TORC1) inhibition. It was shown that metformin inhibits Ras-related GTP binding (Rag) GTPases, thus directly regulating mTORC1 activity (Kalender et al., 2010). Secondly, the downstream targets of mTOR, S6K1 and 4E-BP1 are directly inhibited by metformin (Dowling et al., 2007). Other hypothesis for metformin's geroprotection are alterations in microbial folic acid and methionine metabolism as well as induction of mitohormesis via H_2O_2 regulated by the peroxiredoxin PRDX-2 pathway (Cabreiro et al., 2013; De Haes et al., 2014).

Beyond evident modulation of key stress pathways altered by CR, alongside increased lifespan and healthspan, metformin demonstrates key candidacy as a CRM through its similarity of transcriptome: microarray analysis showed marked overlap between gene expression patterns induced by both metformin and CR (Dhahbi et al., 2005). Whilst further mechanisms remain elusive, it is likely that metformin suffers from the same pleiotropy as CR. Research is beginning to highlight key flaws in the utility of metformin including a propensity to reduce visual acuity and spatial memory of aged male mice and also the exacerbation of damage caused to sciatic nerve fibers and incidence of porcelain gallbladder (Ciric et al., 2018; Dorvash et al., 2018; Thangthaeng et al., 2017). Nonetheless, funding has been granted to begin "the first-in-class study enrolling patients who have been diagnosed with one single age-associated condition and designed to detect the capacity of metformin to delay the manifestation of a second, equally age-associated disorder" (Palliyaguru et al., 2019). At present, metformin is a well-tolerated intervention in humans, including the elderly and non-diabetic which has expedited this clinical trial. However, the doses used to induce lifespan extension in rodents generally exceed the recommended daily metformin therapy in patients with type 2 diabetes, and may correlate to an increased risk, putting into question the worthwhileness of metformin treatment given its relatively modest effects on lifespan in rodents.

Metformin is the only potential CRM to date that has been enrolled in a clinical trial with the sole intention of decelerating ageing. However, rapamycin, the other CRM candidate discussed in this thesis, correspondingly has approved efficacy in clinical trials. Rapamycin, also known as sirolimus, is a complex macrolide antibiotic, produced by *Streptomyces hygroscopicus* strain isolated on Easter Island in 1972 (Seghal et al., 1975). It was initially developed as an antifungal agent, but is now used in clinic by reason of its immunosuppressive effects to prevent renal transplantation rejection and to treat

autoimmune disorders (Lamming et al., 2013). The primary mechanism for this indication is due to the inhibition of interleukin-2 signalling and other cytokine-receptor-dependent signalling pathways by acting on mTOR and preventing the activation of T and B cells (Thomson et al., 2009).

Upon the discovery the TOR pathway as an important regulator of ageing in yeast and *C. elegans*, the potential anti-aging properties of rapamycin began to be seriously explored (Arriola Apelo and Lamming, 2016). Interest was further spiked following research in 2009 by the National Institute on Ageing Interventions Testing Program reporting that dietary supplementation with rapamycin increased mean lifespan 28–38% compared to control animals and also maximum lifespan in heterogeneous mice (Harrison et al., 2009). Importantly, and in contrast to metformin, treatment was initiated at 20 months of age, corresponding to approximately 60 years of age in humans. This late-life prolongevity effect can also be elicited in mice when initiated at 9 months or 19 months as well (Miller et al., 2011; Zhang et al., 2014). Since the initial discovery, numerous other rodent studies have replicated the pro-longevity effect of rapamycin supplementation, even when combined with poor diet (Leontieva et al., 2014). Research has shown rapamycin to also delay ageing-related changes in mice, including tendon stiffening, accumulation of sub-cellular alterations in the myocardium, endometrial hyperplasia, liver degeneration, and decline in physical activity (Wilkinson et al., 2012). Moreover, Rapamycin has been shown to ameliorate morbidity and mortality in mouse models of several neurodegenerative diseases and also progeroid syndromes (reviewed in Lee et al., 2019).

With that said, research has not been unequivocal. Neff et al., although inducing lifespan extension, failed to successfully manipulate most healthspan parameters of aged mice (Neff et al., 2013). In fact, they noted that the putatively age-related traits improved by rapamycin are similarly affected in both young and old mice. This has brought into question the benefit of rapamycin in humans, and led many to suggest that rapamycin is not an anti-aging intervention per se (Richardson, 2013). This is view is in part driven by parallel oncological research indicating rapamycin to extend lifespan through a reduction in cancer mortality rather than a deceleration of ageing (Ciuffreda et al., 2010). Indeed, rapamycin has significantly improved survival in cancer-prone strains of mice and its analogues are clinically used to treat a number of cancers such as: follicular lymphoma or mantle cell lymphoma, pancreatic neuroendocrine tumours, soft-tissue sarcomas, B-cell non-Hodgkin lymphoma, advanced hepatocellular carcinoma, paediatric solid or central nervous system tumours (Kopeina et al., 2017). However, the mechanisms modulating the anti-tumour and anti-ageing aspects of rapamycin are thought to both be due to the direct inhibition of the mTOR pathway which upregulates autophagy (Perluigi et al., 2015), as well as immune-modulating and anti-inflammatory activity beyond that of tumour suppression (Araki et

al., 2009). Furthermore, the inhibition of mTOR can be thought to mimic at least some of the aspects of calorie restriction.

Firstly, as in CR, rapamycin precipitates similar FOXO-mediated apoptotic effect in human cancer cell lines, however in healthy human cells, the apoptosis-promoting properties of FOXO are attenuated by SIRT1 promoting cell survival rather than death (Giannakou and Partridge, 2004; Gillespie et al., 2016). Equally, as stipulated in Ingram and Roth's prerequisites, rapamycin upregulates key stress pathway genes associated with CR that protect against oxidative stress: SOD1, glutathione reductase, δ-aminolevulinate dehydratase (Kofman et al., 2012). Also, rapamycin appears to attenuate the production of ROS *in-vitro* (Shin et al., 2011). Physiologically, similarities between CR and rapamycin can be observed: rapamycin leads to decreased body weight and adiposity and in turn induced improved endocrine function as measured by reduced serum leptin (Arriola Apelo et al., 2016; Carter et al., 2016; Miller et al., 2011).

Rapamycin is the only CRM that has reproduced a similar lifespan increase to that of CR (Arriola Apelo and Lamming, 2016). Unquestionably, rapamycin is a powerful inducer of cellular change and as mechanisms are still being elucidated, numerous safety concerns have emerged. Oncological use of rapamycin in humans comes with significant risk of metabolic complications such as hyperglycaemia, hypercholesterolaemia and hypertriglyceridemia (Sivendran et al., 2014). This adverse metabolic profile is equally present during lifespan studies in mice treated with rapamycin. Numerous authors have reported insulin resistance, hyperlipidaemia and glucose intolerance (Neff et al., 2013; Wilkinson et al., 2012). Further research suggests this is likely due to dysfunctional hepatic gluconeogenesis induced by chronic rapamycin treatment eventually inhibiting mTORC2 (Lamming et al., 2012). Of note though, rapamycin-induced metabolic impairments appear to be fully reversible in both lean and obese mice, raising possibilities and indeed successes in intermittent dosing (Arriola Apelo and Lamming, 2016; Liu et al., 2014).

More concerning though in lifespan studies with rapamycin is the repeated observation of testicular degeneration and increased incidence of cataracts (Richardson, 2013). Additionally, the immunosuppressive properties of rapamycin will continue to raise concerns on its applicability in vulnerable populations. These issues, particularly metabolic ones may resolve as rapalogs more specific for mTORC1 are developed, and indeed rapamycin remains a key candidate to become a *bone fide* CRM. However, clinical applicability of rapamycin remains uncertain especially as no long-term studies have been conducted in large non-human primates. Following on from concerns that rapamycin may not actually slow ageing alongside research also showing a longevity selection bias towards

female mice. Moreover, acute rapamycin treatment in humans cells induces a far more aggressive transcriptional change than in mice indicating that rapamycin may not be as beneficial to humans as it is to lower order species (Fok et al., 2014; Gillespie et al., 2015). Thus, a rational approach to rapamycin must be made beyond the hype.

Both rapamycin and metformin have shown promise and at least demonstrated potential in mimicking the geroprotective properties of CR. However, they both come with substantial criticism. Metformin may provide a still unsubstantiated pleotropic effect whilst rapamycin appears to aggressively over-downregulate one pathway associated with CR. Thus, much work is still required to identify compounds which provide a safe and tolerable profile that extends lifespan and healthspan in a manner similar to CR.

1.4. Allantoin and Other Imidazoline Receptor Agonists as Pro-longevity Calorie Restriction Mimetics

Expediated by recent advances in genomic and transcriptomic sequencing, alongside high throughput genetic and compound screens, a wealth of public data is available to researchers to assist in understanding the discrete complexities of calorie restriction on a genomic, transcriptomic and proteomic level. This has empowered researchers to greatly improve the reverse pharmacological screening process to find and test direct targets of calorie restriction. Efforts have been made in the past to identify a CR gene signature that overlapped relevant compounds but this did not seek to identify novel compounds (Dhahbi et al., 2005). Despite this research, and encouragement from Spindler and Mote, (2007), little has been done to capitalise on this potential.

However, In 2015, our lab exploited Gene Expression Omnibus (GEO), a public database of gene expression files, to isolate an *in-vitro* transcriptome of calorie restriction, derived from rat cell lines exposed to rhesus monkey sera from CR states (Calvert et al., 2016). This transcriptome was then uploaded to a now-outdated Connectivity Map to isolate drugs with an overlapping gene signature (Lamb et al., 2006). Following up these potential drug hits to assess *in-vivo* effects was essential.

Calvert's connectivity map results revealed 5 compounds with a high similarity to the CR gene signature and these were subsequently screened in *C. elegans* for lifespan and healthspan effects. Reassuringly, this method returned an internal control, rapamycin, which duly extended lifespan in a solid media lifespan assay. One particular compound, allantoin, which prior to this study, had not been studied in the context

of ageing, extended lifespan by 21.9%, in a CR dependent way, and significantly improved healthspan defined by pharyngeal pumping.

Calvert et al. speculated that allantoin, as a product of free-radical uric acid oxidation, may exert its lifespan extension through upregulating stress responses in reaction to perceived changes in oxidative stress (Artyukin, 2013). Like humans, *C. elegans* lacks uricase, an enzyme responsible for oxidation of uric acid to allantoin (Ramazzina et al., 2006). Furthermore, in nematodes nitrogen excretion occurs only in the form of ammonia and amino acids, while no significant amounts of urea, uric acid, allantoin, or creatinine have been recorded (Rothstein, 1963). Thus, it is unlikely, allantoin remains as the final step in uric acid metabolism. The oxidative stress feedback hypothesis proposed by Calvert et al. becomes even more untenable when considering that allantion levels are negatively correlated to lifespan with high urate:allantoin ratio being observed among long-lived species (Ma and Gladyshev, 2017). Moreover naked mole rats have a particularly low expression level of uricase, corresponding to lower production of allantoin, and interestingly, humans, as the longest-lived primates have the highest serum urate level (Cutler, 1984; Ma et al., 2015).

Another hypothesis explaining the prolongevity effect of allantoin involves its affinity to the imidazoline type 1 receptor (I1R). This receptor is part of a broader family of imidazoline receptors discovered by Bousquet and Schwartz in 1983, who demonstrated that clonidine, a hypotensive agent, depressed sympathetic tone independently of the canonical adrenoreceptor (Bousquet and Schwartz, 1983). Unlike α2 adrenoreceptors, imidazoline receptors do not show affinity towards catecholamines, but instead bind imidazoline and guanidinium compounds of which clonidine and allantoin are derivatives of the latter (Dardonville and Rozas, 2004).

Yang et al., (2012) discovered that allantoin was able to activate I1Rs in animal models and cell lines. Furthermore, 5mg/kg intraperitoneal injections of allantoin significantly reduces body weight of high-fat diet (HFD) mice in comparison to control HFD; however, this effect was abrogated following antagonism of IR1s by efaroxan (Chung et al., 2013). Other research has demarcated allantoin to exert anti-asthmatic (Lee et al., 2010), antidiabetic (Niu et al., 2010), antihypertensive activities (Chen et al., 2014), as well as memory-enhancing effects in AD (Ahn et al., 2014). Furthermore, allantoin appears to upregulate key genes associated with CR-induced longevity and, recent research has highlighted a transcriptional similarity between metformin and allantoin, including increased AMPK activation (K.-C. Lin et al., 2012; J. Ma et al., 2018). Lastly, recent studies, profiting from our lab group's initial studies have since confirmed increases in *C. elegans* lifespan in response to allantoin (Admasu et al., 2018).

Unfortunately, allantoin lacks convenient translatability. Allantoin is degraded by the microbial flora of the GI tract, and orally administered allantoin fails to be metabolised and excreted in the urine (Kahn and Nolan, 2000). Thus, allantoin requires intravenous or intraperitoneal administration which would significantly disincentivise its use in humans; furthermore, rodent lifespan studies would become inconvenient and expensive owing to regulated daily/weekly injections. Perhaps more concerningly, F344 rats fed rat chow with (n=20) and without 0.2% (n=24) allantoin (2,000 ppm) demonstrated no difference in lifespan when the experiment was aborted after 106 weeks (Lijinsky, 1984).

Given that allantoin has a relatively weak affinity to I1Rs, it is possible that more specific I1R agonists may be able to confer an improved geroprotection from oral administration (Dardonville and Rozas, 2004). Notably, Ye et al., found guanfacine hydrochloride and guanbenz acetate, both guanidium-based I1Rs agonists to extend mean lifespan in *C. elegans* by 15 and 12% respectively (Ye et al., 2014). In a separate paper, the endogenously produced imidazoline receptor ligand agmatine was able to extend lifespan in *C. elegans* by 16% at concentrations of 1mM (Edwards et al., 2015a). Despite its endogeneity, agmatine has the same antihypertensive properties as centrally acting hypertensives, mediated through the imidazoline binding site. Finally, another endogenous imidazoline receptor agonist known as harmane has the propensity to extend mean lifespan in *C. elegans* bred in pathogenic environments by an average of 13 days (Jakobsen et al., 2013). However, neither rilmenidine nor moxonidine, both clinically approved anti-hypertensives, imidazolines with high affinities to I1Rs, have ever been tested in nematodes at all, and in other model organisms for longevity effects.

With this in mind, It was prudent to capitalise on an important opportunity to firstly examine whether the repurposement of clinically approved, more specific imidazoline receptor agonists such as rilmenidine, may be able to extend healthy lifespan in *C. elegans* through the safe mimicry of calorie restriction; and secondly establish through which molecular mechanisms and pathways this longevity effect may occur. Such repurposement and screening represents a reasonable and low-cost identification of easily implementable and translatable CRMs for anti-ageing clinical trials which should serve to reduce the suffering of old-age and improve wellbeing in later life: a moral obligation of the medical and scientific community. By establishing whether this potential CRM could slow down the processes of ageing in *C. elegans*—even by a moderate amount— would instigate a pipeline of research that could expediate the current ageing field and yield dramatic financial and societal benefits to society.

In order to do this, publicly available transcriptomes of cell lines exposed to rilmenidine and other I1Rs need to be mined to indicate whether rilmenidine may affect ageing pathways. If so, these bioinformatic results will require rapid high-throughput screening in *C. elegans* to assess for longevity affects. I will

then dissect the signalling pathway of rilmenidine in *C. elegans* to ascertain whether any longevity effects are mediated via two common ageing pathways associated with calorie restriction: mTOR and AMPK. Then finally it is important to assess the translatability of any longevity effects onto common chronic disease states to validate and discuss whether rilmenidine likely has any clinically geroprotective efficacy.

2. Methods

2.1. Bioinformatics

2.1.1. Probing the Connectivity Map with Age-associated Transcripts

Representative non-tissue specific ageing transcription signatures were gathered from two sources. First, we used a gene signature generated from a meta-analyses of 127 multi-tissue datasets using previously described methods (de Magalhães et al., 2009; Fabris et al., 2018). This is the largest known meta-analysis of multi-publication datasets, and includes datasets used in de Magalhães et al., (2009) and Voutetakis et al., (2015). A sub-analysis was performed using just brain tissue samples within the selected datasets. Notably, this meta-analysis did not include any data generated by the Genotype Tissue Expression Project. There is a similar meta-analysis of differential expression that tackles this data (Mele et al., 2015). They too used a linear regression model. We combined these two sets to establish a consensus gene list of DEGs from both analyses which could then be used to probe the connectivity map.

As performed in other research, the touchstone L100 Gene Expression cMap (Subramanian et al., 2017) was individually queried with each signatures using up to the top 150 recognised protein-coding overexpressed genes and top 150 recognised protein-coding underexpressed genes (Dönertaş et al., 2018). All genes were converted to Entrez IDs. The mean *tau* score and rank of each perturbagen across each signature was recorded. Tau scores over 90 or -90 were considered significant (Broad Institute, 2017).

For the list of DEGs in the Rostral Ventral Lateral Medulla of young and old F344 rats (Balivada et al., 2017), data was fetched from GSE90956 and the top 250 DEGs (as defined by log-fold change) were calculated using GEO2R R script which utilises GEO query and limma topTable function (Barrett et al., 2012)

2.1.2. Meta-analyses of Transcriptional Profiles and Functional Enrichment

Meta-analyses of the rilmenidine treated cell lines was conducted using .GCT files of normalised gene expression from the NIH LINCS Common Fund Programme. Cell line data was filtered to contain only 57 cell lines exposed to rilmenidine at a concentration of 10 μM for 24h. For the meta-analyses of combined imidazoline receptor agonists, the same criteria was applied, using 9 drugs against a total of 221 cell line arrays, producing 2,940,626 gene scores. Thresholds for differential expression values (robust z-ratios calculated by comparing the gene's expression value in the given sample to that same gene's expression

values in all other samples in the cohort and then collapsed replicate samples) were set at >2/-2 as previously defined (Koido et al., 2018). Whilst only 991 landmark genes have been arrayed, a further 12,000 genes have been assigned two levels of inference confidence: well-inferred and inferred. All inferred genes were included in the analysis as in (Wilson et al., 2015). Meta-analyses was done following methods described in de Magalhães et al., (2009). To identify genes consistently under/over expressed in response to rilmenidine and/or imidazoline receptor agonists, I calculated the number of times a gene exceeded the differential gene expression thresholds in each direction for every tested cell line. This tally was then subject to cumulative binomial distribution analysis and Benjamini–Hochberg correction to ascertain the probability of observing an equal or higher under or overexpression by chance; FDR of <0.05 was considered significant (Benjamini and Hochberg, 1995). Transcriptional signature pairwise comparisons were made between relevant gene lists using Fisher's Exact Test and p-values were subsequently adjusted for multiplicity using the Bonferroni correction which was represented by Venn Diagrams created using EuLerr (Larsson, 2018).

Gene ontology (GO) enrichment analysis on DEG lists was carried out using the R package 'topGO' (Alexa and Rahnenfuhrer, 2016) using the weight01 algorithm and Fisher's exact test, to identify enriched Biological Process GO terms. This method accounts for the hierarchical structure of the GO terms and so gives more specific terms than a simple enrichment analysis would. The resultant list of significant Gene Ontology IDs and their respective p-values as derived from 'topGO', were inputted into REViGO (Supek et al., 2011). A similarity threshold of 0.5 was set against a UniProt default background and SimRel semantic similarity.

2.1.3. *C. elegans* Microarray Analysis

6 Gene expression .SOFT file datasets were downloaded from GEO: GSE8058, GSE16753, GSE54024, GSE34113, GSE32521, GSE39252

Datasets were chosen if they contained at least 3 replicates of NT worms no older than 3 days into adulthood and provided gene expression data had already been normalised.

WT transcriptomes were extracted and mean expression for each probe was calculated for each dataset. Given the wide variability in expression units, including some negative values, data was normalised for each probe so that it is between 0 and 1 for all datasets. This is represented as:

$$Z_i = X_i - min(X) \max(x) - \min(x)$$

Where Z_i is the normalised expression value for probe i, X_i is the raw expression value for probe i, and x is all the expression values. Probes were then ranked according to expression and plotted against expression using the R package "ggplot2". Probes targeted to *f13e9.1* are labelled on the graph to indicate spliced expression profiles for *f13e9.1*.

2.2. Reagents

2.2.1. *C. elegans* Strains

Table 1. C. elegans strains.

Strain	Genotype	Outcrossed	Source	Reference	Use
AM141	*rmIs133 [unc-54p::Q40::YFP]*	x5	CGC	(Morley et al., 2002)	PolyQ Protein Aggregation Assay
DA1116	*eat-2(ad1116) II*	x2	CGC	(Avery, 1993)	Dietary Restriction
GR1307	*daf-16(mgDf50) I*	x3	CGC	(Ogg et al., 1997)	Lifespan Analysis
KWN190	*rnyEx109 [nhx-2p::D3cpv + pha-1(+)]*	x0	CGC	(Coburn et al., 2013)	Calcium Signalling
WT Bristol	*WT*	N/A	CGC	(Brenner, 1974)	Control Strain
PHX116	sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry]	0	SUNYBioTech	This Thesis	Phenotyping
PHX117	sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry]	0	SUNYBioTech	This Thesis	Phenotyping
PHX893	*F13E9.1(syb767)IV*	x4	SUNYBioTech	This Thesis	Phenotyping
PHX945	*F13E9.1 (syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry]*	x4	SUNYBioTech	This Thesis	Phenotyping
PHX946	*F13E9.1 (syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry]*	x4	SUNYBioTech	This Thesis	Phenotyping
TG38	*aak-2(gt33) X*	x3	CGC	(Lee et al., 2008)	Lifespan Analysis
VK1093	*vkEx1093 [nhx-2p::mCherry::lgg-1]*	x0	CGC	(Gosai et al., 2010)	Autophagy Measurement

2.2.2. Reagents and Media

Table 2. Reagents and media

Compound Name	In-Text Abbr	Supplier	Cat. No	Working Concentrations	Storage	Use
2',5'-Dideoxyadenosine	DDE	Enzo Life Sciences	CN110	1mM in ddH2O	100mM in DMSO at -20°C	cAMP Decrease
5-Fluoro-2'-deoxyuridine	FUDR	Alfa Aeasar	L16497	100µg/ml in dH2O	10mg/ml in dH20 at 4°C	Lifespan Assays
Agarose Molecular Grade	Agarose	Bioline	BIO-41026	N/A	RT	Gel Electrophoresis
Allantoin	Allantoin	Sigma	05670	250µM in DMSO	25mM in DMSO at -20°C	Tested Compound
BAPTA AM	BAPTA AM	TOCRIS	2787	100µM in DMSO	10mM in DMSO at -20°C	Calcium Measurements
Carbenicillin .Disodium salt	Carbenicillin	Enzo Life Sciences	M250	100-500µg/ml in dH20	100mg/ml in dH2O at -20°C	Autophagy Measurement
Chloroquine Diphosphate Salt	Chloroquine	Alfa Aeasar	J64459	20mM in dH20	100mM in dH20 at -20°C	Autophagy Measurement
Cyclic AMP Sodium Salt	cAMP	Santa Cruz	37839-81-9	5mM in dH2O	100mM in ddH2O at -20°C	cAMP Increase
Dimethyl Sulfoxide	DMSO	Sigma	D8418	1%	RT	Solvent
Efaroxan Hydrochloride	Efaroxan	Sigma	E3263	2mM in ddH20	66mM in ddH2O at -20	Imidazoline Receptor
Ethidium Bromide	Ethidium Bromide	Sigma	E1510	38mg/100ml	10mg/ml at RT	Gel Electrophoresis
Forskolin	Forskolin	Alfa Aeasar	J63292	500µM in DMSO	50mM in DMSO at -20°C	cAMP Increase
Hydrogen Peroxide	H2O2	Sigma	88597	1mM to 5mM	3% at RT in dark	Oxidative Stress
Ionomycin	Ionomycin	Caymen Chemicals	CAY10004974	10µM in DMSO	100µM in DMSO at -20°C	Calcium Measurements
Isopropyl-beta-D-thiogalactopyranoside	IPTG	Thermo Fisher	R0392	100µM in dH2O	10mM in dH2O at -20°C	Autophagy Measurement
Loading Dye	Loading Dye	Thermo Fisher	R0611	x1	x6	Single Worm PCR
L-Proline	Proline	Sigma	P0380	5mM in dH2O	500mM in H2O at -20°C	Thermotolerance
Moxonidine hydrochloride	Moxonidine	Tocris	2282	200µM in DMSO	20mM in DMSO at -20°C	Tested Compound
N-Acetyl-L-cysteine	NAC	Sigma	A7250	5-10mM in dH20	1M in dH2O at -20°C	Oxidative Stress
Nuclease Free Water	Nuclease Free Water	Qiagen	129115	N/A	RT	Single Worm PCR
Nystatin Suspension	Nystatin	Sigma	N1638	50IU/mL in DPBS	10,000 unit/mL in DPBS at 20°C	*C. elegans* Culture
Penicillin-Streptomycin	Penicillin-Streptomycin	Sigma	P4333	50IU/mL	citrate buffer at -20ºC	*C. elegans* Culture
Phusion HF Buffer	Phusion HF Buffer	New England Biolabs	B0518	x1	x5 at -20°C	Protein Extraction
Protease Inhibitor Cocktail	Protease Inhibitor Cocktail	Sigma	COEDTAF-RO	1 tablet per 12mL RIPA	Solid tablets at 4°C	Protein Extraction
Proteinase K Solution	Proteinase K Solution	Ambion	AM2548	700µg/ml	20mg/ml at -20°C	Protein Extraction
Radioimmunoprecipitation assay buffer	RIPA Buffer	Cell Signalling	9806	x1	x10 at -20°C	Protein Extraction
Rapamycin	Rapamycin	Alfa Aeasar	J62473	100µM in DMSO	10mM in DMSO at -20°C	Autophagy Measurement
Resveratrol	Resveratrol	Alfa Aeasar	J60790	100µM in DMSO	10mM in DMSO at -20°C	Lifespan Assays
Rilmenidine Hemifumarate	Rilmenidine	Tocris	790	100-400µM in DMSO	40mM in DMSO at -20°C	Tested Compound
Tetracycline	Tetracycline	Sigma	87128	12.5µg/ml in EtOH	5mg/ml in EtOH at -20°C	Autophagy Measurement
Tetramisole Hydrochloride	Tetramisole Hydrochloride	Sigma	T1512	20mM in dH20	100mM in dH20 at 4°C	Microscopy
Thapsagarin	Thapsigargin	Sigma	T9033	1-5µM in DMSO	Solid Film at -20°C	Calcium Measurements

Nutrient Growth Medium Agar (NGM)

17mg/mL Agar

3mg/mL NaCl

2.5mg/mL Bacterial Peptone

100µM $CaCl_2$

5mg/mL Cholesterol

100µM $MgSO_4$

25µM potassium phosphate buffer (pH6)

LB Agar

37g/L LB Broth with agar (Lennox)

LB Broth

25g/L LB Broth (Lennox) Powder

M9 Solution

6.78g/L Na_2HPO_4

3g/L KH_2PO_4

1g/L NH_4Cl

500mg/L NaCl

2.3. Bacterial Preparation

2.3.1. Generation of Live *E.coli* Stocks

A starter culture of *E. coli* OP50 obtained from the CGC (https://cgc.umn.edu/strain/OP50), as advised (Stiernagle, 2006), arrived on NGM agar (see section 2.2.2) and was stored at 4°C for no more than one week. This culture was aseptically streaked onto LB Agar plates and incubated at 37ºC overnight. The following day, 200mL of Lennox LB broth per single colony was inoculated and further incubated at 37°C for no more than 20 hours. Resultant OP50 *E.coli* cultures were aliquoted into 50ml falcon tubes and centrifuged for 10 minutes at 3000rpm: 90% of the supernatant was then discarded to yield a x10 concentrate (Sutphin and Kaeberlein, 2009). These stocks were stored at 4ºC for no more than one month (Stiernagle, 2006).

For RNAi experiments, sequence verified HT115 *E.coli* strains transformed with either the plasmids CUUkp3301P034Q (*lgg-1)* or CUUkp3303WT 02Q (*bec-1)* were purchased from the Ahringer RNAi feeding library available at SourceBioscience (Kamath et al., 2003): an empty HT115 vector strain was also purchased from CGC https://cgc.umn.edu/strain/HT115(DE3). Upon receipt, the strain was stored for no more than one week at 4°C before being streaked onto LB agar plates containing 100µg/ml carbenicillin (Enzo Life Sciences # M250) and 12.5µg/ml tetracycline (Sigma #87128), to prevent plasmid loss as outlined by Maher et al. (2013). Plates were then incubated at 37ºC in the dark overnight. The following day, isolated single colonies were grown up in Lennox LB Broth containing 500µg/ml carbenicillin for no more than 16 hours (Kamath et al., 2001). Resultant stocks were centrifuged at 3000rpm for 10 minutes and supernatant lessened to yield x10 concentrate. These stocks were then stored at 4ºC for no more than one month.

A stab culture of the *E.coli* strain EPI300 carrying a sequenced validated WRM0616C_F02 (*f13e9.1)* clone was purchased from *C. elegans* TransgeneOme Resource (Sarov et al., 2012) and shipped to SUNYBiotech (https://transgeneome.mpi-cbg.de/transgeneomics/public/clone.html?wellId=32643519). The culture was inoculated onto LB agar w/ 12.5µg/mL chloramphenicol and 50 µg/mL nourseothricin, and incubated at 37ºC overnight. A stable, low copy number culture was cultured in LB broth and subsequently induced with Fosmid Autoinduction Solution (Epicentre #AIS107F) before DNA extraction and purification by SUNYBiotech.

2.3.2. Freezing of *E.coli* Stocks

All stocks were frozen as described in (Gott, 2004). Single colonies of *E.coli* were picked and inoculated into 2.5 ml Lennox LB broth, with the addition of 12.5µg/mL of tetracycline and 100µg/ml carbenicillin for HT115 strains and left to grow overnight for ~18 hours at 37ºC. 0.25 ml 80% glycerol and 0.75 ml of overnight culture were combined per screw-cap freezer tube and stored at -80°C.

2.3.3. Generation of UV-Killed OP50 *E.coli*

Unconcentrated live OP50 *E.coli* cultures were generated as described in section 2.3.1. 32 mL of culture were aliquoted to each 50mL falcon tubes, mixed with absolute ethanol (8mL) to yield a 20% ethanol solution as described (Calvert et al., 2016) and pipetted into T160 flasks to a volume of 150ml. Flasks were transferred to a UV-Linker machine (CL-1000 Ultraviolet crosslinker UVP) and irradiated for 120 minutes at 999,900 microjoules/cm^2. 50ml aliquots of UV-killed *E.coli*-ethanol solution was pelleted at 3000rpm for 10 minutes and resuspended to a x10 concentrate in 5mL Lennox LB broth.

2.4. C. elegans Husbandry

2.4.1. Culture and Stock Maintenance

All worms were cultured on NGM agar supplemented with 50IU/mL penicillin (Sigma #P4333), 50µg/ml streptomycin (#P4333), and 50IU/mL Nystatin (Sigma #1638) to prevent contamination as previously described (Stiernagle, 2006). Worm populations are routinely cultured in such an antimicrobial environment, which as corroborated by my own experiences, did not affect OP50 growth nor development of worms (Garcia et al., 2007; Ludewig et al., 2013; Savion et al., 2018; Scott et al., 2017). The N2 bristol strain was selected as WT for all experiments and any reference to WT *C. elegans*, unless otherwise stated, refers to this strain henceforth. Worms were grown on 60mm NGM plates seeded with x10 Live OP50 *E.coli* for at least 4 generations to minimise transgenerational effects of antecedent starvation or contamination (Greer et al., 2011; Jobson et al., 2015; Rechavi et al., 2014). All worms were grown on laboratory table tops during cultivation at RT and exposed to artificial light; however they were transferred to 20ºC incubators and kept in the dark during assay preparation and execution to mitigate the effects of varying light on worm phenotypes (De Magalhaes Filho et al., 2018). No generations that had undergone bleach synchronisation or a previous assay were reused for population propagation. Stock worms were not cultivated for more than 1 month before being replaced by a thawed central stock. Before any assay, worms were grown in well-fed uncontaminated conditions and any assays or populations that developed contaminations were abandoned.

2.4.2. Freezing and Thawing of *C. elegans* Strains

Upon receipt, strains were picked onto NGM plates seeded with x10 live OP50 *E.coli*. They were then grown on those plates for 4 days before being picked onto 4 fresh 60mm NGM plates again seeded with x10 live *E.coli.* Strains were then left to populate the plates and consume all food inducing acute and mild starvation such that L1 arrest occurred in recently hatched eggs. At this point, worms were collected in M9, pelleted at 1500rpm and resuspended in 3mL M9 solution. The worm suspension was mixed with equal volume sterile freezing solution (5.8g/L NaCl, 50mM KH_2PO_4 (pH 6.0), 25% (w.v) glycerol, 300mM $MgSO_4$,) and aliquoted into 1.5mL freezing vials. Worms were immediately transferred to -80ºC for indefinite storage. Vials could be thawed at room temperature, emptied directly onto an NGM plate surface and allowed to dry for 30 minutes in a tissue culture hood. Plates were then seeded with a small amount of x10 concentrated OP50 and left overnight. Surviving worms were picked onto fresh agar plates and cultured as in Section 2.4.1.

2.4.3. Drug Treatments

All drug treatments, unless otherwise stated, were administered to *C. elegans* via the addition of the respective compound to NGM, which was then spotted with UV-killed OP50. Whilst, different routes of administration are available, it has been established that dissolution of drugs in NGM agar offers the best subsequent bioavailability and accumulation in nematodes which peaks at 24h (Zheng et al., 2013).

Drugs were dissolved upon receipt into aliquoted stock concentration in their relevant solvents and appropriately stored for no more than one month before use (see 2.2.2). Drugs that were dissolved in DMSO, were stored in concentrations such that the final volume of drug solution requiring addition to the NGM plates did not exceed 1% DMSO. DMSO in concentrations other than 1% have been shown to significantly alter lifespan and other ageing markers (Wang et al., 2010). All plates, unless otherwise stated, were prepared one day before use in sterile conditions. NGM agar was cooled to below 65°C after same-day autoclaving, and respective drugs were concomitantly added to the plates as 3mL NGM was pipetted into 35mm petri dishes. Plates were gently swirled to homogenise the solution and left to dry for 1 hour in a hood. 60µl of x10 concentrated OP50 *E.coli* was then added to the centre of the plate and again the plates were left to dry for 30 minutes in a hood. Plates were wrapped in parafilm and stored at 20°C before use.

2.4.4. Alkaline Hypochlorite-Based Synchronisation of Gravid Hermaphrodites

All worm populations were synchronised using an alkaline hypochlorite treatment based on methods described by Porta-de-la-Riva *et al.* (2012). For each population, well-fed gravid hermaphrodites were recovered and pooled in M9 buffer from at least 3 confluent 100mm NGM agar plates previously seeded with live x10 concentrated *E.coli* OP50. Worms were pelleted by centrifuging at 1500rpm for 2 minutes at room-temperature in 15ml falcon tubes and supernatant was discarded. 2ml of alkaline hypochlorite solution (NaOH 1 M, NaOCl ~1.6%) was added and tubes were agitated for around 10 minutes until only embryos remained visible in the solution. The reaction was stopped by addition of 12ml M9 solution and centrifuged at 1500rpm for 2 minutes at room-temperature. The supernatant was discarded and the pellet was rewashed a further 3 times in M9 buffer before being incubated overnight with gentle rocking in 2ml M9 solution. The following day, synchronised L1 nematodes were recovered by centrifugation and left to develop for requisite times on NGM plates seeded with x10 concentrated live OP50 *E.coli*.

2.5. Nematode Transgenesis

The *f13e9.1* locus was viewed using WormBase with the protein motifs tracker enabled to allow visualisation of protein domain regions to be excised (Sigrist et al., 2010; Zerbino et al., 2018). Homozygous PHX893 (F13E9.1(syb767)IV) strains were created using CRISPR/Cas9 technology and performed by SunyBiotech. The 3rd exon, 4th exon and part of 5th exon was deleted and replaced by a splice site and stop codon that terminated transcription and resulted in mutant strain carrying an 873bp homozygous deletion in the *f13e9.1* gene. This strain was subsequently outcrossed four times against an WT background using PCR screening at each stage and the final outcrossed strain was confirmed by sequencing.

Second, the transgenic strains PHX117 sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry] and PHX116 sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry] containing and integrated full-length rescue of the *f13e9.1* locus within the fosmid were generated on WT backgrounds and created by SUNYBiotech. Specifically, the integrated rescue contained the WRM0616C_F02(pRedFlp-Hgr)(*F13E9.1*[33366]::S0001_pR6K_Amp_2xTY1ce_EGFP_FRT_rpsl_neo_FRT_3xFlag)dFRT::unc-119-Nat) fosmid construct derived from the copy-number inducible vector pCC1Fos grown up in *E.coli* strain EPI300 (See section 1.3.1) . Fosmid DNA was extracted and added to a 20ng/µL DNA mixture containing both the purified fosmid and pCFJ90 Pmyo-2::mcherry. The solution was microfinjected into 10-30 adult WT worm gonads to generate a line of extrachromosomal transgenic arrays. Stable lines were then irradiated with γ ray dose of 40 GRAY to produce two lines of the same genotype. The previously produced, PHX893 (F13E9.1(syb767)IV) was then crossed 4 times with PX116 to generate the two rescue lines: PHX945 and PHX946 F13E9.1 (syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry].

2.6. Biochemical Methods

2.6.1. Protein Extraction

Whole worm protein lysates were prepared as in Hu *et al.* (2017) . ~500 day 1 adult worms per condition and genotype were transferred to either empty NGM plates to induce starvation (+ve control) or to UV-killed OP50 *E.coli* NGM plates containing requisite drugs or vehicle (1% DMSO). After 24 hours, worms were suspended in M9 buffer and centrifuged at 1000rpm for 2 minutes to yield a wet pellet. Worms were then re-suspended on ice in 50µl of RIPA buffer with added phosphatase inhibitors (Cell Signalling Technologies #9806) and cOmplete EDTA-free protease inhibitor cocktail (Roche # 04693159001) before sonication: 10 sec ON/1 minute OFF, 14% amplitude 3 times until the vast majority of animals

were dissolved. Lysates were then spun down twice at 13,000g/20minutes 4° C and immediately frozen at -20°C for no more than one week before use.

2.6.2. Western Blot Analysis

Total protein lysate concentrations were quantified using a bicinchoninic acid (BCA) assay (BioRAD) according to the manufacturer's protocol.

Protein lysates were heated for 5 minutes at 95°C, cooled on ice for 2 minutes and pelleted for 3 minutes at 13,000g. 25µg of protein was loaded per well into an 18 well 10% TGX™ Precast Gel (BioRad), submerged in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and separated at 300V for approximately 30 minutes. Gels were transferred via Trans-Blot® Turbo™ Transfer System to nitrocellulose membranes and blocked for 60 minutes in blocking buffer (Licor Odyssey® Blocking Buffer #P/N 927-40100). Blots were incubated overnight with the primary antibody Phospho-ERK (Cell Signalling Technology, Catalogue number: 9101) at 1:1000 in blocking buffer (Licor Odyssey® Blocking Buffer #P/N 927-40100) (Gee et al., 2013) with gentle rocking. The following morning, blots were washed three times in TBST before being incubated for 1 hour with the secondary antibody IRDye® 800CW Goat anti-Rabbit IgG at a concentration of 1:10,00 in blocking buffer w/ 0.1% tween. This process was then repeated for the loading control, α-tubulin, using anti- alpha Tubulin (ab72910) and IRDye® 680RD Goat anti-Mouse IgG. Changes in ERK phosphorylation are often calculated using phospho-ERK:Total ERK ratios (Kao et al., 2004; Nykamp et al., 2008). However, given the frequent use of ERK as a loading control (H. Huang et al., 2004; Hwang et al., 2005), we followed previous examples in both worms (Chen et al., 2008; Villanueva-Chimal et al., 2017) and other models (Redshaw and Loughna, 2012; Zhou et al., 2015) and calculated changes in phospho-ERK via the surrogate loading control α-tubulin. In a subset of experiments, executed by Shanghai Model Organisms, lysates from PHX945 and PHX946 strains were probed with monoclonal FLAG-tag antibodies (Sigma #F1804) to verify for fosmid *F13E9.1* rescue.

Bound antibody was detected using Odyssey® CLx Imaging System. For all immunoblotting, three independent trials for each genotype and/or condition was completed and mean densiometric ratios between loading control and pERK or FLAG minus local median background were calculated using Image Studio Lite as per software guidelines (Licor Biosciences, 2013) . Results were considered significant if the magnitude of the %change exceeded the CV by at least x1.5.

2.6.3. Genomic DNA Extraction

Single worms were picked and dropped into individual PCR tubes on ice containing 10µl of freshly aliquoted proteinase K (400µg/ml) in Phusion HF Buffer. Tubes were immediately transferred to -80ºC overnight. The following morning, tubes were thawed on ice before being added to a thermocycler set to

the following: 65ºC for 90 minutes and 90ºC for 15 minutes. Crude DNA extract was then stored for no more than one week at 4ºC before use in PCR.

2.6.4. Single Worm PCR

Single Worm Polymerase Chain Reactions were made using Phusion® High-Fidelity PCR Master Mix (New England Biolads #M0531S) to a final volume of 50ul per reaction made up of the following: 3ul template (crude DNA extraction), 5µl of 10µM primer mix 1:1, 17µl of nuclease-free water (Qiagen), and 25µl of Phusion® High-Fidelity PCR Master Mix with HF Buffer. Reaction mixtures were then added to a thermocycler to undergo the following cycles:

All primers (40nM synthesis scale) were designed using PrimerBlast (Ye et al., 2012) and ApE v2.0.55 for genotyping WT and *f13e9.1 -/-* worms. Primer were purchased from Eurogentec Ltd at a 100µM stock concentration in nuclease-free H_2O. 10µM aliquots were stored at -20ºC until use.

The primers used were as follows:

Gene Target	Primer Name	Sequences
f13e9.1	F13_1	F: 5'-AGGACCACCAGGAGCTACAT-3'
		R: 5' TCACTGTCGGCGATCTGAAA- 3'
f13e9.1	Int	F: 5'-CATGCGATTACCGAACGACT-3'
		R: 5'-CCATTCGTACGCGTACAGTA- 3'
f13e9.1	Ext	F: 5'- GCTTGAGAGCAGTCAGACGT-3'
		R: 5'- TTACGAAGGGCCATTCGTAC- 3'
f13e9.1	F13_2	F: 5'- GAGCGGCAGAAGTAATCCGT-3'
		R: 5'- AGTGAACGACAAGACGACGAA- 3'
WRM061C_F02	FOSMID	F: 5'- TCCTATCAGAAAACTGGTCG -3'
		R: 5'- GTGGTCTCTCTTTTCGTTGG- 3'

2.6.5. Gel Electrophoresis

1g agarose (Bioline # BIO-41026) was dissolved in 100µl x1 TAE buffer (2mM Tris HCl, 50mM EDTA, pH 8.0) and microwaved for 1 minute until solution was clear. After cooling, 0.03mg ethidium bromide was added (Sigma Aldrich #E1510) and cast into a mould for electrophoresis. 10ul of x6 DNA Gel Loading Dye (Thermo Fisher #R0611) was added to each PCR tube and then 10ul of PCR product was loaded into each well. Gels were submerged in x1 TAE buffer and ran at 100v for 40 minutes before being visualized under UV on a ChemiDoc XRS+ (BioRad). Images were analysed and inverted in Image Studio Lite.

2.7. Lifespan Analyses

For all strains and each assay, worms were cultured in the same conditions as described in Section 2.4.1. Well-fed gravid hermaphrodites were recovered and pooled in M9 buffer from at least 3 confluent 100mm plates and bleach syncronised as described in Section 2.4.4. Larval crowding is well known to influence development rate and lifespan (Ludewig et al., 2017), such that wet worm pellets never exceeded 500µL and remained comparable between groups. Resultant hatched embryos were halted at L1 overnight in M9 buffer to maximise time-synchrony, before being added to NGM seeded with x10 OP50 *E.coli* concentrate and left to develop for 52 hours at 20°C until late L4/adult. One day prior to L4 molt, 6-well plates containing 3ml of NGM per well were made up in a sterile hood, with the addition of requisite test compounds when required as in section 2.4.3. Furthermore,100µg/ml FUDR was also added as in Calvert et al., (2016) at recommended concentrations (Gruber et al., 2009; Sutphin and Kaeberlein, 2009), to circumvent confounding issues of egg-laying and subsequent worm development. Despite research extoling the negligible effect of FUDR on lifespan (Gandhi et al., 1980; Hosono et al., 1982), studies have noted FUDR to influence lifespan in a number of mutants (Kato et al., 2016; Van Raamsdonk and Hekimi, 2011). The seemingly scant evidence for the effect of FUDR on the lifespan of strains used and generated herein combined with the ubiquity of FUDR in *C. elegans* research permitted its use in this thesis.

At L4/adult molt, worms were transferred to previously made 6-well lifespan assay plates and maintained at 20°C in the dark, wrapped in parafilm to moderate humidity. Worms were counted every 2 to 3 days and dead worms (failure to respond to no more than 3 "prods" with a platinum worm pick) were removed at each inspection. 60 µL of UV-killed OP50 was added three times a week to all plates until day 15 of the assay by which point low-food consumption negated the need for additional *E.coli* (Calvert et al., 2016).

For late administration lifespan assays, worms were prepared and added to lifespan assay plates as described in this section. At day 12, requisite worms were washed of lifespan assay plates with M9 and carefully pipetted onto new drug plates prepared on day 11, seeded with UV-killed *E.coli*, and contained freshly prepared test compounds and 100µg/ml FUDR.

Survival results attained during this research were verified by automated lifespan assessment using the lifespan machine at Ewald lab in the Swiss Federal Institute of Technology (Stroustrup et al., 2013). Additionally, a small proportion of lifespan assays, indicated where necessary, were conducted by

Shanghai Model (Cuong et al., 2018; Fei et al., 2017; Hou et al., 2009; Weidong et al., 2018; Yang et al., 2013; Ying et al., 2015; Zhu et al., 2015)

For all lifespan assays, unless otherwise stated, at least 150 worms per genotype and/or condition were used across at least 3 independent trials. Worms that crawled into the agar, or experienced matricidal events were removed from the assay and not included in the lifespan calculations as in previous research (Bridi et al., 2015; Fleckenstein, 2014; Gusarov et al., 2017; Hsu et al., 2009; Schulz et al., 2007; Slaughter, 2013). The biases accrued by the removal of censored animals from lifespan is well documented, and indeed the Kaplan-Meir survival curve was designed as a nonparametric method for estimating survival curves that considers the contribution of these worms (Kaplan and Meier, 1958). That said, such events occurred rarely (less than 1%) across treatment and genotype. Furthermore, the unchanging incidence of matricide and desiccation across groups suggests that the lifetime (age at death) of each worm was independent of the potential loss time of censorship and would contribute little to the final analyses as evidenced in Petrascheck and Miller, (2017). For each condition and/or genotype, survival results were pooled. Lifespan statistics were calculated using the OASIS2 online tool (Han et al., 2016). Specifically, mean lifespan was measured as the no. days when survivorship reached 50%. Furthermore, maximum lifespan was defined as the mean lifespan of the final 10% of survivors in a given population. P-values relating to survival difference between populations were calculated using log-rank (Mantel-Cox method) test.

2.8. Autophagy Studies in C. elegans

2.8.1. Measurement of Autophagy *in-vivo*

The vKEx1093 reporter strain generated by Gosai et al. (2010) was purchased from the CGC (https://cgc.umn.edu/transgene/23476) and frozen upon receipt. One week before each trial, aliquots of vkEx1093 worms were thawed and selectively picked on uncontaminated NGM agar seeded with live OP50 for 7 days to establish a stabilised line. Gravid hermaphrodites were then bleach synchronised as in section 2.4.4. and resultant embryos were left to grow for 52 hours at 20°C on NGM plates seeded with live OP50 *E.coli* (Papandreou and Tavernarakis, 2017). At late-L4 stage, worms were transferred to either empty NGM plates to induce starvation (+ve control) or to UV-killed OP50 *E.coli* NGM plates containing requisite drugs or vehicle (1% DMSO). Worms were then incubated at 20°C for 24 hours to maximise drug absorption and efficacy(Zheng et al., 2013). As described in previous assays (Burkewitz et al., 2012; Chapin et al., 2015), 20mM chloroquine was also added to the NGM for the drug incubation period when measuring autophagic flux. After 24 hours, day 1 adult worms were immobilized and mounted onto glass slides in 20mM solution of tetramisole hydrochloride. Approximately 10 worms per

condition were imaged at a x10 objective on a Zeiss Axio Observer using a 150ms exposure time. Each worm was imaged within 5 minutes as tetramisole is known to induce puncta accrual after this time (Zhang et al., 2015). In total, at least 25 images across 3 independent trials were collected and pooled for each condition, in line with previous measurements of autophagy (Eisenberg et al., 2009; Morselli et al., 2010). Levels of autophagy induction were quantified based on a previously used technique (Martinez et al., 2015). Using Zen software, three 1mm^2 boxes were sequentially assigned to the most fluorescent areas of posterior intestine for each animal. With set histogramic parameters (black: 0 gamma:1.0 white: 1000 or 5000 for highly fluorescent animals), the total number of visible puncta were manually counted in each box, and a mean number of puncta calculated per mm^2 of animal intestine. Limiting measurement to fluorescent areas of the posterior intestine reduces the mosaic effect of extrachromosomal expression *in-vivo* and using three individual boxes limits the accumulation of subjective counting disparity. Median number of puncta per mm^2 posterior intestine for all animals in each condition was calculated and compared using one-factor (ANOVA) variance analysis corrected by the post hoc Bonferroni test.

2.8.2. RNAi Autophagy Knockdown Lifespan Analysis and Validation

For RNAi lifespan analyses, F1 synchronised WT *C. elegans* were grown on live OP50 *E.coli* until late L4-stage. Nematodes were then transferred to NGM plates (penicillin-streptomycin omitted) containing 100µM IPTG, 400µM FUDR and 50µg/ml carbenicillin with either 200µM rilmenidine hemifumrate or 1% DMSO Vehicle. Plates had previously been seeded with live *E.coli* (HT115) expressing an empty vector, *bec-1* dsRNA or *lgg-1* dsRNA (section) and incubated for 1–2 days at room temperature prior to induce dsRNA expression.

Worms were maintained on the same drug plates for the entirety of their lifespan and scored for survival every 2-3 days as previously described (Seo et al., 2018) with the addition of requisite *E.coli* as and when it was consumed. Fresh strains were prepared for each assay. A caveat must be noted that Hansen *et al.*, (2008) initially used FUDR on single plates during autophagy knockdown assays but stopped following a failure to induce the requisite phenotype with *bec-1*. Nonetheless, this was not experienced during our assays, and moreover, researchers have continued to use FUDR with *bec-1* RNAi (Edwards et al., 2015b; Hashimoto et al., 2009a; Seo et al., 2018). A total of over 100 worms per condition were assayed across 3 independent trials and analysed as in section 2.7.1.

Knockdown was validated by measuring autophagy inhibition of vKEx1093(*nhx-2*::*mCherry*::*lgg-1)* worms rather than RT-PCR; a decision based on previous methods (Miedel et al., 2012; Pietrocola et al., 2018; Schipanski et al., 2013). Autophagy was pharmacologically upregulated in F1 day-1 adult vKEx1093 worms by the addition of rapamycin 100µM to RNAi feeding plates and incubated at 20^{o}C (Ikenaka et

al., 2013). After 3 days the worms were observed for autophagy knockdown and images were acquired as described in previous sections (section 2.81). At least 12 images across 3 independent trials were collected for each condition. The number of visible puncta per mm^2 posterior intestine of each animal was manually counted and calculated. Given the variable penetrance of RNAi in *C. elegans* (Kamath et al., 2001) and consequent non-normal data distribution, a Krushal test was performed across all conditions (Han et al., 2017).

2.9. Stress Assays

2.9.1. Oxidative Stress (H_2O_2) Resistance Assay on Solid Media

Survival of day-1 worms exposed to varying concentrations of hydrogen peroxide (H_2O_2) was assessed as an index of oxidative stress resistance based on methods previously employed using solid media (Ludewig et al., 2013; Oláhová and Veal, 2015). Well-fed gravid hermaphrodites were bleach synchronised as in section 2.4.4. and resultant embryos were left to grow for 52 hours at 20°C on NGM plates seeded with live OP50 *E.coli*. Bleach treatment has not been proven to affect survival rates in oxidative stress conditions and as such this protocol has previously been used in a number of similar *C. elegans* assays (Bou Dib et al., 2014; Izumikawa et al., 2016; Smith et al., 2014). At late-L4 stage, worms were transferred to seeded UV-killed OP50 *E.coli* NGM plates containing 200µM-400µM rilmenidine hemifumarate, or 5-10mM NAC (+ve control) or vehicle (1% DMSO) for 24h. Such concentrations of NAC have previously been used to reduce oxidative stress in *C. elegans* (Yang and Hekimi, 2010) and also NAC has been used to ameliorate superoxide-induced phenotypes (Sha et al., 2017). After 24h, worms were transferred to freshly prepared 3ml NGM plates containing requisite drugs (rilmenidine, NAC, 1% DMSO) and 1mM, 2mM or 5mM H_2O_2 (made from 3% stock H_2O_2 purchased the same week to prevent degradation (Sigma # 88597)). A subset of worms were removed from drug exposure whilst incubated on H_2O_2 plates in order to account for any confounding external reactivities of H_2O_2, rilmenidine and NAC, not related to endogenous superoxide scavenging. Survival of at least 60 worms per condition across 3 independent trials was scored every 2 hours for 10 hours based on published guidelines (Senchuk et al., 2017) and previous methods (Jansen et al., 2002). Death of each animal was assessed at the indicated times by microscopic examination of ability to respond to no more than 3 "prods" with a platinum worm pick. Survival and mean lifespan was estimated from a Kaplan-Meir plot for each condition using OASIS2 (Han et al., 2016), and statistical differences between populations compared by way of log rank test and Bonferroni correction.

2.9.2. Thermotolerance and Recovery Assay

Survival of day-1 adult worms exposed to an acute bout of temperature up-shift while exposed to varying drugs was assessed as an index of thermotolerance resistance based on published guidelines (Zevian and Yanowitz, 2014). Well-fed gravid hermaphrodites were bleach synchronised as in section 2.4.4. and resultant embryos were left to grow for 52 hours at 20°C on NGM plates seeded with live OP50 *E.coli*. Whilst, Zevian and Yanowitz, (2014) advise against the bleach syncronisation of worms due to the acquired increase in basal stress resistance, this technique has been widely for such assays (Kumsta et al., 2017; Raynes et al., 2012). At late-L4 stage, worms were transferred to seeded UV-killed OP50 *E.coli* NGM plates containing 200µM-400µM rilmenidine hemifumarate, or 5mM L-proline (+ve control) or vehicle (1% DMSO) for 24h. Proline has been shown to improve thermotolerance by ~30% in *C. elegans* through the improvement of protein stabilisation and membrane integrity (Edwards et al., 2015b). The thermotolerance assay was started at day 1 adulthood, to avoid premature age-related proteostastic collapse (Ben-Zvi et al., 2009) and to negate the need for FUDR which has been shown to interfere with thermotolerance and proteotoxic stress (Angeli et al., 2013) . After 24h of respective drug exposure, worms were added to an incubator preset to 37ºC to induce heat-shock whilst still exposed to the respective drugs (Zevian and Yanowitz, 2014). After 3 hours, worms were removed from the incubator and being kept on the same drug plates, shifted to 20ºC and scored for survival after a ~20h "recovery period" (Kumsta et al., 2017). This period prevents false categorization of worms as visual inspection of worms immediately after heat shock is not a reliable determinant of their ultimate survival (Zevian and Yanowitz, 2014). At least 100 worms per condition across 3 identically designed independent trials were scored as either dead or alive by their ability to respond to no more than 3 "prods" with a platinum worm pick. Percentage survival was calculated and populations compared by one-way ANOVA of variance with Tukey post-hoc correction.

Rilmenidine increases thermotolerance but not oxidative stress resistance dependent on *f13e9.1*. Survival of WT (WT,WT), *f13e9.1* (-/-) mutants, and two *f13e9.1* rescue mutants (PHX945 and PHX946) in the presence or absence of rilmenidine following exposure to either heat-stress or oxidative stress.

2.9.3. Assessment of *F13E9.1*::eGFP localisation

The strains WT , PHX893, PHX945 and PHX946 were synchronized to adulthood (day 1) and exposed to drugs as in section 2.4.3. No less than 10 worms per genotype were immobilised in 20mM tetramisole pipetted into a glass-bottomed dish and observed under a Zeiss 710 confocal microscope at 20 °C with a Fluar 20x/0.75 lens. Images were acquired as in Teuscher and Ewald, (2018) which recommends a super-thin bandpass green filter (520 +/- 20nm) to overcome inherent autofluorescence within worms.

2.9.4. PolyQ Protein Aggregation Assay

The *C. elegans* strain AM141 {*rmIs133*[P(*unc-54*)*Q40*::*YFP*]} (Morley et al., 2002) was purchased from the CGC (https://cgc.umn.edu/transgene/23476) and frozen upon receipt. One week before each trial, aliquots of AM141 worms were thawed and picked on uncontaminated NGM agar seeded with live OP50. Strains were cultured for one week before being used for the polyQ aggregation assay, of which is based on previously employed methodologies (Xiao et al., 2014; Zhang et al., 2012). Gravid hermaphrodites were bleached synchronized and the yielded eggs were incubated overnight in M9 medium. Syncronised L1 nematodes were then transferred to NGM plates containing 200µM-400µM rilmenidine hemifumuarate concentrations or a 1% DMSO vehicle. After incubation for the indicated periods of time, the nematodes were collected and immobilized in 10µl of 20mM tetramisole hydrochloride onto microscope slides. Approximately 30 animals per condition across 3 trials were imaged under a Zeiss Axio Observer microscope at either a x10 and x20 objective. The number of polyQ40::YFP aggregates in body wall muscle was counted using ZenBlue software with set histogramic parameters (black: 500 gamma:1.0 white: 5000). Approximately 50 animals across 3 independent trials were randomly selected for each treatment group and scored for number of aggregates. Groups were compared by two-factor repeated measures ANOVA and Tukey post-hoc correction.

2.10. Body Size and Developmental Phenotyping

2.10.1. Body Size Phenotyping

Synchronised day 1 hermaphrodite worms cultured on live *E.coli* NGM plates were measured for body length and width in brightfield at x10 objective on a Zeiss Axio Observer following paralysis in 20mM tetramisole. It is well established that in *C. elegans*, prolonged exposure to tetramisole induces acute shrinkage and changes in the skin elasticity (Galimov et al., 2018; Glenn et al., 2004; Woodruff et al., 2018), however, the attainment of images that provide accurate determination of the body size change is difficult. As such, the acute use of tetramisole is still commonplace when imaging worms for body length measurement (Lant et al., 2015; Li et al., 2018; Mörck and Pilon, 2006; Shaohe Wang et al., 2015). Between 10-20 worms per data point were used in line with previous body length analyses (Bar et al., 2016). Due to the significant transparency and animation of the tails obscuring the tail end-point, animal length was measured from head (most visibly anterior buccal line) to a position where the tail tapered to a 10-μm ±0.5μm diameter (Petzold et al., 2011). Body width measurements were taken from the posterior vulval peak to the corresponding outer edge of the intestinal cuticle (Mörck and Pilon, 2006). Measurements was made using the segmented lines function on Image J software (Collins, 2007)

normalised to the scale bar as implemented in previous work (Bar et al., 2016; Dineen and Gaudet, 2014; Mörck and Pilon, 2006).

2.10.2. Developmental Measurements

Bleach synchronised worms that had been halted in L1 starvation for 24h were measured for development following exposure to food source as described in (Schindler et al., 2014). ~ 500 worms per genotype and condition were placed onto NGM plates containing requisite test compounds and spotted with live OP50 *E.coli*. It is well established that live *E.coli* often metabolises drugs dissolved in NGM, reducing their efficacy (Zheng et al., 2013); however we were unable to elicit reliable and measurable development in worms grown on dead OP50 *E.coli,* which is in line previous observations (Lenaerts et al., 2008) . Worms were added to a 20ºC incubator and left to develop. After 24 hours, measurement of worm development was conducted within another 24 hour window loosely based on previous reports (Schindler et al., 2014): 0 hours (24 hours exposure to food source), 3 hours, 6 hours, and then 24 hours (48 hours exposure to food source). Per trial and timepoint, 10 worms were immobilized and mounted onto glass slides in 20mM solution of tetramisole hydrochloride. Animals were then imaged at a x10 objective for body length and x60 objective for vulval development in brightfield on a Zeiss Axio Observer. Vulval development was scored using visual identification of late larval stage vulval checkpoints detailed by Schindler, Baugh and Sherwood, (2014). Statistical significance of differences in vulval development was determined by 2 way ANOVA.

2.11. "Healthspan Measurements"

2.11.1. Age-related autofluorescence

Autofluorescence of the intestine was measured as an index of healthspan based on previously published guidelines (Bansal et al., 2015). Well-fed gravid hermaphrodites were bleach synchronised and resultant embryos were left to grow for 52 hours at 20°C on NGM plates seeded with live OP50 *E.coli*. At late-L4 stage, WT or mutant worms were transferred to NGM plates containing requisite drugs or vehicle (1% DMSO) spotted with UV-killed OP50 *E.coli* and left for either 1 day or 10 days at 20ºC to age, based on previously used time points (Pluskota et al., 2009). 10 animals per data point were immobilized and mounted onto glass slides in 20mM solution of tetramisole hydrochloride (Yan et al., 2017) before being imaged through both brightfield and DAPI filter using a Zeiss Axio Observer (300ms exposure, x10 objective) (Bansal et al., 2015). A total of at least 30 worms per condition were imaged across 3 independent trials.

Intestinal florescence was quantified using mean pixel intensity. Intestinal areas from pharyngeal-intestinal valve to rectum were traced using the spline mode in Zen Blue v2.3 and raw mean pixel intensity was calculated (Hill et al., 2014). This figure was then normalised against the average pixel intensity of 3 300μm^2 boxes placed in non-intestinal areas of each worm (distal portions of the tail and head). Net mean pixel intensity was calculated for each sample and median sought for each group. Statistical difference between day 1 and day 10 for each condition was independently calculated by 2-way Mann-Whitney U to account for outliers.

2.11.2. Motility Assay

Motility assay was based on a modified protocol of Huang, Xiong and Kornfeld, (2004) used by Calvert *et al.*, (2016). NGM agar plates were firmly tapped onto the microscope to stimulate movement. In responding worms, not impeded by OP50[1], body bends (defined in Calvert *et al.*, (2016) as movement of the head in a half sigmoidal wave that propelled movement and/or resulted in a bend in the body that moved halfway down the length of the worm) were counted for 30 seconds in 10 worms for each condition at each time point (5, 10 and 15 days post-L4 moult) per trial across 3 independent trials to a total of 30 worms per genotype and condition. Mean deterioration in motility per genotype and/or condition was compared by 2- factor repeated ANOVA and Tukey post-hoc correction whilst individual time-point comparisons were tested by student's T-test.

[1] Worms exhibit slowed and staggered locomotion when in the presence of OP50

3. Results: Rilmenidine Opposes the Transcriptional Direction of Ageing And Improves Survival in *C. elegans*

3.1. Introduction

Ageing has been previously defined as a vulnerability to disease and death characterised by 9 hallmarks (López-Otín et al., 2013). The molecular hallmarks of ageing both affect and are affected by transcriptional changes (Stegeman and Weake, 2017). As such, researchers have considered ageing to be a consequence of genomic instability manifesting itself as lack of dynamic control over the expression of genes (Tarkhov et al., 2019). Furthermore, the inability to effectively regulate homeostasis can be hypothesised to result in global dysregulation in gene expression, consistent across cell-type and represented by an ageing transcriptional signature or "fingerprint".

That said, the magnitude of such dysregulation is surprisingly small and targeted. Only 4% of genes show age-related changes in expression in either human brain or kidney tissue, or in monkey (Macaca mulatta) skeletal muscle; and meta-analysis of approximately 1500 human blood samples identified 12.5% of expressed genes as being differentially expressed with age (Berchtold et al., 2008; Kayo et al., 2001; Lu et al., 2004; Peters et al., 2015; Racette et al., 2017; Rodwell et al., 2004). Importantly, such specific changes reproducibly occur in stress-related pathways with noticeable upregulation of DNA repair and inflammatory pathways across multiple species, tissues and cell types (Stegeman and Weake, 2017).

Thus, this more narrowed field of change offers reduced noise and heterogeneity which may be beneficial in identifying targetable pathways dysregulated by age-driven transcriptional changes. This is bolstered by evidence showing the power of human transcriptome changes as a robust metric for ageing which can be conveniently, quickly and reliably employed to test anti-ageing treatments before being validated in a laboratory setting (Fleischer et al., 2018; Peters et al., 2015).

Previous work has identified that calorie restriction can elicit distinct and widespread changes in gene expression in a number of biological pathways, including growth hormone signalling, lipid metabolism, immune response, retinol metabolism, copper ion detoxification, and circadian rhythms (Swindell, 2009; Whitaker et al., 2014). Moreover, murine studies have suggested that CR might be able to reverse age-associated gene expression changes (Lee et al., 1999). Specifically, mice initiated on CR at middle age demonstrate a profound transcriptional reprogramming that retards age associated gene expression changes in cardiac tissue (Lee et al., 2002). In murine hippocampal tissue excised from mice treated with

life-long CR demonstrated a reversal of age-associated changes in mitochondrial dysfunction, inflammation, and stress response gene expression patterns (Zeier et al., 2011).

It is rational to propose that drugs which elicit a similar transcriptional profile to that of calorie restriction could reverse ageing signatures which may confer geroprotection. Indeed this was previously attempted using the connectivity map (cMAP) by our lab (Calvert et al., 2016). Initially developed by the Broad Institute, the cMAP is a database resource comprised of 6,000 transcriptomes of various compound treatments (drug perturbations) performed on a 5 different human cell lines (Lamb et al., 2006). Users upload 2 gene sets representing the top downregulated and the top upregulated genes of their chosen transcriptome. The CMap then implements a modified two-tailed non-parametric Kolmogorov–Smirnov statistical test to calculate the similarity of drug-perturbed expression profiles to the gene sets used to query the database. The primary aim is to identify drugs which oppose or mimic a transcriptome, such that they may be repurposed to induce or reverse complex phenotypes. This bioinformatic approach has successfully identified a number of compounds that have subsequently been tested for the treatment of a number of conditions such as obesity (Liu et al., 2015), irritable bowel disease (Nair, 2013), and cancer (Wei et al., 2006). In the ageing field, the prototypical version of cMAP has been used numerous times to identify prolongevity drugs that might oppose a queried ageing signatures derived through various bioinformatic approaches (Dönertaş et al., 2018; Gillbro et al., 2015; Janssens et al., 2019). However, in collaboration with Library of Integrated Network-based Cellular Signatures consortium, the number of cell lines and compounds in the cMAP database was recently and dramatically expanded to include a total of 476,251 gene expression profiles generated using 27,927 perturbagens, including the loss of function (LOF) or gain of function (GOF) mutations across nine cell lines (Subramanian et al., 2017). The magnitude of this project is capacitated by the algorithmic reduced-representation prediction of genome-wide transcriptional changes from a set of 1000 “landmark” genes. This next generation cMAP has now been cited over 200 times across broad disease biology fields.

Only one paper, thus far, has utilised the updated cMap to find anti-ageing compounds, wherein authors derived a *C. elegans* ageing signature to identify and validate heat-shock protein inhibitors as pro-longevity compounds (Tarkhov et al., 2019). To date, no-one has published a CR gene set query to identify possible CR mimetics using the updated cMap. This could serve to re-validate allantoin as a potential CR mimetic whilst also offering a more detailed transcriptional analysis of imidazoline receptor agonists, such as rilmenidine, in relation to CR and ageing.

Furthermore, upon the potential validation of bioinformatic insight garnered from the cMAP analysis, it is pertinent to assess the *in-vivo* propensity of any hit compounds, to ameliorate the ageing process. Indeed,

ageing is a complex phenotype manifesting in functional declines at many levels of biological organization. As such, measuring ageing, or indeed a rate of ageing, is a significant challenge. Pathology and degeneration occurs at different times and rates, even within nematodes, making direct assessment even more challenging: however, any stochastic demise must eventuate in death and determine organismal lifespan (Ezcurra et al., 2018; Stroustrup et al., 2013). Thus, the measurement of lifespan, has for the last few decades, become the most common, robust and accepted metric of the ageing process, determining the consequence of ageing and death (Collins et al., 2006; Kirkland and Peterson, 2009; Newell Stamper et al., 2018). Measurements of ageing by lifespan assays are critical for determining the geroprotective properties of a compound and often form the initial assessment any gerontological drug studies (Collins et al., 2006).

Among the aforementioned benefits that have proliferated the use of *C. elegans* in the ageing research field, is its short lifespan, and large, manageable cohorts making it amenable to robust longitudinal study. The acquisition of statistical lifespan signatures has become a mainstay in defining the anti-ageing effect of chemical intervention (Stroustrup et al., 2013). This is commonly represented by means of survival curve: a monotonically decreasing function that quantifies the surviving fraction of a population vs time. Importantly, calorie restriction, in almost all model organisms, routinely elicits significant perturbations in survival (Liang et al., 2018). In *C. elegans,* survival/lifespan data is advisably processed by Kaplan-Meir analysis to demonstrate any significant efficacy an intervention may have in improving mean and/or maximum lifespan (Lionaki and Tavernarakis, 2013).

3.2. Results

3.2.1. Probing of the Connectivity Map With Age-Related and Calorie Restriction Transcripts Reveals Rilmenidine To Have a Significant Ageing and Senescence-associated and Gene Expression Profile

I probed the connectivity map with 8 different gene sets (Table 3). First, an ageing gene signature generated by our lab from a meta-analyses of 127 multi-tissue datasets of aged animals was used (Fabris et al., 2018) and from another meta-analysis of GTex ageing data (Mele et al., 2015). DEGs from both datasets were converted to Entrez IDs and filtered to generate a consensus gene list that was differentially expressed in both datasets. This represented our global ageing signature as well as the two individual meta-analyses being queried separately.

Second, imidazoline receptor expression is most pronounced in the brain, corresponding to the centrally-acting hypotensive effect of I1Ras (Bousquet et al., 2020; Ding et al., 2013). I1RAs may therefore elicit a brain-specific transcript, so I generated a gene set of DEGs from a sub-analysis using just brain tissue

samples within the selected datasets using the Fabris et al. meta-analysis gene-signature. Then I further compartmentalised to include only Rostral Ventral Lateral Medulla (RVLM) gene expression changes with age. I harvested the only published and normalised gene expression data from the RVLM of young and old F344 rats using GSE90956 series matrix text files to produce a DEG set list defined by z-score. Rilmenidine and moxonidine are anti-hypertensives that exert their hypotensive effect through inhibition of the sympathetic nervous system. Rilmenidine acts primarily on imidazoline receptors in the RVLM to reduce sympathetic tone and to modulate sympathetic baroreflexes agonism (Head et al., 1998).

Thirdly, given that cMAP works on an *in-vitro* level, I utilised a meta-analysis of publicly available datasets to identify a cellular senescence signature, which may represent more cell-specific ageing change (Chatsirisupachai et al., 2019).

Lastly, I probed the cMAP with two separate dietary restriction profiles. We used the original transcriptional profile from published data in rat cells exposed to sera from rats or rhesus monkeys undergoing CR used in Calvert et al. (2016). However, this yielded a relatively low number of DEGs which would be vulnerable to noise and cross-species differences. Thus it was decided to upload an alternative CR gene set, derived using the same methods as for GSE90956, using human foreskin fibroblasts cultured in media supplemented with human sera obtained from: 1) healthy lean and weight-stable volunteers who were consuming calorie-restricted diets, for an average of 7 years, and 2) age-, sex-matched individuals eating a Western Diet (Omodei et al., 2013).

cMAP groups pharmocological classes which represent a collection of bioactive drugs with similar mechanism of action. Likewise, genetic perturbation classes represent GOF of LOF mutations that elicit a set ontology. Given that the purpose of this was not to identify new individual drugs but to validate process and the suitability of rilmenidine for further study, only the mean *tau* score "connectivity" of each perturbagen class was recorded corresponding to the fraction of reference gene sets with a greater similarity to the perturbagen class than the current query. We used a tau threshold of -90 for significantly opposing perturbagens and +90 for similar profiles as recommended by the database developers and as utilised elsewhere (Ferguson et al., 2018; Liao et al., 2019) .

First and foremost, allantoin did not show significant connectivity to any of the signatures we queried; even the original de Cabo 2003 CR dataset used to initially identify the CRM properties of allantion. This highlights artefactual differences in perturbagen gene profiles between the prototypical and existing cMAP builds. An interesting pattern emerged from the data demonstrating that perturbagen classes that have been previously and routinely associated with amelioration of ageing phenotypes, were positively correlated with ageing and senescent signatures. Likewise, those age ameliorating classes were

negatively correlated to the DR transcripts used to query the cMAP. This further highlighted the unintuitively contrary results from the cMAP query. Specifically, mTOR inhibitors and IGF-1 inhibitors, classes of drugs which are routinely demonstrated to extend lifespan and confer geroprotection, appeared to elicit a significantly similar transcriptional profile to all 4 ageing and senescence signatures (Harrison et al., 2009; Mao et al., 2018). Moreover, mTOR inhibitor classes, despite their forerunning as CRMs, elicited a counterintuitive opposition to human CR gene expression patterns. With that said, using genetic perturbagens, the queries reinforced previously published observations that ageing and senescence is associated with an activation of the NFκB pathway (Haustead et al., 2016; Helenius et al., 1996). Both the universal ageing signature and also the gene signature derived from the senescence meta-analysis, resulted in significant connectivity of *tau* 94.3 and 99.24 with NF-κB activation. Furthermore, and validating the legitimacy of this bioinformatic approach, the senescence gene signature yielded a perfect and identical 100 score for cell-cycle LOF perturbations.

Perturbagen classes that yielded a significantly dissimilar signature to the universal ageing signature and thus offered evidence of potential geroprotective properties, were bile acid and fibroblast growth factor receptor (FGFR) inhibitors. Bile acids have long been recognized as endocrine regulators beyond their role as emulsifiers in the gut lumen, through escapement into systemic circulation. Through interaction with farnesoid X receptors or by the G-protein coupled bile acid receptor (GPBAR1, formerly TGR5), bile acid supplementation interacts with ageing-relevant pathways involved in neuroprotection and glucose homeostasis (Ackerman and Gerhard, 2016; Shapiro et al., 2018). Interestingly, FGFR inhibitors were the only pharmacological class that elicited significant (*tau* -92.59) opposition to the universal signature whilst offering significant similarity to long-term human CR gene expression changes. FGFR GOF mutations are oncogenic and are present in a number of human cancers; as such, FGFR inhibitors are routinely employed in chemotherapy (Katoh, 2016). However, relevantly, FGFRs are upregulated by fasting in the liver and adipose tissue, and FGFR have been demonstrated to increase bile acid secretion improve insulin resistance and glucose metabolism (Ge et al., 2014; Hagel et al., 2015; Katoh, 2016). Thus FGFR inhibitors represent a promising target for CRM identification in future research.

To assess the prolongevity potential of I1RAs, we compared rilmenidine and moxonidine to the 8 gene sets. Moxonidine failed to have a significant connectivity to any of the queried gene sets. However, rilmenidine significantly opposed the all-tissue GeTex ageing signature (*tau* -93.24) and the gene signature of senescence (*tau* -99.68). Unfortunately, rilmenidine failed to elicit any significant transcriptional similarity to either of the CR gene sets, although some insignificant positivity was apparent

between rilmenidine (*tau* +74) and long-term human CR gene expression changes. Nonetheless, rilmenidine represents the more promising I1RA for anti-ageing intervention.

Nischarin, is the only mammalian putative translated protein which expresses the I1RA binding site. LOF mutations significantly opposed the gene signature of hepatocytes exposed to CR serum. In theory, Nischarin LOF should correspond to a reduced I1RA agonism including those such as agmatine which are endogenously derived. Thus, it is plausible to hypothesise that I1RAs may play a part in some CR signalling. This is contrary to previous findings demonstrating Nischarin mutant mice to have better fasting insulin and glucose tolerance, eventuated by an increase in AMPK phosphorylation (Dong et al., 2017).

Notably, both brain ageing signatures failed to generate any significant signals. Given the significant physiological consequences of rilmenidine activity on RVLM neurons, it was surprising to not observe a transcriptional overlap as seen in the universal ageing signature. This suggests that the potential suppression of an ageing signal by rilmenidine is lost in the brain and that this signal may be uncoupled from rilmenidine's primary hypotensive effect. Thus, the potential anti-ageing transcriptional profile of rilmenidine warranted further bioinformatic investigation.

Table 3. cMAP analysis reveals drug classes which share significantly opposing or similar transcriptional profiles to 8 queried gene sets. *The top 150 upregulated and 150 downregulated differentially expressed genes (DEGs) for each of the 8 datasets (3 all-tissue ageing signatures, 2 brain-specific ageing signatures, 1 senescence signature and 2 CR signatures) were individually inputted to the online drug repurposing tool cMap. Those signature inputs were compared to a database of 27,927 perturbagens, including the loss of function (LOF) or gain of function (GOF) mutations across nine cell lines. A numerical median connectivity or tau score was generated derived from a Kolmogorov–Smirnov enrichment statistical evaluation of each gene expression profile: Perturbational classes (grouped compounds or mutations by mechanism of action or ontology) were recorded based on a summary tau connectivity score. Perturbagens with a tau score >90 demonstrate a significantly similar transcriptional profile to their corresponding gene set query, whilst a tau score <-90 represents a significant opposition to the inputted transcriptional direction.*

	Ageing Genetic Sig.			Brain ageing Sig.		Senescence Genetic Sig.	Calorie-Restriction Genetic Sig.	
Author	**Fabris (2018)**	**Mele (2015)**	**Combined**	**Balivada (2017)**	**Fabris (2018)**	**Chatsirisupachai (2019)**	**Omodei (2013)**	**de Cabo (2003)**
No. Differentially Expressed Genes	**1024**	**1573**	**583**	**540**	**580**	**1261**	**357**	**41**
Tissue	**All-Tissue**	**All-Tissue**	**All-Tissue**	**RVLM**	**Whole Brain**	**All-Tissue**	**Foreskin Fibroblasts**	**Hepatocytes**
Perturbagen Class: Pharmacological								
Bile Acid	-59.16	-95.37	-72.95	6.58	-1.62	-98.13	79.82	-73.12
Bromodomain Inhibitor	18.2	17.12	66.79	2.21	-0.26	99.57	-96.23	99.24
CDK inhibitor	7.85	95.8	29.39	3.41	-0.03	99.83	-97.12	-89.54
DNA dependent protein kinase inhibitor	95.7	97.47	94.23	-0.03	-0.01	99.32	-96.3	74.66
FGFR inhibitor	-58.91	-92.96	-92.59	-0.01	7.27	-19.51	97.72	-96.8
FLT3 Inhibitor	74.74	95.74	93.83	0.57		99.7	-91.61	-76.87
HMGCR Inhibitor	52.5	98.54	98.21	-4.6	1.25	96.65	-70.62	21.63
IGF-1 Inhibitor	91.77	98.12	97.01	10.38	0.42	99.65	-94.6	-8.29
IMPDH Inhibitor	81.28	96.25	80.63	2.39	38.93	97.89	-97.83	-88.12
Jak Inhibitor	28.84	80.08	92.56	2.66	3.46	99.97	-93.8	-68.05
MTOR Inhibitor	99.19	99.52	99.33	1.26	28.2	98.62	-96.75	-45.97
PI3K Inhibitor	98.34	99.32	99.09	3.55	1.22	99.23	-97.63	-23.84
PDGFR/KIT inhibitor	67.4	94.74	96.83	-0.05	1.11	84.32	-81.62	-92.71
Protein Synthase Inhibitor	1.17	34.08	94.26	-57.77	2.38	94.08	12.36	-96.18
Topoisomerase inhibitor	36.06	98.06	84.74	6.3	0	99.39	-94.48	-61.81
T-type calcium channel blocker	94	90.59	96.05	-0.06	3.75	97.64	-54.92	-60.86
Perturbagen Class: Genetic								
Cell Cycle Inhibition GOF	90.04	71.72	85.71	43.44	0.2	100	-96.37	-58.99
NFKB Activation GOF	94.74	78.41	94.3	-18	7.39	99.24	-25.48	-80.81
Perturbagen Class: Imidazoline Receptor Related								
Rilmenidine	-67.49	-93.24	-85.51	22.14	-10	-99.68	74	-32.61
Moxonidine	-10.66	-29.266	-7.29	-6.27	-3.95	-13.23	-1.11	21.39
Nischarin GOF	-0.93	14.11	-1.76	0	13.43	-0.49	5.79	16.61
Nischarin LOF	2.87	-38.34	12.95	-46.92	-34.9	14.03	-2.73	-93

3.2.2. Meta-Analysis of *In-vitro* Imidazoline Receptor Agonist Treatment To Derive a Transcriptional Profile For Gene Set Enrichment Analysis

To ascertain a consensus rilmenidine transcriptome for further study, a meta-analysis was performed to identify universally DEGs induced by *in-vitro* rilmenidine treatment. Using the L-1000 database (clue.io), normalised gene expression data was downloaded representing the gene expression changes (z-scores) of 61 cell lines treated with 10μM of rilmenidine for 6 hours in comparison to cell-line-matched, DMSO-treated wells. In the L-1000 database, all cell lines were treated with 10 μM rilmenidine for either 6h or 12h. 6h was chosen because it has been previously used as a time point for phenotype induction with rilmenidine in both human and rodent studies (De Visser et al., 2002; Perez et al., 2012).

Meta-analysis was performed via a value counting method as in de Magalhães et al., (2009), and is detailed in the methods section. In order to account for the subtilties of transcriptional change, a relaxed z-ratio threshold for differential expression was set at -1.96 and +1.96 (Cheadle et al., 2003). Overall, I identified 50 genes across the cell-lines consistently overexpressed with rilmenidine treatment, using an adjusted Benjamini–Hochberg cut-off of P <0.01. Conversely, we found 154 genes consistently underexpressed with rilmenidine treatment. *SERPINA3* was the most consistently overexpressed gene, being differentially expressed in 15 of the 61 trials (adj. p-value 1.63E-09). *SERPINA3* is a member of the serine protease inhibitors (SERPINs) superfamily. It is secreted as an acute phase protein to inhibit cathepsin G, limit inflammation and contribute to improved wound healing (Chelbi et al., 2012; Horvath et al., 2005; Whisstock and Bottomley, 2006). Importantly, gene expression of *SERPINA3* has been reported to decrease with age (Glass et al., 2013). *WIF1* is an inhibitory extracellular protein with a WNT inhibitory factor (WIF) domain and 5 epidermal growth factor (EGF)-like domains; this was the 2nd most consistently over expressed gene in response to rilmenidine, but is also known to be downregulated with ageing (Makrantonaki et al., 2012). Of the remaining top 10 expressed genes (SERPINA3, WIF1, MAL, FBXO11, SNX11, KYNU, SPP1, RRP8, RRS1, TLR4), all were landmark or well-inferred genes. Furthermore, a number of genes are involved in immune function (SPP-1, *MAL, TLR-4)* and a further 3 were involved in protein processing (SNX11, FBXO11 and RRS1).

Of the 154 genes consistently under-expressed in rilmenidine treated cell-lines, CHMP4A was the most significant, being differentially expressed in 13 of the 61 trials (adj. p-value 1.01E-10). CHMP4A belongs to the chromatin-modifying protein/charged multivesicular body protein (CHMP) family. Evidence suggests, CHMP4A is a key facilitator in degradation of surface receptor proteins and formation of endocytic multivesicular bodies (MVBs) (Tsang et al., 2006). Elsewhere, CHMP4A has been thought to accelerate cell cycle progression, and is accordingly differentially expressed during senescence (Rapisarda et al., 2017). Of interest, CHMP4a possesses a coiled coil domain, which has been

hypothesised to carry potential imidazoline receptor binding site (Katoh et al., 2003). The second most under-expressed gene was calpastatin (CAST). This gene encodes a calpain inhibitor, which supresses proteolytic activity of calpains (Mikosik et al., 2013). Dysregulation of calpains, key cleavage molecules, has been shown to influence cell migration and that calpain inhibition blunts tumour growth (Raimbourg et al., 2013). Thirdly, PPP2R5E was consistently underexpressed in 12 of the 61 trials (adj. p 1.44E-09). PPP2R5E is a gene that encodes a regulatory subunit of the tumor suppressing protein phosphatase 2A. Not only is its downregulation associated with cancer development and poor outcome, it's downregulation is also shown in high-risk for AD APOE4 patients (Grochola et al., 2009; Theendakara et al., 2017). However, conversely, activated mTORC1 induces protein phosphatase 2A (PP2A)-mediated dephosphorylation of the transcription factor forkhead box K1 (FOXK1) and thus its downregulation may be associated with reduced mTOR functioning (Nakatsumi et al., 2018). Of the other top ten most underexpressed genes, kinectin 1 was included. This gene is known to become overexpressed in ageing and its expression can be modulated by CR (Chang et al., 2015; Prolla et al., 2006). Moreover, included in the top ten was the consistent underexpression of C2CD5, a phospholipid binding and insertion protein. As such, C2CD5 contributes to insertion of GLUT4 into the plasma membrane and is thus associated to a "low glucose response" (Xie et al., 2011).

To elucidate the collated effect of rilmenidine on cellular processes and gain some more functional insight, the differentially expressed gene lists of rilmenidine were enriched using a TopGO r-script and reclassification with Revigo (see materials and methods). This returned key processes affected by rilmenidine treatment. 166 biological processes (GO terms) were returned to be significantly enriched in the overexpressed rilmenidine gene list following TOPGO analysis using a weighted Fischer p value <0.05. This more relaxed threshold was employed to account for the focus on enriched functional categories rather than on individual genes. This resultant list was further filtered by Revigo, a software which summarises gene lists by removing redundant annotations to generate term subsets using a simple clustering algorithm that relies on semantic similarity measures (Supek et al., 2011). Using a p value <0.01 threshold the analysis returned 22 GO terms that were enriched in the overexpression gene-list for rilmenidine (see Table 4). Results suggest that rilmenidine drives an overexpression in genes associated with development (GO:0060433: bronchus development, GO:0003179: heart valve morphogenesis, GO: 0060231: mesenchymal to epithelial transition, GO:0035162: skeletal muscle tissue regeneration, GO0048701: Embryonic cranial skeleton morphogenesis). Furthermore, and relevant to calorie restriction and geroprotective properties, rilmenidine treatment appeared to reduce expression of inflammation associated genes (GO:0032689, negative regulation of interferon-gamma production; GO:1903556,

negative regulation of tumor necrosis factor superfamily cytokine production). Interestingly, rilmenidine treatment also associated with GO:0042149 cellular response to glucose starvation.

Table 4. Gene Ontology (GO) terms for biological processes that are over represented in the lists of significantly underexpressed genes following meta-analysis of rilmenidine treated cell line transcripts. *P-values obtained using topGO to derive Fischer's exact values and adj.p values following redundancy reduction using REViGO. Shading pertains to extracellular matrix associated GO terms. Shading pertains to immune associated GO terms.*

Underexpressed Rilmenidine		
GO Term	**Description**	**REVIGO Log10 P-value**
GO:0070486	leukocyte aggregation	-3.9586
GO:1903543	positive regulation of exosomal secretion	-3.7696
GO:0022617	extracellular matrix disassembly	-3.6383
GO:0043312	neutrophil degranulation	-3.4559
GO:0042060	wound healing	-3.2676
GO:0045445	myoblast differentiation	-3.1427
GO:0033572	transferrin transport	-3
GO:0051093	negative regulation of developmental process	-2.8996
GO:0043062	extracellular structure organization	-2.857
GO:0050900	leukocyte migration	-2.8539
GO:0006027	glycosaminoglycan catabolic process	-2.8447
GO:0045214	sarcomere organization	-2.8239
GO:1902117	positive regulation of organelle assembly	-2.7055
GO:0048146	positive regulation of fibroblast proliferation	-2.699
GO:0042130	negative regulation of T cell proliferation	-2.6498
GO:0045600	positive regulation of fat cell differentiation	-2.6326
GO:0035722	interleukin-12-mediated signalling pathway	-2.5884
GO:0071230	cellular response to amino acid stimulus	-2.5452
GO:0098609	cell-cell adhesion	-2.341
GO:0071407	cellular response to organic cyclic compound	-2.3344
GO:0007160	cell-matrix adhesion	-2.2967
GO:0009894	regulation of catabolic process	-2.2211
GO:0001523	retinoid metabolic process	-2.2147
GO:0001657	ureteric bud development	-2.1512
GO:0006024	glycosaminoglycan biosynthetic process	-2.0768

Table 5. Gene Ontology (GO) terms for biological processes that are over represented in the lists of significantly overexpressed genes following meta-analysis of rilmenidine treated cell line transcripts. *P-values obtained using topGO to derive Fischer's exact values and adj.p values following redundancy reduction using REViGO. Shading pertains to GO terms associated with developmental processes. Shading pertains to anti-inflammatory associated GO terms.*

Overexpressed Rilmenidine		
GO Term	**Description**	**REVIGO Log10 P-value**
GO:0030501	positive regulation of bone mineralization	-3.7959
GO:0060433	bronchus development	-3.2676
GO:0003179	heart valve morphogenesis	-3.0862
GO:0042447	hormone catabolic process	-3.0706
GO:0030277	maintenance of gastrointestinal epithelium	-3.0132
GO:0060231	mesenchymal to epithelial transition	-2.8153
GO:0035162	embryonic hemopoiesis	-2.7721
GO:2000311	regulation of selective glutamate receptor activity	-2.7721
GO:0090303	positive regulation of wound healing	-2.71
GO:1904646	cellular response to beta-amyloid	-2.6576
GO:0045992	negative regulation of embryonic development	-2.6216
GO:0045671	negative regulation of osteoclast differentiation	-2.4634
GO:0032689	negative regulation of interferon-gamma production	-2.4353
GO:0043403	skeletal muscle tissue regeneration	-2.382
GO:0048701	embryonic cranial skeleton morphogenesis	-2.3487
GO:0042149	cellular response to glucose starvation	-2.2848
GO:0072160	nephron tubule epithelial cell differentiation	-2.2434
GO:1903556	negative regulation of TNF family cytokine production	-2.2426
GO:0001709	cell fate determination	-2.2182
GO:0030890	positive regulation of B cell proliferation	-2.1979

The pipeline was repeated for the rilmenidine underexpressed gene list. Again 138 GO terms were derived from TOPGO analysis of the underexpressed gene list which was further filtered to 25 GO terms by RIVIGO. In agreement with results demonstrated for the upregulated genes, biological processes that were significantly downregulated were immune related (GO:0070486, leukocyte aggregation; GO:0043312, neutrophil degranulation; GO:0050900, leukocyte migration; GO:0035722 interleukin-12-mediated signalling pathway). Additionally, and again relevant to calorie restriction, rilmenidine causes a down regulation of genes that respond to amino acid stimulus (GO:0071230).

To further clarify whether the transcriptional perturbations induced by rilmenidine treatment were symptomatic of imidazoline receptor activity, we repeated the meta-analysis to pool gene-expression data from 9 different known imidazoline receptor agonists (Table 6). Strikingly, despite rilmenidine being only 1 of 9 imidazoline receptor agonists, every single overexpressed gene in rilmenidine treated cells was also overexpressed in the combined drug meta-analyses, with a total of 267 genes being overexpressed when all imidazoline receptor agonists were analysed together. Conversely, 586 DEGS were

underexpressed in the combined drug signature of which 129 were also significantly underexpressed in rilmenidine treatment; only 3 genes were not subsumed by combined treatment. Thus it can be reasonably concluded that imidazoline receptor activation is the likely cause of transcriptional perturbations by rilmenidine.

Table 6. Imidazoline receptor agonists included in the combined drug gene expression meta-analysis.

Drug	Rilmenidine	Moxonidine	Clonidine	Guanabenz	Tizanidine	Guanfacine	Cimetidine	Naphazoline	Cirazoline	Lofexidine
No. Cell Lines Tested	31	18	38	27	10	23	9	10	9	11

Next, it was important to establish whether rilmenidine was able to rescue any of the transcriptional alterations observed in ageing which might elucidate geroprotective mechanisms. Overlapping of DEGs in rilmenidine and DEGs from the Fabris et al. whole tissue ageing signature highlighted significantly opposing transcriptional direction, such that genes that became overexpressed in ageing were underexpressed in rilmenidine treatment (Fischer's Exact; p= 7.72E-07). This suggested rilmenidine might be able to rescue transcriptional overactivity in ageing. To identify another potential transcriptional opposition, genes consistently underexpressed with ageing were compared to genes consistently overexpressed in rilmenidine, however this yielded no significance.

It was important to establish which biological processes might be involved in the proposed rilmenidine induced rescuing of age-related overexpression. Firstly, rilmenidine did not match the transcription of the DR dataset in any direction (see supplementary; Fischer's exact; over vs over p=1.0; under vs under p=0.7). Thus to gain further insight, gene enrichment as described above using TOPGO and REViGO was performed against the differentially expressed gene intersect for ageing overexpression and rilmenidine underexpression. 129 different biological processes were enriched in the intersect. Following reclassification by REViGO, 16 biological processes remained enriched and these are listed in Table 7. The most significantly enriched term was GO:0006027 glycosaminoglycan catabolic process. Glycosaminoglycans are abundant structural components of the extracellular matrix (ECM) and their catabolism forms part of a more global ECM breakdown in ageing (D. H. Lee et al., 2016). This GO term is related to 4 other GO terms associated with extracellular matrix function that are enriched in the ageing over/rilmenidine under gene list (GO:1903543 positive regulation of exosomal secretion; GO:0001523 retinoid metabolic process; GO:1905048 regulation of metallopeptidase activity; GO:0022617 extracellular matrix disassembly. Another collection of terms that were enriched were involved with immune response and inflammation (GO:0050900 leukocyte migration; GO:0035722interleukin-12-mediated signalling pathway; GO:0016032 viral process; GO:0042060 wound healing; GO:0070486

leukocyte aggregation). In fact, ECM and immune function GO terms dominated more than half of the significantly enriched biological processes. When the same gene enrichment pipeline of gene list intersects was repeated for combined imidazoline receptor drugs, in lieu of rilmenidine, ECM terms and immune terms were consistently within the top 20 GO terms (See Supporting Table 1).

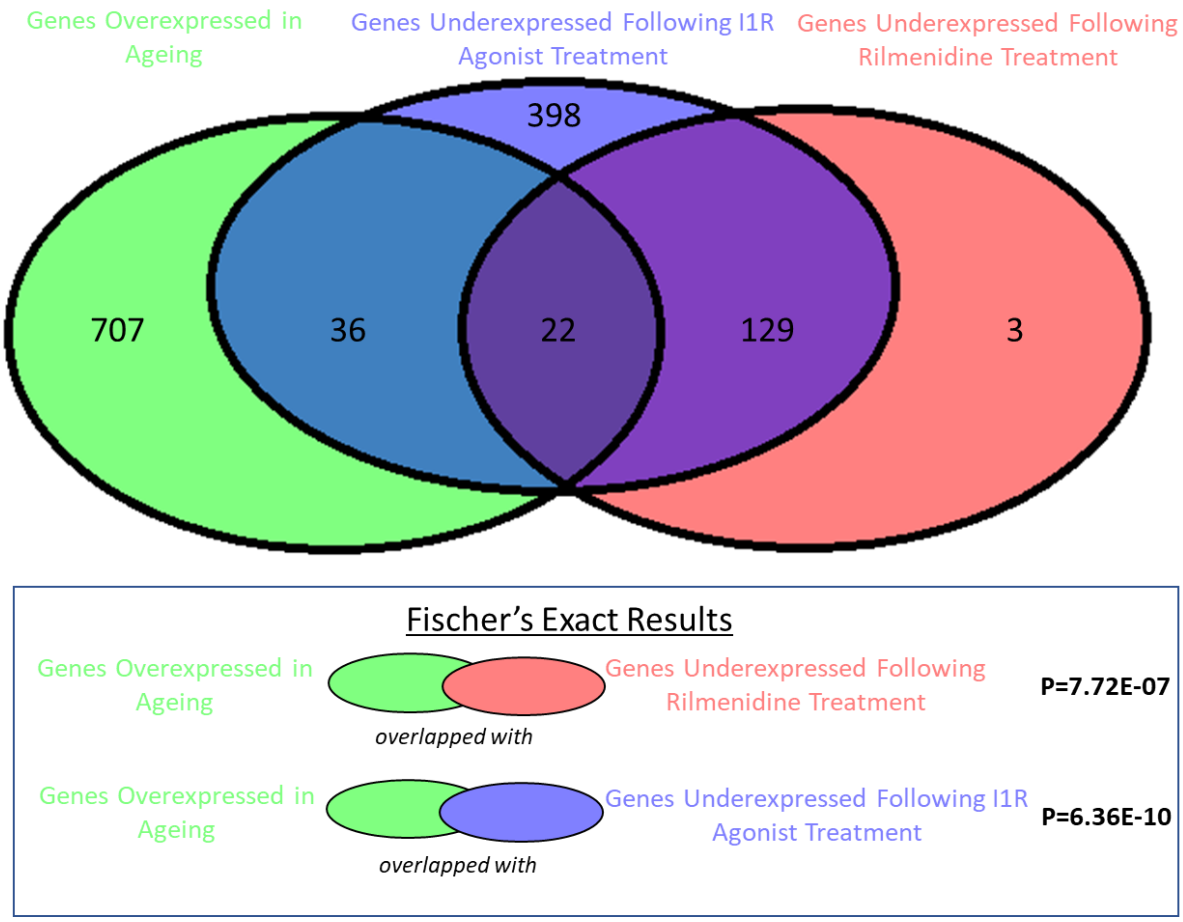


Figure 3. Venn diagrams demonstrating the combined overlaps of 3 different gene-sets*. 1. Genes consistently overexpressed in ageing. 2. Genes consistently underexpressed in cells exposed to rilmenidine. 3. Genes consistently underexpressed following a meta-analysis of a range of cells exposed to 9 different known imidazoline receptor agonists. Fishers exact test using a human exome background used to establish significant overlaps.*

Table 7. Revigo recategorised GO terms derived from TOPGO gene enrichment of DEGS that intersect both rilmenidine-induced underexpression and age-induced overexpression.

Underexpressed in Rilmenidine And Overexpressed In Ageing		
GO Term	**Description**	**REVIGO Log10 P-value**
GO:0006027	glycosaminoglycan catabolic process	-3.9586
GO:0016032	viral process	-3.7696
GO:0042060	wound healing	-3.6383
GO:0070486	leukocyte aggregation	-3.2676
GO:1903543	positive regulation of exosomal secretion	-3.1427
GO:0097284	hepatocyte apoptotic process	-3
GO:0045445	myoblast differentiation	-2.8996
GO:0001523	retinoid metabolic process	-2.857
GO:0010988	regulation of low-density lipoprotein particle clearance	-2.8539
GO:1905048	regulation of metallopeptidase activity	-2.8447
GO:0033572	transferrin transport	-2.699
GO:0043687	post-translational protein modification	-2.5884
GO:0050900	leukocyte migration	-2.5452
GO:0035722	interleukin-12-mediated signalling pathway	-2.2211
GO:0022617	extracellular matrix disassembly	-2.2147
GO:0016540	protein auto-processing	-2.1512

3.2.3. Rilmenidine and Other Imidazoline Receptor Agonists Improve Survival in *C. elegans*

With the knowledge that rilmenidine and, more generally, imidazoline receptor agonists seemingly oppose the transcriptional direction of ageing, we exposed WT adult hermaphrodite *C. elegans* to varying concentrations of rilmenidine or moxonidine, to assess changes in survival. We utilised a popularised solid media NGM based lifespan assay using both FUDR and UV-killed OP50 *E.coli* (Sutphin and Kaeberlein, 2009). At L4/adult molt, worms that had previously developed on FUDR-free NGM and live OP50 *E.coli* were transferred to, and remained on thereafter, NGM plates that had been supplemented with both FUDR and a homogenously dissolved DMSO solution to 1% containing either rilmenidine (0μM, 100μM, 150μM, 200μM, 300μM, 400μM), or moxonidine (0μM, 100μM, 150μM, 200μM, 250μM). Additionally, we reassessed the previously demonstrated pro-longevity dose of allantoin (250μM) as well as the oft-cited pro-longevity dosages of resveratrol (100μM) and rapamycin (100μM) to establish the reliability of the assay (J. Lee et al., 2016; Robida-Stubbs et al., 2012; Viswanathan et al., 2005; Wood et al., 2004).

Dosages for I1RAs were established around the most efficacious dose administered by Calvert et al. when using the same solid media platform (Calvert et al., 2016). Given that guanidium compounds such as allantoin exhibit a more subtle agonistic affinity to I1RAs, we hypothesised a reduced dosage of rilmenidine or moxonidine, more specific I1RAs, would confer similar lifespan extension (Dardonville and Rozas, 2004).

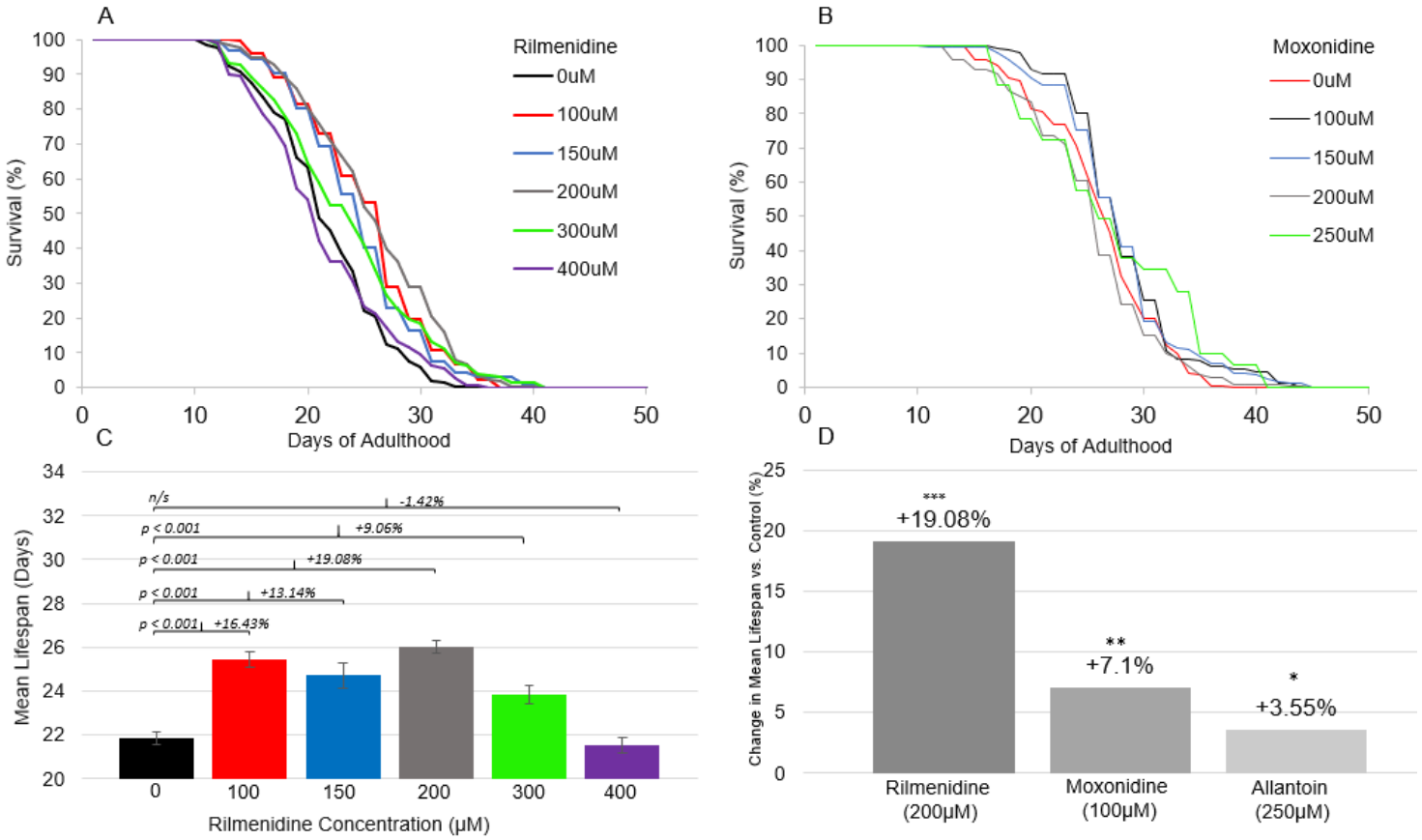


Figure 4. Improved survival of C. elegans treated with varying concentrations of imidazoline receptor agonists. *A) Pooled survival curves of adult WT worms fed UV-killed OP50 also treated with increasing concentrations of rilmenidine (100uM-400uM) compared to control animals treated with a 1% DMSO vehicle. All tested concentrations except 400uM significantly improved survival (adj. p<0.001) as measured by log-rank test and Bonferroni corrections. n>90 animals across at least 3 independent trials.B) Pooled survival curves of adult WT worms fed UV-killed OP50 also treated with increasing concentrations of moxonidine (100uM-200uM) compared to control animals treated with a 1% DMSO vehicle. 100uM, 150uM, and 250uM. Moxonidine treatment significantly improved survival (adj. p<0.01) as measured by log-rank test and Bonferroni correction. n>190 animals across at least 2 independent trials. C) mean lifespan (days\ of pooled trials measuring the survival of rilmenidine treated with increasing concentrations of rilmenidine (100uM-400uM) compared to control animals treated with a 1% DMSO vehicle. Error bars represent SEM; adjusted p-value derived from log-rank test and Bonferroni correction. D) % change in mean lifespan of the most potent concentration of each imidazoline receptor agonists (IRA) vs trial-specific control animals treated with a 1% DMSO vehicle. Data represented as means of at least three independent experiments (Bonferroni adjust ed log-rank test, *p < 0.05, **p < 0.01, and ***p < 0.001 IRA-exposed worms compared to 1% DMSO counterparts).*

Table 8. Differences in mean and maximum lifespan of WT C. elegans exposed to various compounds relative to the control within the same sets of trials. *Mean lifespan in days is the pooled mean of treatment cohort and the standard error (± S.E.M). Maximum lifespan defined as the day at which 90% cohort mortality was reached. Lifespan assays performed at 20 °. Adjusted p-value was determined by log-rank test using the Kaplan-Meier survival analysis and subsequent bonferonni correction. Pooled n = number of deaths observed across all respective trials.*

WT WT worm treatment	*Pooled n=*	No. trials	Mean lifespan (days)	Std. error	% Chg Vs. Ctrl.	90% Mortality (days)	Adj. p-value
DMSO							
WT WT	651	6	24.03	0.19	0.00	30	n/a
1% DMSO	806	6	23.84	0.17	0.01	30	n/s
Rilmenidine							
0µM	374	6	21.85	0.26	0.00	29	n/a
100µM	205	3	25.44	0.37	16.43	33	p<0.001
150µM	92	3	24.72	0.58	13.14	31	p<0.001
200µM	325	5	26.02	0.32	19.08	33	p<0.001
300µM	222	3	23.83	0.44	9.06	33	p<0.001
400µM	262	3	21.54	0.36	-1.49	30	n/s
Moxonidine							
0µM	331	3	26.41	0.29	0.00	33	n/a
100µM	196	3	28.31	0.36	7.19	33	p<0.01
150µM	198	3	27.98	0.39	5.94	35	p<0.005
200µM	372	3	25.58	0.30	-3.14	32	n/s
250µM	61	1	27.48	0.93	4.05	36	p<0.05
Allantoin							
0µM	238	3	25.05	0.32	0.00	32	n/a
250µM	195	3	25.94	0.38	3.55	32	p<0.05
Resveratrol							
0µM	100	1	20.97	0.50	0.00	27	n/a
100µM	48	1	25.73	0.94	22.70	35	p<0.001
Rapamycin							
0µM	109	3	22.07	0.49	0.00	28	n/a
100µM	223	3	27.35	0.50	23.92	39	p<0.001
Time Dependent Rilmenidine							
0µM	165	3	22.08	0.38	0.00	29	n/a
Single Dose at Day 1 (200µM)	144	3	29.51	0.56	33.65	39	p<0.001
Single Dose at Day 12 (200µM)	83	3	29.39	0.66	33.11	34	p<0.001
Day 1 and Day 12 Dose (200µM)	68	3	29.15	0.78	32.02	38	p<0.001

Wild-type (WT) adult hermaphrodite *C. elegans* are reported to have an average lifespan of 23 days at 20ºC when fed UV killed OP50 *E.coli* (Sutphin and Kaeberlein, 2009). In agreement, and under our standard laboratory conditions, control WT animals had mean lifespan of 24.03 ± .19 days (n=651) and a maximum lifespan of 30 days. Furthermore, to address any confounding effects of DMSO on lifespan interventions, the post-developmental addition of 1% DMSO to the NGM media did not significantly affect the mean or maximum lifespan ($p<0.43$) of WT worms (n=806). Confirmatively, both resveratrol (100µM) and rapamycin (100µM) significantly extended lifespan similar to previously published observations (J. Lee et al., 2016; Robida-Stubbs et al., 2012; Viswanathan et al., 2005) (Table 9).

Rilmenidine increased mean lifespan at all but the highest tested dosage (400µM); dosages increases were discontinued thereafter (Figure 4A). Lower dosages of 100µM and 150µM extended mean lifespan by +17% ($p<0.001$) and +13% ($p<0.001$) respectively (Figure 4C). Mean lifespan changes were most pronounced at 200µM with a +19% ($p<0.001$) increase. Higher dosages of 300µM increased mean lifespan, to a lesser extent, by 9% ($p<0.001$), and 400µM insignificantly reduced mean lifespan by -1.42%. Lifespan extending dosages of rilmenidine unanimously increased maximum lifespan by 14% ($p<0.001$). Thus rilmenidine neither follows a dose-dependent curve nor a non-linear J-shaped hormetic curve. Instead rilmenidine elicited a somewhat gaussian function on lifespan with a peak at 200µM.

Similarly, animals treated with moxonidine did not exhibit a dose-dependent curve nor a non-linear J-shaped hormetic curve (Figure 4B). In fact, the largest increases in mean lifespan (+7.1%) was observed at the lowest concentration range (100µM) of moxonidine treatment ($p<0.006$), however this dosage failed to increase maximum lifespan. Both 150µM and 200µM elicited significant increases in mean lifespan of 7% ($p=0.0047$) and 5% ($p=0.0105$), and also maximum lifespan 6% and 9% respectively. 200µM moxonidine failed to significantly alter mean or maximum lifespan in either direction.

Despite a reported increase in mean lifespan of over 20% in our lab's previous work, in this study, only a 3.55% ($p<0.05$) change in mean lifespan was observed following 250µM allantoin treatment; and no change in maximum lifespan was elicited (Figure 4D). Taken together, these three compounds display a capacity to extend lifespan correlatively to their imidazoline binding site affinities. Rilmenidine exerts the most pronounced binding affinity in literature, whilst also displaying a largest increase in lifespan at the tested dosages (Nikolic and Agbaba, 2012). This is line with preceding evidence showing a more significant transcriptional correlation between rilmenidine and ageing in comparison to moxonidine and allantoin. Thus from hereon, rilmenidine will be tested as a potential geroprotective CRM.

To confirm the efficacy of rilmenidine to extend lifespan in *C. elegans*, a multi-centred approach was taken. It is well established that inter-laboratory variation and idiosyncrasies can significantly affect

lifespan. To ascertain the validity of our initial findings, we invited an independent lab to test the pro-longevity effect of rilmenidine without prior knowledge of our results. The lab of Colin Ewald tested our hypothesis using an automated imaging and analysis platform called the *C. elegans* "lifespan machine" (Stroustrup et al., 2013), which has repeatedly been proven to be a reliable tool (Lin et al., 2018a; McEwan et al., 2016; Stroustrup et al., 2016). They followed the same solid agar NGM drug protocol as in our methods. Ewald's lab independently reported significant lifespan extension by rilmenidine at the optimum dose (200μM) when treatment was initiated at L4/adult molt (Ewald lab: mean/max lifespan change $p<0.05$) (data not shown). 200μM was the dose, unless otherwise stated, administered to worms hereafter.

Rilmenidine should ideally work when administered later in life. Although pharmacological interventions may prove to ameliorate the effects of ageing in humans when initiated in early adulthood, the application of this would present significant difficulties. Untenably protracted longitudinal survival-outcome clinical trials alongside, the ethical and compliance concerns of life-long administration precludes the clinical usage of any anti-ageing intervention for such times. Therefore, researchers have been keen to establish if potential compounds can extend lifespan when treatment is initiated later in life and the ageing process has considerably progressed (Cabreiro et al., 2013; Guha et al., 2014). This is particularly important given the difficulties of eliciting CR-mediated longevity in aged animals.

WT worms grown throughout adulthood on NGM agar plates seeded only with UV killed OP50 *E.coli* were transferred, on day 12 adulthood, to NGM agar plates containing the most 200μM rilmenidine, or vice versa. At day 12, worms exhibit significant age-related deterioration. Specifically, by day 12 pharyngeal pumping, a routine measurement of animal health, has typically ceased alongside egg-laying (Collins et al., 2008). Furthermore, animals also exhibit dramatically altered metabolism, increased age-associated autofluorescence and proteostatic collapse: a key event in organismal ageing (Chapin et al., 2015; Collins et al., 2008; Rollins et al., 2017). Rilmenidine treatment initiated from day 12 significantly extended lifespan ($p>0.0001$) in line with early-life treatment (Figure 5).

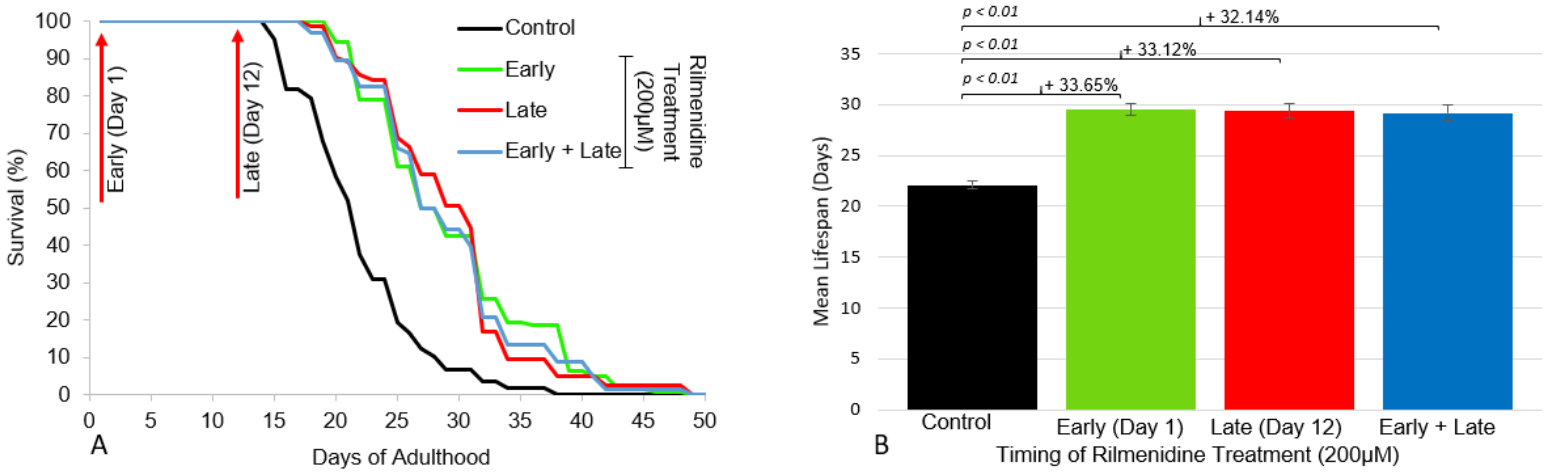


Figure 5. Effect of different rilmenidine administration times on C. elegans survival. *A) Pooled survival curves of adult WT worms fed UV-killed OP50 also treated at different time points (day-1 or day-12 adulthood) with 200uM rilmenidine compared to control animals treated with a 1% DMSO vehicle from day-1 adulthood. Each lifespan experiment was repeated at least three independent times with similar results. Quantitative data and statistical analyses for the representative experiments are included in* ***Table 9****. B) % increase in mean lifespan of pooled trials measuring the survival of adult WT worms fed UV-killed OP50 also treated at different time points (day-1 or day-12 adulthood) with 200uM rilmenidine compared to control animals treated with a 1% DMSO vehicle from day-1 adulthood. Error bars represent SEM; adjusted p-value derived from log-rank test and Bonferroni correction.*

Our solid media drug administration approach may lend to decompositional loss of potency towards the conclusion of a lifespan assay and thus an unrealised pro-longevity capacity of rilmenidine. However, the ability of rilmenidine to extend lifespan late in life is not additive to any pro-longevity effect exerted by early life treatment. Worms transferred from rilmenidine drug plates to fresh rilmenidine drug plates at day 12, lived no longer than worms grown exclusively on one rilmenidine drug plate from L4/adult molt. Therefore, it is possible any cellular mechanism by which rilmenidine exerts its geroprotection, is conserved through adulthood and ageing, and, it is unlikely that rilmenidine concentrations significantly erode during the lifespan assay. However, maximum lifespan was not as dramatically affected when treatment was initiated at day 12 (+17%) in comparison to complete lifespan exposure (+31.03%).

3.3. Discussion

In this chapter the recently updated cMAP was queried with 2 universal ageing signatures, a senescence signature and two dietary restriction signatures in a bid to identify potential calorie restriction mimetics which could show similarity to CR transcripts but opposition to ageing transcripts. This is the first recorded attempt to identify compounds that are significantly correlated to several simultaneously queried gene lists. The cMAP confirmed that rilmenidine was significantly opposed to the transcriptional direction of ageing, which was also mirrored in senescence. Specifically, the gene expression changes induced by *in-vitro* rilmenidine treatment, as recorded in the cMAP database, elicited a significantly negative *tau* connectivity score. Furthermore, rilmenidine displayed a transcriptional similarity to dietary restriction however this was not significant. Importantly, neither allantoin or moxonidine, I1RAs with a lower binding affinity, showed any directional connectivity with either ageing or dietary restriction.

The confirmation that rilmenidine, as measured by the transcriptional connectivity of the cMAP, seemed to oppose the transcriptional direction of ageing, led to further investigation into specific gene changes. A meta-analysis of LINCS data containing 57 cell lines exposed to rilmenidine at a concentration of 10μM for 24h demonstrated rilmenidine to significantly overexpress genes related to developmental processes, as well as genes related to the downregulation of immune response. Additionally, rilmenidine treatment appeared to underexpress genes related to active immune response and inflammation, extracellular matrix function and response to amino acids. The gene expression changes identified by this meta-analysis were indeed similar to the cMAP findings and clarified that rilmenidine likely rescues the gene expression perturbations of ageing, specifically those that become overexpressed in ageing. These genes correlated to immune hyperactivation in ageing and furthermore there was a strong enrichment of genes related to extracellular matrix disassembly. Moreover, these transcriptional relationships were

mirrored in all imidazoline receptor agonists, highlighting an imidazoline receptor agonist specific effect on ageing. To test the potential geroprotection of rilmenidine *in-vivo*, WT *C. elegans* were fed increasing concentrations of rilmenidine to establish an optimum pro-longevity dose (200μM). This dosage extended mean and maximal lifespan in WT worms, when administered throughout life or once the worm had aged. This capacity demonstrates important translational potential that warrants further investigation.

The identification of potential pro-longevity compounds is a popular endeavour within the gerontological community. DrugAge (Build 3), a database which I and other lab members have collated, lists 567 different compounds purported to extend lifespan in model organisms (Barardo et al., 2017). Not only does this show a wide interest in the field but compendiously highlights the vast targetability of the heterogeneities and multiplicities of ageing, which had previously made the actual identification of translationally precise anti-ageing therapies difficult. Thus, researchers have turned to "omics" based approaches to aggregate such ageing pleiotropism into universal signatures of ageing which can help identify compounds that can more broadly oppose ageing in favour of individual pathway targeting. Transcriptional signatures using harvested dermal fibroblasts report to recapitulate biological ageing more precisely than other published metrics (Fleischer et al., 2018). The pattern of global transcriptional changes in ageing is conserved and an increase in age-associated expression of stress response and immune response pathways is reproducibly observed in ageing studies (Stegeman and Weake, 2017).

Cell cycle inhibition also significantly correlated with the Fabris et al. ageing meta-analysis used from our lab. This added legitimacy to the ageing signature given this phenomenon is characteristic of cellular senescence which accumulates in aged tissue through replicative termination and stress induction. Accordingly, the transcriptional signature of senescence which was queried also revealed a perfect connectivity score to cell cycle inhibition. Thus, the similarity is potentially explained by aged tissue from the datasets used in the ageing meta-analysis being derived from heterogenous tissue that will contain a growing quantity of senescent and quiescent cells (Childs et al., 2015). Furthermore, a comparatively diminished stem cell pool in sample tissue may also contribute to a positive connectivity to cell cycle inhibition

The above pattern observation may potentially explain why perturbagen classes that have been previously and routinely associated with amelioration of ageing phenotypes, were positively correlated with ageing and senescent signatures in the cMAP analysis. This contraindication to current ageing literature was apparent in several classes including a positive association to IGF-1 inhibition and also mTOR inhibition. Aged tissue that chronically accumulates damage prompts the accumulation of quiescent cells which display reduced AKT (Segrelles et al., 2014; Wei et al., 2016) and increased PTEN

phosphatase activity (Yue et al., 2017), which, in turn, suppresses mTOR signalling (Gan and DePinho, 2009). Quiescent cells are transcriptionally active and downregulate proliferation-related genes and remain hyporesponsive towards growth factors and other external stimuli (Matsuda et al., 1992; Seshadri and Campisi, 1990; Yao, 2014). Hence the downregulation in growth factor signalling is transcriptional hallmark of ageing (Frenk and Houseley, 2018). This is a similar notion to long-lived post mitotic cells such as neurons, myotubes, or cardiomyocytes that enable advanced mammalian function which will have been enriched in brain and muscle of aged tissue used to derive the aged signature. Transcriptional analysis has revealed post-mitotic tissue to be associated with reduced cell cycle gene expression and a reduced responsiveness to amino acid stimulation (Castillo-Morales et al., 2019). Thus, compared to the cancer cell lines used in the cMAP RNA-seq data acquisition of perturbagens, aged tissue is much more likely to show comparably reduced expression of mTOR, IGF-1 and PI3K stimulated genes which may mirror the effects of mTOR, IGF-1 and PI3K inhibitors in immortalised cell lines.

Conversely, rilmenidine demonstrated a strong negative correlation to the ageing gene sets as well as the senescence signature, implying that rilmenidine is unlikely to significantly influence factors pertaining to cell cycle and proliferation. Nonetheless, it still showed significant transcriptional opposition to senescence implying that a relationship exists between the ancillary functions of senescence beyond mere cell cycle arrest. No significant connectivity was however observed in any aged brain tissue, even RVLM tissue which is primarily post-mitotic. Whilst one is keen to deduce that the age-related gene expression perturbations by rilmenidine are decoupled from its sympatholytic function in the brain and RVLM, it is more likely that given none of the cell lines used to generate the cMAP data originate from the brain, the results reflect a poorly transcriptional compatibility when querying brain tissue (Dönertaş et al., 2018).

Additionally, cMAP analysis failed to demonstrate any significant connectivity to either gene lists of dietary restriction, and this was the same with moxonidine and also allantoin. Moreover, a similar paradoxical pattern occurred whereby mTOR inhibitors and IGF-1 inhibitors, routinely touted as calorie restriction mimetics elicited, significantly opposing profiles to CR (López-Lluch and Navas, 2016). This is comparable to observations that long term CR restriction as seen in the datasets used herein, elicit an opposite gene expression profile to short-term starvation which is commonly used to provoke changes in mTOR and IGF activity (Whitaker et al., 2014). Strikingly, neither of the DR gene lists returned any perturbations which showed the same connectivity direction. This limits any inference that could be made, but excellently highlights the difficulty in applying gene expression-based approaches to a non-binary spectrumed phenomena vulnerable to external artefacts such as ageing and calorie restriction. To query

the cMAP, *in-vitro* calorie restriction datasets were explicitly chosen rather than tissue derived from calorie restricted model organisms specifically to minimise confounding factors. However, the translation of CR into *in-vitro* culture through the infusion of CR sera is likely to dilute the already modest global gene expression effects of calorie restriction (Gillespie et al., 2016). And, the fact that allantoin had been previously correlated to CR in preceding cMAP analysis on an outdated version, highlights artefactual differences in perturbagen gene profiles between the prototypical and existing cMAP builds.

With that said, gene expression meta-analysis of rilmenidine treated cell lines revealed several enriched biological pathways that are conserved in CR. A 2012 meta-analysis of dietary restriction gene expression studies, using the same methods as in this thesis, equally revealed upregulation of retinol metabolism as seen in response to rilmenidine treatment in this chapter (Plank et al., 2012). The authors referenced another paper showing that physiological retinol levels are altered during CR, and also suggesting retinol to have anti-inflammatory properties (Berggren Soderlund et al., 2003). Also, rilmenidine treatment consistently overexpressed genes associated with glucose starvation whilst consistently underexpressing cellular response to amino acid stimulus; both processes are definitely tied to CR response (Soultoukis and Partridge, 2016).

The rilmenidine meta-analysis produced a pattern of differential expression that was consistently enriched in both directions for downregulation of immune response and inflammation and also extra-cellular matrix function. When this was overlapped with the ageing gene signature, these processes remained enriched. Notably, the relationship between ageing and rilmenidine was unidirectional and only genes that were overexpressed in ageing could be rescued by rilmenidine – overexpression of genes by rilmenidine did not significantly intersect genes underexpressed in ageing.

Ageing has a complex phenotype likely reflected in its transcriptome, complicated by mutational and dysregulational epigenetic noise. This causes difficulty in differentiating between the targetable, detrimental effects which occur with age and the potentially beneficial responses to those changes enacted by cells to survive. Do maladaptive detrimental programmes become overexpressed in ageing? Indeed, global hypomethylation occurs with age that may give rise to pathological hyperfunctional overexpression but, it is likely that ageing interferes with the transcriptional machinery in multiple ways, increasing diversity and heterogeneities. There are numerous genetic pathways that become downregulated in ageing such as those encoding mitochondrial proteins and downregulation of the protein synthesis machinery (Frenk and Houseley, 2018).

This accumulating evidence warranted survival analysis in *C. elegans* specifically aimed at testing imidazoline receptor compounds as a preliminary investigation into their CRM properties. Indeed, both

moxonidine, rilmenidine and allantoin all extended lifespan proportionally to their binding affinities which suggested that imidazoline receptor agonism may play a part in the lifespan extension of these compounds rather than a broader sub-lethal hormetic response. Importantly, this prolongevity effect was equally prominent when worms were fed rilmenidine in late life. Furthermore, moving worms treated from day 1 onto fresh plates containing rilmenidine at day 12 did not additively affect lifespan compared to worms exposed to the drug only from day 12. This suggests that rilmenidine may influence the same ageing programme in early and late life. This offers significant translation potential for the treatment when considering the preference for treatments starting in late life that can shorten longitudinal clinical trials alongside reducing the ethical and compliance concerns of life-long administration. When considering rilmenidine as a potential CRM, this late-life benefit significantly bolsters its preferential candidacy given that both mice and rats initiated on CR in late-life demonstrate negligible effects or indeed reductions in longevity (Ingram and de Cabo, 2017). This compounds concerns that dietary restriction in the elderly would contribute to the development of sarcopenia and bone loss, and likely precipitate the genesis of frailty in vulnerable individuals (Batsis et al., 2015).

It could be argued that rilmenidine treatment was not started sufficiently late to qualify for a geriatric intervention. Treatment was started at day 12, 10 days prior to the mean age of mortality, which would translate to 15 months of age in C57BL/6J mice, 3 months premature of “old age” qualification (Fox, 2007). However, ageing rates between taxonomical rank is diverse and ageing biomarkers such as proteostatic collapse are evident by day 3 in *C. elegans* (Ben-Zvi et al., 2009). Moreover, by day 12 pharyngeal pumping, a routine measurement of animal health, has typically ceased alongside egg-laying (Collins et al., 2008). Furthermore, animals also exhibit dramatically altered metabolism, increased age-associated autofluorescence and autophagic malfunction: a key event in organismal ageing (Chapin et al., 2015; Collins et al., 2008; Rollins et al., 2017). Practically, the transfer of worms between plates when significantly frail would confound the fitness of the animals.

The ability for animals to survive is often dependent on their existential biome, which in the laboratory setting is dominated by the interaction with *E.coli* - their primary food source but also an infectious agent. Further work should confirm rilmenidine does not reduce the consumption of *E.coli* to mimic a completely upstream DR state and thus not meet Ingram’s criteria for a CRM. Secondly, despite worms grown in a pathogen free environment through the addition of nystatin and penicillin/streptomycin to all NGM plates as recommended, it is possible rilmenidine is still modulating the nematode’s innate immune response to prevent pathological infection (Lionaki and Tavernarakis, 2013; Stiernagle, 2006).

Future bioinformatic work should focus on delineating the expression changes induced by rilmenidine from ageing and senescence. Genes differentially expressed in senescence should be compared to rilmenidine to assess whether there is equal enrichment in comparison to ageing. Furthermore, given the utilisation of worms in this thesis as a model organism additional work comparing *C. elegans* specific transcriptional changes in ageing would be beneficial. Very recently, a paper has been published which developed a transcriptional age signature in *C. elegans* to probe the cMAP, but could also be compared to rilmenidine (Tarkhov et al., 2019).

4. Results: *F13E9.1* is an IRAS Ortholog Containing Similar Functional Domains and Potential Rilmenidine Binding Sites

4.1. Introduction

In order to assess the mechanisms by which rilmenidine may exert its prolongevity effect, we sought to identify a putative nematode receptor capable of binding rilmenidine and inducing bioactivity. At the time of writing, no imidazoline receptors have been characterised or indeed experimentally tested in *C. elegans.* Of course, clinically, rilmenidine is classified as a potent imidazoline type 1 receptor agonist. This is predicated by early radioligand-binding studies in bovine brain membranes showing high-affinity sites that recognized rilmenidine and other imidazoline adrenergic agents (Ernsberger et al., 1987). These imidazoline binding sites, when agonised in CHO cell lines, could induce specific cell-signalling responses such conversion of extracellular signal-regulated kinase 1 (ERK1), ERK2, and c-jun kinases to their active forms (Li et al., 2006). In 2000, a gene encoding an I1RA candidate protein, named imidazoline receptor antisera-selected protein (IRAS) was isolated (Piletz et al., 2000). The subsequent transfection of hIRAS cDNA into Chinese Hamster Ovary (CHO) and PC12 cells resulted in high-affinity I1-receptor like binding sites which induced greater ERK activation, when stimulated with rilmenidine than in transfected controls; this effect was repeated following overexpression of the hIRAS in HEK293 kidney cells (Piletz et al., 2003b). This relationship is confirmed by evidence that antisense inhibition of hIRAS in PC12 cells, reduced imidazoline binding affinity and moxonidine-induced ERK1/2 activation (Sun et al., 2007).

4.2. Results

4.2.1.*f13e9.1* is Orthologous to IRAS

It was logical to search for a potential IRAS homolog in *C. elegans*. Firstly, using a standard NCBI nucleotide megablast search with default parameters, the entire *C. elegans* genomic sequence was analysed for significant alignment homology. This returned no similar *C. elegans* sequences. Previously published work had hinted that the *C. elegans* genome may encode a putative IRAS homolog, however, this has never been explored (Piletz et al., 2000). Specifically, Alahari et al (2000), when initially characterising IRAS, noted that the N-terminal sequence of IRAS displayed strong homology to a *C. elegans* protein coded for at the gene locus *f13e9.1*. Additionally, two other papers have assumptively referred to *f13E9.1* as *NISCH* (the murine homolog of IRAS) in spite of any experimental evidence (Kinchen et al., 2008; Lu et al., 2011). Notwithstanding, using a direct approach, both the human IRAS and the *f13e9.1* sequence were blasted against each other – this also returned no significant results. Finally, the protein sequence alignment was considered. *F13E9.1* is a wholly uncharacterised 57.9kDa protein which has never been directly studied. The only seemingly available literature to date is at a genetic level in the form of a systematic RNAi screen which suggested the *C. elegans* protein may be involved in cell migration networks, although this was never validated in nematode models (Cram et al., 2006). Lastly, a microarray to measure transcriptional response to heat-stress implicated *f13e9.1* as a possible responsive element – again this was never followed up (Jovic et al., 2017).

A NCBI BLASTp query was performed with parameters set to search all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects but limited to records that include: *C. elegans* (taxid:6239) (See Figure 7) (Johnson et al., 2008). IRAS is known to have a number of transcript variants encoding 4 different isoforms (Piletz et al., 2003a). The full-length human IRAS (167kDa and 1504aa) is poorly expressed in all examined tissues, suggestive of a normally fragmented protein (Piletz et al., 2003a). We therefore queried the BLAST server with isoform 3, the 3rd longest isoform. This isoform is a c-terminally truncated isoform of 63kDa and 583aa which is identically coded to the full sequence except for a small region 511-583 AAs IMFVQEEALA...PEVQVVPGSG → NRVCTLLLVE...CLLGEDSQLL, which does not contain any functional domains. It is a similar length to the suggested IRAS homolog, *f13e9.1.* The BLASTp query returned the strongest similarity and top-hit alignment to the translated full length *f13e9.1* sequence (e-value 2e-80) with only 6% of residues not sharing physico-chemical similarity (See Supporting Figure 2) ; the full length IRAS sequence also returned significant similarity to *F13E9.1* (e-value 2e-80). Additionally, two other proteins, T05H4.3 and K10D2.8

were reported to have similarity (e-value < 1e-5); therefore, we cannot exclude the existence of additional, even less conserved, IRAS orthologs in *C. elegans*.

Secondary structure similarity between IRAS and *F13E9.1* was then assessed using the Protein Structure Prediction (PSIPRED) Server, which predicts secondary structure based on an artificial neural network tested against the DSSP algorithm (Buchan et al., 2013) (Figure 7). In the N-terminal, *F13E9.1* shared 9 helices with IRAS, located in identical residues, including a 20 amino acid region (88-108) of exactly equal identity. Moreover, beta-sheets were commonly observed with IRAS in all but 1 location within the *F13E9.1* 250aa N-terminus. In the C-terminus, *F13E9.1* shared large helical homology between aa438-452, and again between aa462-495. Within the entire *F13E9.1* sequence, all 12 beta-sheets were identically present within human IRAS isoform 3 (Figure 7).

To define whether such secondary structure similarity corresponded to functional domain conservation we analysed both protein sequences using the web application InterproScan 75.0 (Jones et al., 2014). *F13E9.1* revealed a PHOX domain, 5 leucine rich repeats and a coiled coil, all of which are conserved within isoform 3 human IRAS (Figure 8). Specifically, a PHOX binding domain was identified between amino acids 13-129. Likewise, in IRAS a PHOX binding domain differing in length by only 7 amino acids is present in the residues 11-121. Congruently, this domain shared 84% sequence identity to the *F13E9.1* PHOX domain, including 8 conserved areas of secondary structure. PHOX (PX) domains possess phosphoinositide-3-phosphate-binding capacity, likely enabling IRAS and, most likely, *F13E9.1*, to be anchored to the intracellular surface of the plasma membrane (Piletz et al., 2003b). Furthermore, a PHOX domain Is likely the necessary component that fulfils the sorting nexin function of IRAS, suggesting *F13E9.1* may potentially exhibit such properties (Lim and Hong, 2004; Reddig et al., 2005). The murine NISCH homolog lacks the N terminal PHOX domain and is thus considered a cytosolic interacting protein involved in cytoskeletal organization (Reddig et al., 2005).

Similarly, both human IRAS and *F13E9.1* encode 5 leucine rich repeats which commence at 286/288 aa and ending at 397 and 425 aa respectively (Figure 8). Of the amino acids which encode the leucine rich repeats only two amino acids differed in *F13E9.1* giving a 98% identity match between the two proteins. Leucine rich repeats are not necessary for the endosomal sorting nexin properties of IRAS, however it is likely they provide a versatile structural framework for the formation of protein-protein interactions between IRAS itself or between IRAS and its potential interacting partners such as integrin a5 (Lim and Hong, 2004).

The last functionally recognised domain from the InterPro analysis was a coiled coil. This domain is predicted to sit at residue 455-482 in *F13E9.1* (Figure 8). Likewise in the full length IRAS, a coiled coil is

present at residues 671-693 with a similar 27 amino acid length (e value 5e-04). The coiled coil is the predicted imidazoline binding site. For a ruthenium binding region is present in the coiled-coil domain of IRAS, and addition of this dye, blocks imidazoline binding (Sun et al., 2007). Piletz et al (2000) deemed the aa 623–700 to be the imidazoline binding site, which is where the coiled coil domain sits containing an acidic domain rich in glu/asp. Indeed, *F13E9.1* maintains a glutamic acid rich and acidic code in its coiled coil domain (See supporting figure 2). Thus, it is probable, *F13E9.1* may have imidazoline binding capacity. Additionally, IRAS homo-oligomerises via its coiled coil region and also this region is vital for its localization to endosomes (Lim and Hong, 2004). Of interest, murine Nischarin that lacks a Phox domain, retains a coiled-coil domain at aa 380–405 within the N terminus, enabling formation of vesicles. In total, *F13E9.1* has significant homology to nishcarin and likely also possesses functional similarity. This is highlighted by gene ontology analyses showing both IRAS and *F13E9.1* to have phosphatidylinositol binding (GO:0035091) which may facilitate membrane anchoring and participate in endosomal sorting and cell signalling, possibly involving leucine rich repeats.

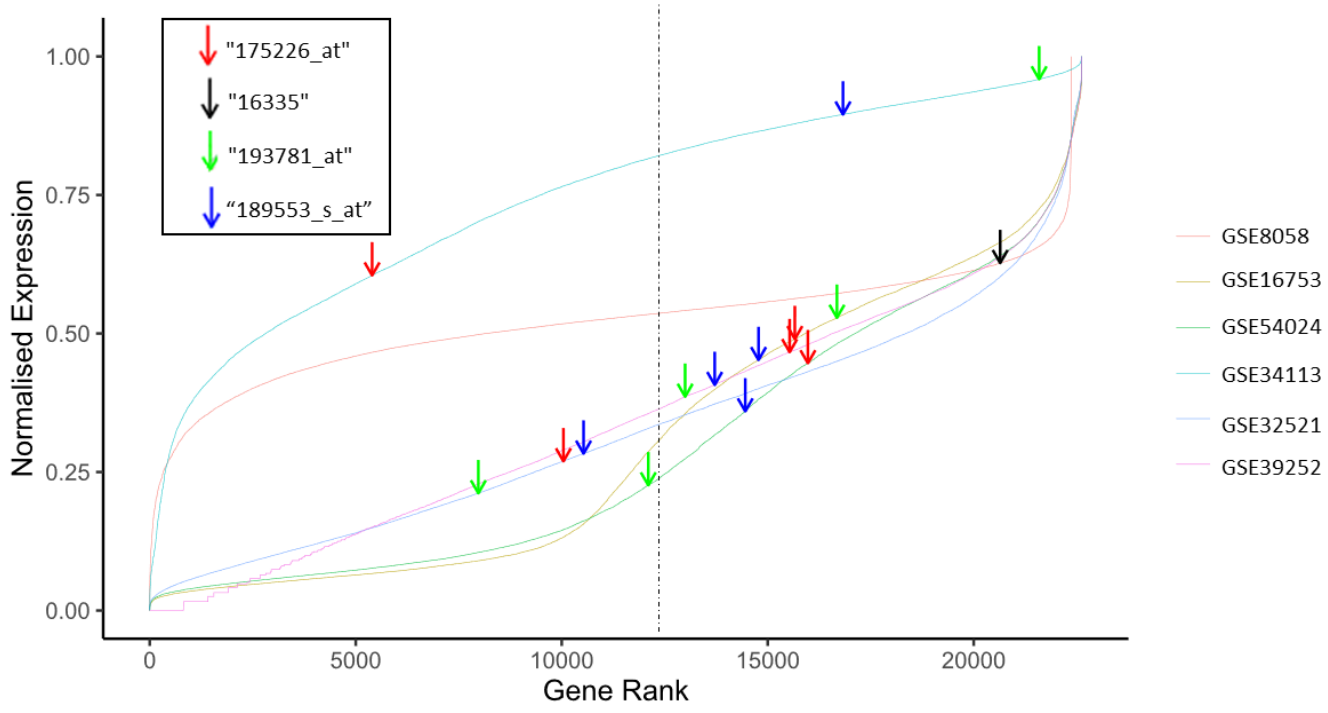


Figure 6. Native wild-type (WT) f13e9.1 gene expression analysed in 6 microarray datasets. *Publicly available GEO datasets were mined (GSE8058, GSE16753, GSE54024, GSE34113, GSE32521, GSE39252) Each array probe was ranked according to its normalised mean gene expression value across all control WT samples within a dataset. f13e9.1-specific probes are annotated by way of coloured arrow.*

To determine subcellular localisation and possible native expression pattern of *f13e9.1,* a transgenic reporter construct was developed (see chapter 2.5). The WRM0616C_F02 (*f13e9.1*) clone fosmid, expressing full length *f13e9.1* with a 3' end fused eGFP, driven by its native *f13e9.1* promoter was coinjected with an mCherry co-marker and integrated onto an WT background to generate 2 similar lines of PHX116 and PHX117 [*f13e9.1*::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry]. eGFP expression in day-1 adult transgenic animals was analysed under confocal fluorescent microscopy, however, endogenous expression levels were untraceable most likely being either under the detection limit or hidden by a super-thin bandpass green filter (520 +/- 20nm) required to overcome inherent autofluorescence within worms (See supporting figure 4). Marker co-injection with myo-2::mCherry produced predictable expression profiles (see supporting figure 4). Furthermore, there was also no detectable expression of *F13E9.1*:eGFP in transgenic non-integrated animals carrying the extrachromosomal array, ruling out chromosomal silencing as a factor. Undetectable GFP fluorescence is a well-documented problem in the worm community (Sarov et al., 2012; Teuscher and Ewald, 2018). FLAG immunoblotting was also carried out in rescue lines PHX945 and PHX946 (See supporting figure 3). No bands were detected for eGFP or x3FLAG in PHX946, and minimally visible bands were observed in PHX945. Therefore, to establish basal *f13e9.1* expression in WT worms, microarray expression probes across 6 publicly available datasets were plotted by rank against their respective whole transcriptome (Figure 6). On the Affymetrix *C. elegans* genome arrays (used in all but GSE8058), 3 probes correspond to *f13e9.1;* and on the University of Washington custom array (used in GSE8058), 1 probe corresponds to *f13e9.1*. Probe expression had significant intra and inter-dataset variability, however, 12 of 16 probes sat within the upper half of the expression ranking. Such native expression would ordinarily be expectantly observed under fluorescent microscopy (Sarov et al., 2012)

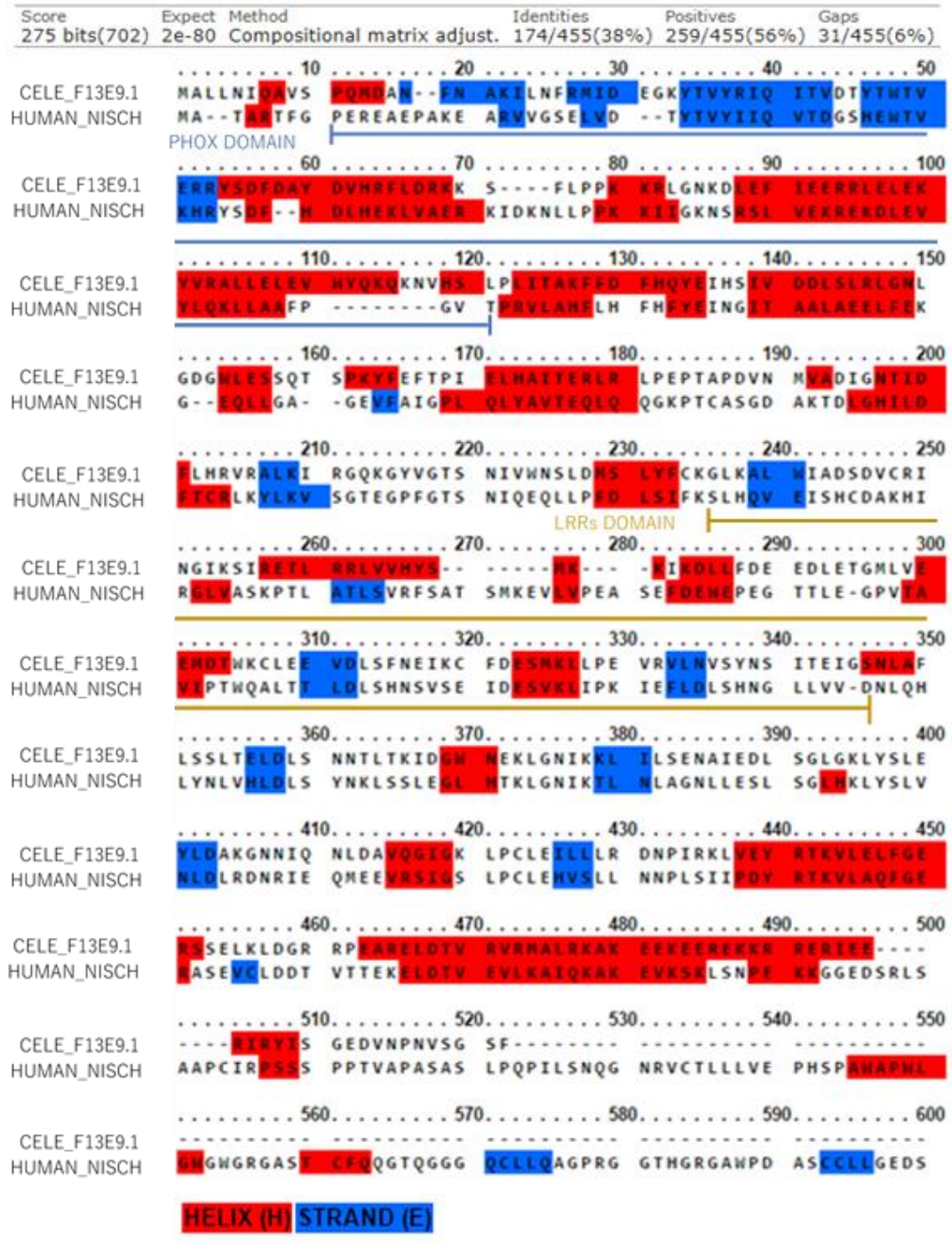


Figure 7. Modified BLASTp Alignment of C. elegans F13E9.1 protein sequence (CELE_F13E9.1 – UniProtKB-K8F807) and truncated human* NISCHARIN *isoform 3 (HUMAN_NISCH – UniProtKB-Q9Y2I1-3) using BLASTp with default parameters. *Sequence similarity measured by e-value >1e – 5. Identity = number of residues encoded by the same amino acid; positives corresponds to the number of amino acids that are either identical between F13E9.1 and the IRAS sequence or have similar chemical properties. Residues that do not correspond are represented by a -. Conserved secondary structure elements were analysed using PSIPRED and highlighted in colour; predicted alpha helices are highlighted in red and extended confirmation (beta-strand) are highlighted in blue. Functional protein motifs of human NISCH isoform 3 (PHOX domain and leucine rich repeats-LRRS) are underlined as defined by InterPro have been inserted at respective residue commencing and finish.*

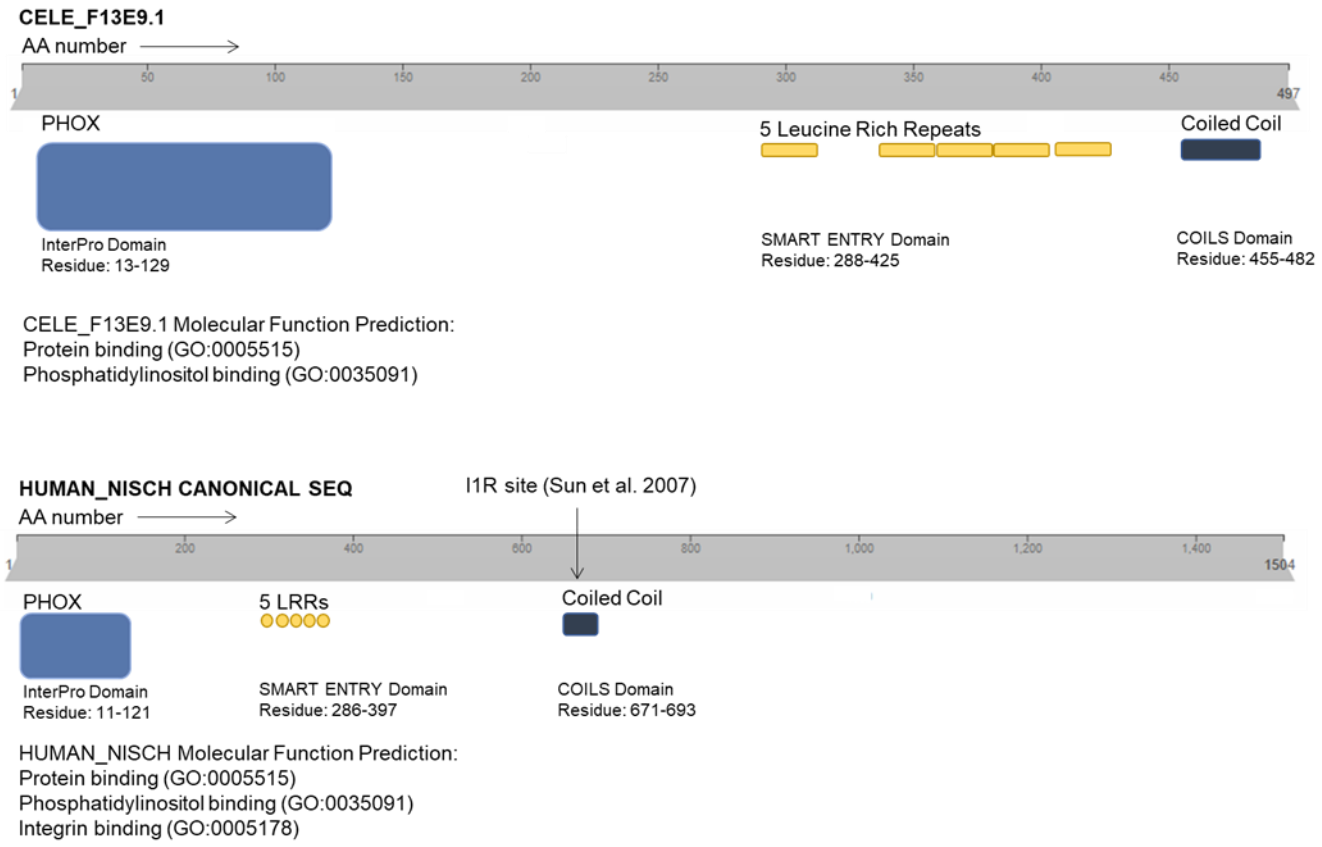


Figure 8. Schematic representation of InterPro domain architecture for the C. elegans F13E9.1 protein sequence and corresponding domains for the canonical full-length human* Nischarin *protein sequence (UniProtKB-Q9Y2I1-1). *Human* Nischarin *and F13E9.1 encode, at proportionally similar residues, three key protein domains: PHOX binding domain, 5 leucine rich repeats (LRRs) and a coiled coil which is also the location of an imidazoline binding site in Human* Nischarin *(hIRAS) (Sun et al., 2007). Interpro also embeds a gene ontology application (InterPro2GO) with 34,213 GO terms mapped to InterPro entries. GO terms relevant to either protein are listed.*

4.2.2. F13E9.1 is an Imidazoline Binding Protein That Mediates Rilmenidine-Induced Bioactivity

To further examine the role of F13E9.1 as a functional homolog of IRAS that could mediate rilmenidine cell signalling, I designed a knockout mutant *f13e9.1 -/-* strain (PHX893) which was generated by SUNYBiotech using the CRISPR/Cas9 system (**Figure 9**) (See Chapter 2.5 for methodological detail). *f13e9.1* is an 8 exon gene on the 4th chromosome: exon 2 and a large intronic site thereafter is overlapped at the 5' end by the gene *f13e9.11*. Thus a 873bp homozygous deletion was initiated at the 3rd exon and replaced by a stop codon to prevent transcription of all subsequent exons. Admittedly, this resulted in a truncated *f13e9.1* gene product which still contained a PHOX domain that may permit IRAS-associated functionality such as endosomal targeting (**Figure 9**). However, 3rd exon deletion and termination abrogates the key signalling domains of leucine rich repeats and the crucial imidazoline binding site in the coiled coil domain.

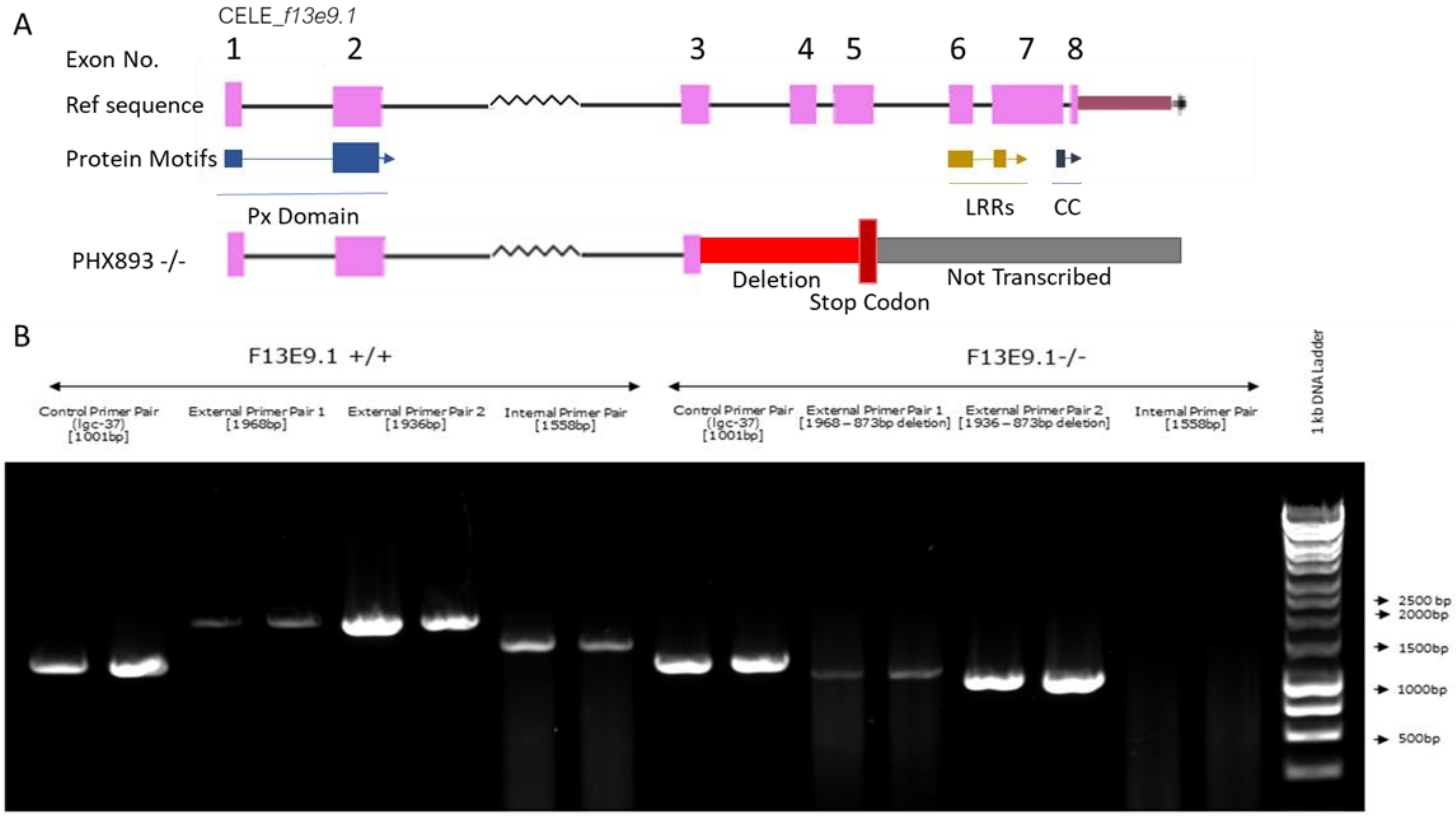


Figure 9. Representation and genotyping of Crispr/CAS9 deletion of the C-terminus f13e9.1. *A) schematic diagram illustrating WT f13e9.1 exons alongside translated protein motifs and the deleted regions in the homozygous mutant PHX893 (f13e9.1-/-). LRRs = leucine rich repeats (PFAM:PF13855); CC = coiled coil domain (ncoils) as defined by wormbase Jbrowse protein motif tracks B) End point single worm PCR genotyping of WT and PHX893 strain following x4 outcrossing. Using oligonucleotides flanking the deletion site (external primer pairs) generated either a 1968bp or a 1936bp amplicon depending on oligonucleotide pair in WT worms or a correspondingly 873bp reduced amplicon size in the genomic DNA of homozygous f13e9.1 -/- (PHX893) mutants (see chapter 2.5). Likewise, an oligonucleotide pair targeted to sequences within the Crispr deletion site produced a 1558bp amplicon in WT worms but no amplicon in homozygous f13e9.1 -/- (PHX893) mutants. Control amplifications were conducted on the lgc-37 gene coded on 3rd chromosome.*

Of course, rilmenidine has never been studied in *C. elegans*. Thus it is essential to establish that rilmenidine is indeed bioactive in *C. elegans* and that lifespan extension by rilmenidine is not merely a hormetic response, instead, eliciting key signalling pathways that are conserved in previously established cell models. Experiments to determine signalling in response to imidazoline agents have highlighted a number of pathways. One particularly well characterised pathway following interaction of rilmenidine with I1-imidazoline receptor leads to activation of PC-PLC and subsequent accumulation of the second messenger diacylglyceride (DAG) from phosphatidylcholine, and the release of phosphocholine. This causes downstream activation of MAPK/ERK1/2, with phosphorylation of ERK1/2 being a common read-out for I1R activation by an I1R agonist (Edwards et al., 2008; Piletz et al., 2003b; Sun et al., 2007). *C. elegans* contains only one ERK gene (*mpk-1*), rendering it a more facile system to measure compared to the six isoforms of PLC. In order to assess if rilmenidine was bioactive in *C. elegans* and that such activity was likely specific to imidazoline receptor activation, the downstream activation of ERK (mpk-1) was measured, by way of pERK (mpk-1) to α-tubulin immunoreactivity, in response to 24h treatment with rilmenidine at varying concentration (200µM, 300µM and 40000µM) to establish a working concentration (Figure 10). Rilmenidine increased ERK phosphorylation most at the concentration of 200µM. Furthermore, 400µM produced an increase in ERK phosphorylation and 300µM did not significantly activate ERK (Figure 10). Researchers using *in-vitro* animal cell lines have also reported a comparable biphasic dose–response relationship.

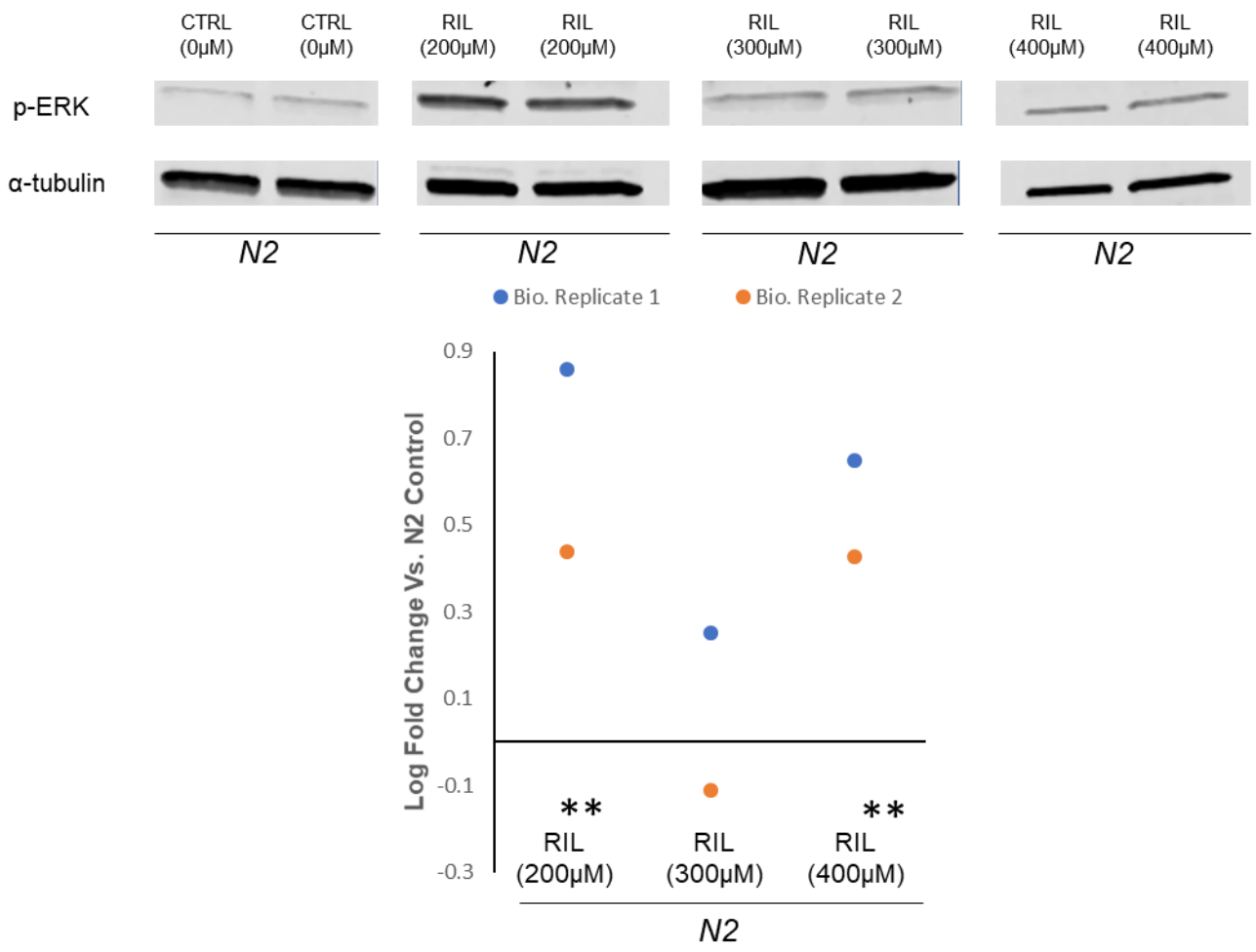


Figure 10. Effect of different concentrations on rilmenidine-induced MPK-1 phosphorylation as measured by Western Blotting. *Phosphorylation of MPK-1 was assessed in wild-type (WT) in response to 24h pharmacological intervention, and subsequent western blotting using the primary antibody Phospho-ERK (Cell Signalling Technology, Catalogue number: 9101) followed by the loading control, α-tubulin. All experiments were run in biological triplicate on separate western blots using different worm populations. Data is semi-quantified as log-fold densiometric ratio change relative to a-tubulin. Data is then expressed as log fold change compared to WT control (1% DMSO). Coefficient of variation (%CV), defined as the percent standard deviation: mean ratio; significant difference in groups if % fold change is x1.5* greater or x2** than % CV. WT WT worms were treated with 3 different concentrations of rilmenidine for 24h. Rilmenidine at 200μM and 400μM significantly increased MPK-1 phosphorylation **(%CV vs. FC = >2).*

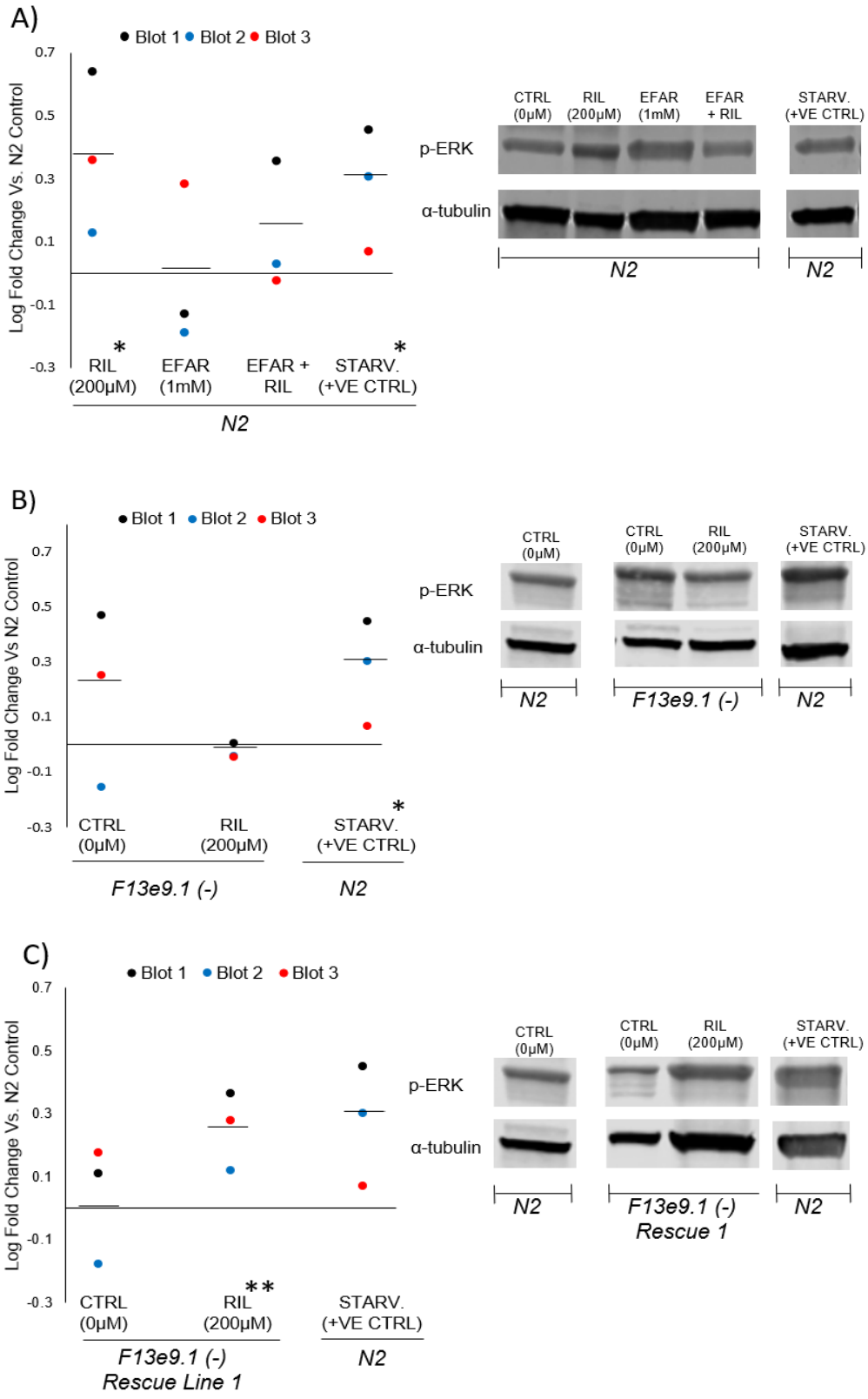
Rilmenidine increases lifespan most significantly at 200µM and also increases ERK phosphorylation in *C. elegans* most prominently at that concentration, hence it was necessary to elucidate the role *f13e9.1* may have in the transduction of a phosphorylative signal to ERK at life-extending concentrations.

Again, when repeated in biological triplicate, 24h 200µM rilmenidine significantly increased ERK (MPK-1) phosphorylation in comparison to 1% DMSO vehicle-treated WT worms by 103% mean fold change (%CV vs. FC = >1.5) (Figure 11A). To establish whether this increase in activity was mediated via rilmenidine binding to a I_1-imidazoline receptor, worms were co-incubated with rilmenidine (200µM) and efaroxan (2mM), an established selective I_1-imidazoline receptor antagonist. The efaroxan concentration used in worms was scaled to previously established ratios used *in-vitro* and *in-vivo* such that efaroxan concentration was x10 higher than the optimal rilmenidine dose (Dahmani et al., 2008; El-Mas et al., 2009; Li et al., 2006). 24h efaroxan treatment alone did not significantly affect baseline ERK activity compared to 1% DMSO vehicle-treated WT worms; however, efaroxan significantly attenuated ERK activation by 200µM rilmenidine (Efar+Ril %CV vs. FC =<1.5) (Figure 11A). This suggests rilmenidine treatment most likely interacts with an endogenous imidazoline binding site in *C. elegans* to elicit increases in ERK activity.

To further investigate whether the effect of rilmenidine on ERK stimulation in *C. elegans* was mediated by the putative IRAS ortholog *F13E9.1*, we also measured ERK phosphorylation in the mutant PHX893 *(f13e9.1-/-)* strain (Figure 11B). Importantly, *f13e9.1* did not interfere with the functionality of ERK: baseline pERK activity was not significantly affected by deletion of *f13e9.1* (%CV vs. FC = <1.5). However, critically, 24h treatment with 200µM of rilmenidine repeatedly failed to increase ERK activity in mutant PHX893 *(f13e9.1-/-)* worms, implying the necessity of *f13e9.1* for rilmenidine induced ERK activation (Figure 11B).

It is conceivable that *f13e9.1* deletion might have led to off-site mutations in other genes that could affect rilmenidine bioactivity. Thus, to confirm the requirement of *f13e9.1* to mediate rilmenidine induced ERK phosphorylation, PHX893 *(f13e9.1-/-)* worms were backcrossed with PHX116 and PHX117 [*f13e9.1*::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry] strains to generate 2 new strains of full length gene rescues named PHX945 and PHX946 [*f13e9.1*::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry]. Whilst the co-injection marker was stably observable in both strains and genotype was confirmed by both sequencing and PCR, there was substantial difficulty in detecting a protein-level expression by western blot at ~100kD (*F13E9.1 is a 57kD protein* + the tag part including the eGFP, FLAG and the linker peptides accounts for a molecular mass of 35 kD = 92kD) (See supporting figure 3). This is likely attributed to the variable basal expression of *f13e9.1* as documented in microarray analyses. Additionally, IRAS the

proposed human ortholog has numerous splice variants; all but the full-length transcript, which is the least expressed isoform, cleave the c-terminal end. Both rescue lines did not interfere with ERK functionality as measured by insignificant changes to basal pERK activity (%CV vs. FC = <1.5) (Figure 11C,D). Furthermore, reintegration of a full length *f13e9.1* sequence in both PHX945 and PHX946 lines significantly restored the propensity of rilmenidine 200μM to increase ERK phosphorylation (%CV vs. FC = >2)(Figure 11C,D). Reintegration of fosmids often leads to increased expressible copy number in rescue lines. Hence it is possible, increased imidazoline binding sites may be available in PHX945 and PHX946 lines, such that it might increase the potency of rilmenidine on ERK phosphorylation. However, neither PHX945 nor PHX946 strains exhibited increased ERK phosphorylation in response to rilmenidine than the WT strain. This suggests PHX945 and PHX946 are suited to further rescue study following demonstration of rilmenidine-induced ERK signalling rescue capacity.


A)
Blot 1 Blot 2 Blot 3
Log Fold Change Vs. N2 Control
0.7
0.5
0.3
0.1
-0.1
-0.3
*
*
RIL (200μM)
EFAR (1mM)
EFAR + RIL
STARV. (+VE CTRL)
N2
CTRL (0μM)
RIL (200μM)
EFAR (1mM)
EFAR + RIL
STARV. (+VE CTRL)
p-ERK
α-tubulin
N2
N2
B)
Blot 1 Blot 2 Blot 3
Log Fold Change Vs N2 Control
0.7
0.5
0.3
0.1
-0.1
-0.3
*
CTRL (0μM)
RIL (200μM)
STARV. (+VE CTRL)
F13e9.1 (-)
N2
CTRL (0μM)
CTRL (0μM)
RIL (200μM)
STARV. (+VE CTRL)
p-ERK
α-tubulin
N2
F13e9.1 (-)
N2
C)
Blot 1 Blot 2 Blot 3
Log Fold Change Vs. N2 Control
0.7
0.5
0.3
0.1
-0.1
-0.3
**
CTRL (0μM)
RIL (200μM)
STARV. (+VE CTRL)
F13e9.1 (-) Rescue Line 1
N2
CTRL (0μM)
CTRL (0μM)
RIL (200μM)
STARV. (+VE CTRL)
p-ERK
α-tubulin
N2
F13e9.1 (-) Rescue 1
N2

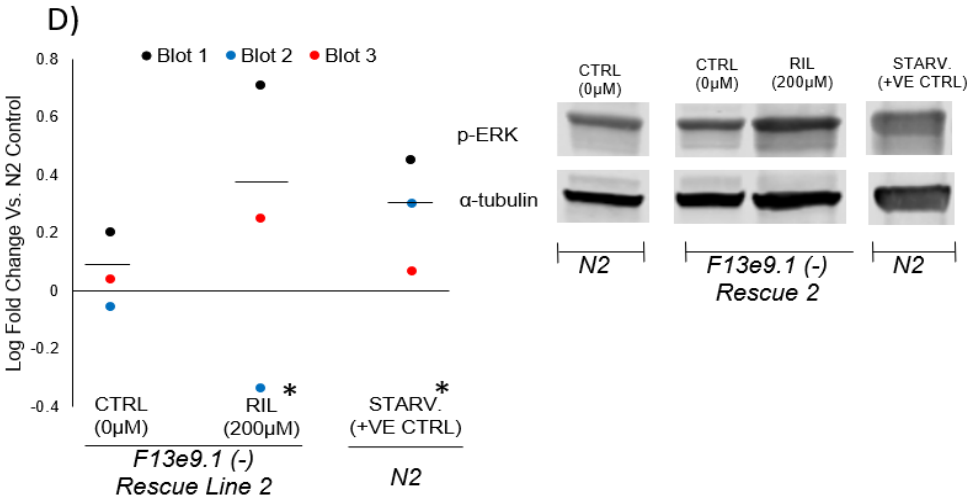


Figure 11. Effect of different genetic backgrounds on rilmenidine-induced MPK-1 phosphorylation as measured by Western Blotting. *Phosphorylation of MPK-1 was assessed in wild-type (WT,WT), homozygous f13e9.1 (PHX893) mutants, or PHX945 and PHX946 both carrying [f13e9.1::EGFP::3xFLAG, unc-119(+)+myo-2::mCherry] in response to 24h pharmacological intervention, and subsequent western blotting using the primary antibody Phospho-ERK (Cell Signalling Technology, Catalogue number: 9101) followed by the loading control, α-tubulin. All experiments were run in biological triplicate on separate western blots using different worm populations. Data is semi-quantified as log-fold densiometric ratio change relative to a-tubulin. Data is then expressed as log fold change compared to WT control (1% DMSO). ---- represents mean log-fold change between 3 biological replicates. Coefficient of variation (%CV), defined as the percent standard deviation: mean ratio; significant difference in groups if % fold change is x1.5* greater or x2** than % CV. A) WT WT worms were treated with either rilmenidine (200µM), efaroxan (2mM) or in combination for 24h. Rilmenidine significantly increased MPK-1 phosphorylation *(%CV vs. FC = >1.5), however neither efaroxan alone nor rilmenidine and efaroxan in combination significantly increased MPK-1 phosphorylation n/s(%CV vs. FC = <1.5). B) f13e9.1 (syb767) were treated with either 200 µM or a 1% DMSO vehicle for 24h. Rilmenidine failed to reproducibly increase phosphorylation of MPK-1 n/s(%CV vs. FC = <1.5). C and D) PHX945 and PHX946 worms were treated with either 200 µM or a 1% DMSO vehicle for 24h. Both strains significantly increased MPK-1 phosphorylation **(%CV vs. FC = >2 in PHX945) and (%CV vs. FC =>1.5 in PHX946).*

4.2.3.*f13e9.1* Mutations Affect Body Size but not Development

Three separate Nischarin transgenic mouse models have been reported, all demonstrating that murine Nischarin mutants are smaller than their wild-type counter parts. Firstly, in 2013, recessive mutants with deletion of the 4th exon with subsequent stop codon insertion were reported (Zhang et al., 2013). These mice were born smaller than WT littermates and at adulthood (12 weeks) remained 22% lighter than WT. Secondly, separate researchers identified a T to C substitution mutation (edsn) within exon 14 of Nisch, which was predicted to cause a Leu972Pro missense change in NISCH protein (Crompton et al., 2017). Importantly this mutation occurred further to the 5' end than key protein motifs coding areas such the PX domain, LRRs and the coiled coil. Nonethess, both male and female *Nisch*$^{edsn/edsn}$ mice were 35% smaller than WT littermates at 20wks of age. Finally, Dong et al. generated Nischarin-mutant mice with deletions in exons 7–10 (encoding 224–395 amino acids containing the five LRR domain) (Dong et al., 2017). Accordingly, these mice also exhibited dramatic growth defects showing a similar ~22% reduction in body weight, which was partly rescued in heterogenous mice.

Comparably, *f13e9.*1 -/- mutants have a deletion and termination that spans predicted LRRs and also coiled coil domain. To further clarify whether *f13e9.1* was a likely ortholog to *NISCH,* body phenotype was measured in *f13e9.1 -/-* worms in comparison to WT. Photographed day 1 adult worms were measured for length and width (see chapter 2.10.2). Day 1 adult WT worms had a mean length of 881µm (±16.95 SEM) Figure 12A. The microscope available had a minimum objective of x10, causing a bias selection to potentially smaller WT worms which could be accommodated within the field of view. As such, WT worms were moderately shorter than those routinely reported in literature (Li et al., 2018; Mörck and Pilon, 2006). Day 1 adult *f13e9.1* mutants were 5.44% shorter that WT worms (mean length at day 1: *WT* 881 ± 16.95 µm, n = 18; *f13e9.1-/-*, 833 ± 17.75 µm, n = 13; T-test: $p<0.05$) (Figure 12C). Similarly, day 1 adult *f13e9.1* worms were 5.3% thinner than WT worm counterparts when measured from posterior vulval peak to the corresponding outer edge of the intestinal cuticle (*WT* 71.28 ± 2.26 µm, n = 18; *f13e9.1-/-*, 67.32 ± 2.10 µm, n = 13; T-test: $p<0.05$) (Figure 12B). Of note, WT width is 15% shorter than equally devised measurements in WT worms published elsewhere (Mörck and Pilon, 2006). In total, day 1 adult *f13e9.1* mutants had an estimated total body area 12.06% smaller ($p<0.05$) than WT worm counterparts (*f13e9.1*=56.12mm ± 2.10 S.E.M vs WT 63.82mm ± 2.14 S.E.M; T-test: $p<0.05$).

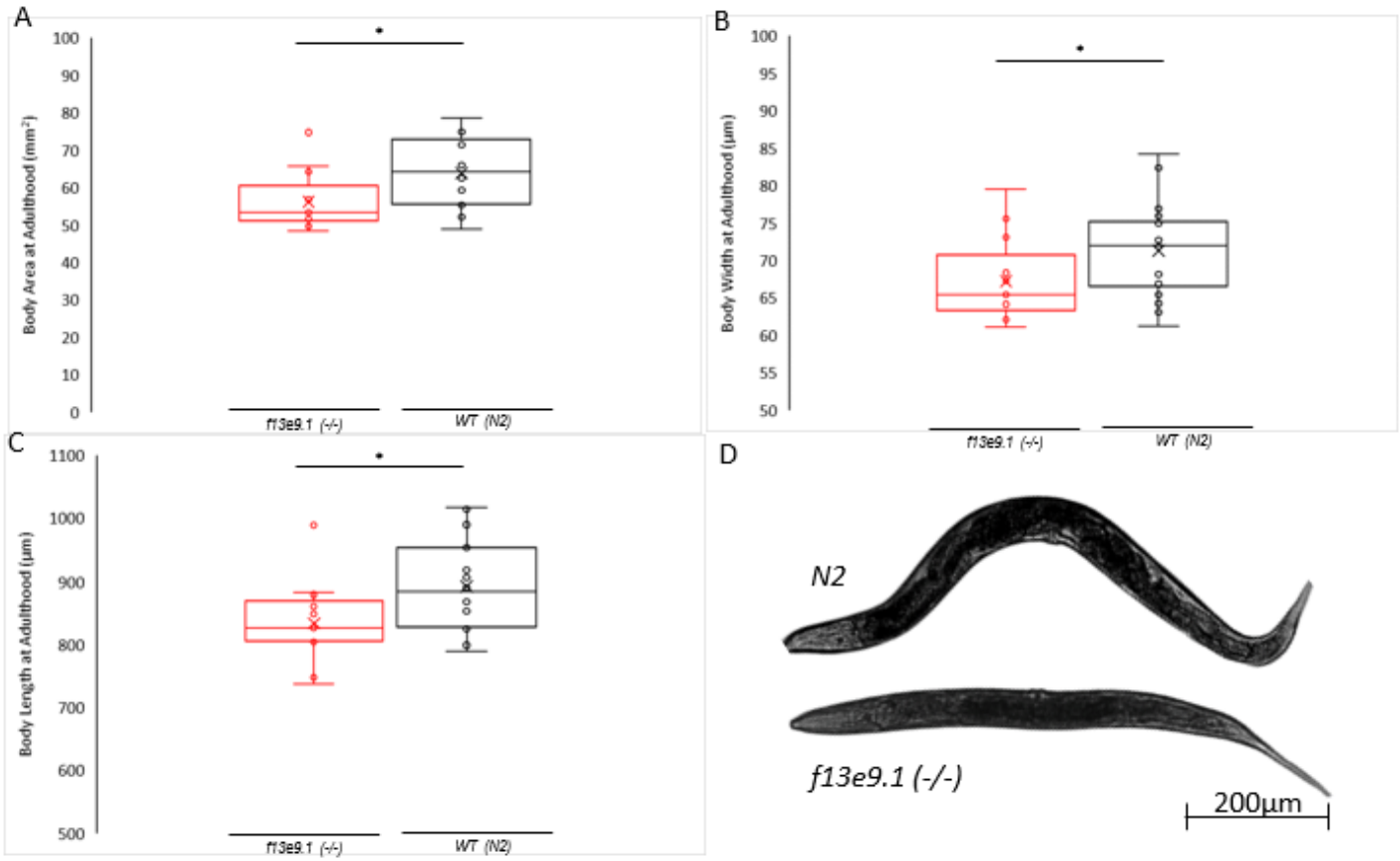


Figure 12. f13e9.1 mutants exhibit reductions in body size at adulthood. *Day 1 f13e9.1 and WT adult animals were bleach synchronised and grown on NGM plates with adequate live E.coli OP50 until day 1 adulthood. Images of animals were captured in brightfield at x10 objective on a Zeiss Axio Observer following paralysis in 20mM tetramisole. Body length and width was measured along the animal's respective axes using NIH image J software. A) Body area quantification for adult WT (n=18) and f13e9.1 (n=13) worms randomly selected from 3 separate trials. Body area defined as widthxlength at day 1 adulthood. The body area f13e9.1 mutants was significantly reduced. 2-way t-test; asterisk (*) indicate p<0.05. B+C) Morphologic data for WT (N=18), f13e9.1 (N=13) animals randomly pooled from 3 separate trials. Length was measured from the head to the region of the tail where the diameter reaches 10 µm. The length of f13e9.1 mutants was significantly reduced Width was measured just behind the vulva region and was reduced in f13e9.1 worms (2-way t-test; asterisk (*) indicate p<0.05.) D) Representative pictures of WT and f13e9.1 animals captured in brightfield at x10 objective on a Zeiss Axio Observer following paralysis in 20mM tetramisole. Animal heads are left. Scale bar: 200 µm.*

Mutant Nischarin mice have previously exhibited comparatively lighter body weights and size consistently from embryos, through parturition and adulthood. Therefore, smaller body size was attributed to developmental abnormalities rather than delays in rate of development.

To clarify that adult size defects in *f13e9.1 -/-* were not a cross-sectional artefact of slowed development, *f13e9.1 -/-* worms were compared to WT animals to establish rate of development to adulthood. It is sensible to decouple measurement of development from metrics of body length, which may lead to confounding results. As such, categorical staging of vulval evolution and outgrowth, as advanced by Ludewig et al. (2017), was used to assess rate of development in worms. Cross-sectionally analysed *F13e9.1-/-* worms from a pooled analysis, developed at a similar rate to WT worms and both strains had reached full vulval development and post L4 molt stage within 48h of bleach synchronised eggs being exposed to NGM seeded with OP50 (Figure 13). Neither strains exhibited any reproductive development abnormalities and staging speed was similar to that recorded in literature (Ludewig et al., 2017).

The well-respected hyper-function theory of ageing suggests that developmental programmes essential for timely growth and maturation run on into adulthood and fundamentally drive ageing through hypertrophy-associated pathologies (Blagosklonny, 2006, 2013). Furthermore, the antagonistic pleotropic supplement to this infers that perturbations that increase reproductive potential early in life increase risk of disease and mortality later in life (Kuningas et al., 2011). de Magalhães, (2012) elegantly refers to this as the "short-sighted watchmaker". Indeed, conversely improved longevity is routinely correlated to delayed fertility: Long-lived animals or those that are subjected to dietary restriction tend to display slow development. Specifically, down regulation of the insulin/IGF signalling in *C. elegans* as observed in DR, delays larval development and increases adult lifespan. Thus, interventions which increase lifespan through the supposed attenuation of "hyperfunctional" pathologies in ageing may be doing so through the down regulation of biosynthetic and proliferative processes that are important during development but detrimental during adulthood. It is logical that calorie restriction mimetics may intervene with such processes and manifest in delayed larval development. However, the exposure of rilmenidine, at a prolongevity dose (200μM) to WT worms after exit from L2 did not significantly affect reproductive development and animals progressed to adulthood at a normal rate (Figure 13).

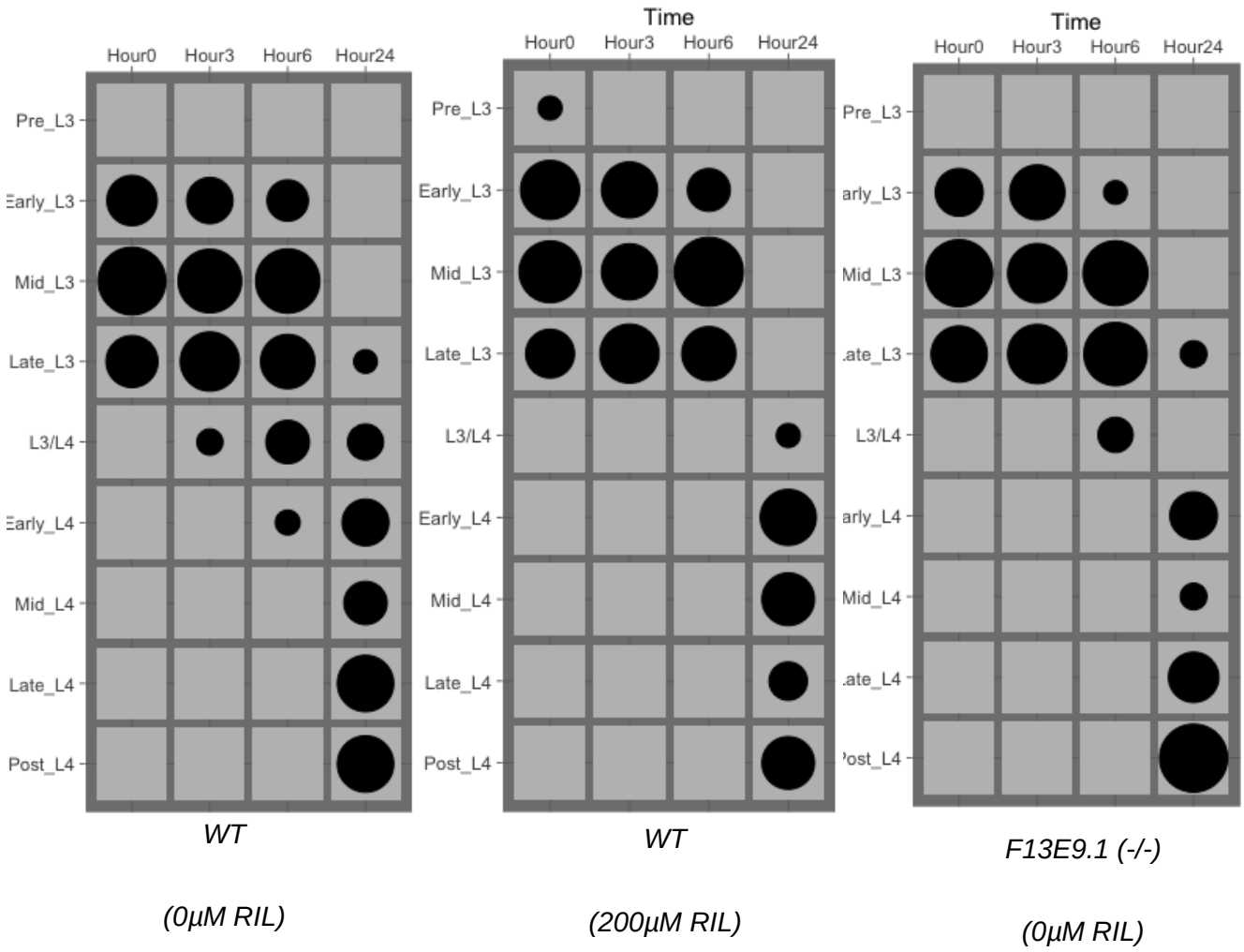


Figure 13. F13e9.1 -/- mutants and rilmenidine-fed WT worms do not exhibit delayed vulval development from the L3 stage. *Animals were bleached and resultant eggs were time-synchronised in M9 buffer overnight. Arrested L1s were added to OP50 E.coli seeded NGM plates at 20°C for 24 hours. From then "hour 0" commenced, and all groups were maintained at 20°C. In the chart, developmental stages are listed on the Y-axis and were determined by the extent of vulval development. The no. of hours after a 24h exposure to food from L1 is plotted on the x-axis. The areas of the circles in the chart reflect the percentage of the population at each stage of development; n≥30 from 3 independent pooled trials for each time point. Statistical difference established by way of 2-way anova wherein a mean development score could be ascertained through pre L3 corresponding to a score of 0, Early L3 is 1 and Mid L3 a 3 etc. No groups displayed any significant difference in development rate.*

4.3. Discussion

In this chapter a bioinformatic approach was taken to highlight a putative *C. elegans* orthlog to the human IRAS which could be phenotypically characterised to assess its candidacy as a potential imidazoline receptor that mediates rilmenidine bioactivity. Comparative protein sequence analyses showed that the predicted *f13e9.1* sequence and IRAS share a similar overall structural organization with conserved PHOX domains, 5 contiguous leucine rich repeats, and a coiled coil domain that all occurred sequentially in both species. Previous research suggests the a type 1 imidazoline receptor is present in an acidic residues rich in glu/asp within the coiled coil domain of IRAS (Piletz et al., 2000; Sun et al., 2007). Accordingly, *F13E9.1* protein charge analysis revealed a series of acidic residues within the coiled coil domain reflective of a proposed imidazoline binding site.

Initial comparative nucleotide sequence analysis revealed no significant alignment between IRAS and any *C. elegans* genes, however significant protein sequence alignment was noted between IRAS and *F13E9.1*, as well as two other uncharacterised proteins: T05H4.3 and K10D2.8 both are which are poorly annotated and lack characterisation.

When comparing phylogenetically distant species such as humans and *C. elegans* nucleotide alignment becomes insensitive in identifying homology. First, DNA-DNA comparisons are largely based on straightforward text matching and thus evolutionary mutations in small nucleotides sequences make alignments rarely able to detect homology after more than 200–400 million years of divergence (Pearson, 2013). Furthermore, long intronic sequences common in humans can easily "throw-off" alignment (Koonin et al., 2003). Contrariwise, the degeneracy of the genetic code, makes protein sequence alignment much better suited for homology searching. There is far less likelihood of different amino acid substitutions occurring during evolution in comparison to single base changes meaning protein:protein alignments routinely detect homology in sequences that last shared a common ancestor more than 2.5 billion years ago (e.g. humans to *C. elegans*) (Pearson, 2013). As such protein:protein alignment showed *F13E9.1* to be significant to IRAS including three keys domain regions.

The PHOX domain occurs within the first 20 amino acids of both IRAS and *F13E9.1*. In humans, 49 genes encode PHOX domains, of which the majority are sorting nexins (Chandra et al., 2019). PHOX domains have diverse functions including cell signalling (e.g., PLD1 and 2, PIKC2α, β, and γ), vesicle trafficking (e.g., SNXs 1, 2, 3, 5, and 6), protein scaffolding (e.g., SH3PXD2A and B), and cytoskeletal transport (SNX23/KIF16B) (Chandra et al., 2019). However, perhaps their most well characterised interaction involves its engagement with anchoring phosphoinositide lipids organelle membranes such

as endosomes (Viaud et al., 2016). The subsequent localisation of PHOX-domain proteins to cell membranes facilitates critical pathways in vesicular trafficking and cell signalling within the secretory and endocytic system (Chandra and Collins, 2018). The PHOX domain in *IRAS,* has been demonstrated to target to sorting/recycling endosomes but must require co-interaction with the coiled coil to oligomerise and execute the function (Lim and Hong, 2004). This suggests that *f13e9.1* likely localises to endosomal membranes. *F13e9.1::eGFP* expression could not be localised in *C. elegans.* Gene expression data suggests wildly variable expression patterns for *f13e9.1* which could preclude observation. However, a number of datasets recorded extremely high expression levels of *f13e9.1* probes but the exact binding site of these probes was not elucidated. *IRAS* undergoes a number of different splicing events, all of which result in removal of the 3' end, and the full length transcript is rarely translated. Thus, the highly transcribed "189553_s_at" and "175226_at" probes may correspond to binding at the 5' end of *f13e9.1* and the lowly transcribed "193781_at" probe may represent the frequently spliced 3' end. Secondly, the fosmid integrated to generate the strains were tagged at the C terminus with a fluorescent and affinity cassette consisting of the Ty1 peptide, eGFP and 3xFLAG. As there are no known splice sites for *f13e9.1* it may be likely these reporters were spliced and degraded prohibiting *in-vivo* localisation of cleaved and functional *F13E9.1*. Lastly, integration into silenced areas of the chromosome is unlikely as the extrachromosomal fosmid strain also demonstrated no measurable eGFP fluorescence.

Little mechanistic and functional information can be derived from PX interactions as the vast majority of IRAS study has been done in murine cell-lines which lack the N-terminus PX domain. However, STRING DB analysis the predicted functions partners of *f13e9.1* to be sorting nexins (Szklarczyk et al., 2015). Nonetheless, the PX domain of IRAS has been demonstrated to target the vesicle formation of Mu opioid receptor (MOR) to ensure it recycling back to the membrane. Agonism of I1RAs has helped reduce opioid dependence and IRAS knockouts accelerate dependence (Li et al., 2016, 2009).

Five contiguous leucine rich repeats have been functionally characterised in IRAS as well, and importantly *F13E9.1* demonstrates a similar pattern of LRR expression. More than 60,000 proteins from viruses to eukaryotes contain LRRs, and are known to have diverse and broad functions through the enablement of protein:protein interactions. In 2017, a mutant mouse model was developed with targeted deletion of the LRR region (Dong et al., 2017). This model demonstrated interaction of IRAS LRRs with AMPK and importantly, LOF in LRRs resulted in defective Nischarin–AMPK interaction leading to increased AMPK activity. This implied that WT IRAS inhibited AMPK normally. As a result, mutant mice showed increased AMPK phosphorylation and decreased mTOR activity and enhanced sensitivity to

AMPK activators. This presents critical associative evidence between IRAS, *F13E9.1* and calorie restriction.

The coiled coil domain of IRAS appears conserved in *F13E9.1*. It is thought that the coiled coil co-functions with the PHOX domain to permit oligomerised binding to endosomes and interaction with phosphatidylinositol 3-phosphate (PI3P) in PI3P-enriched endosomes (Lim and Hong, 2004). As discussed, murine IRAS does not possess a PHOX domain but the coiled-coil region in the amino terminus of IRAS may be sufficient for regulation of translocation in mouse cells (Ding et al., 2008). A large number of other functions have been characterised in IRAS including interaction with integrin alpha 5 however, their binding sites reside in the C-terminus of IRAS which is not conserved in *C. elegans* and thus are unlikely to play a role in the *F13E9.1* functionality. Nonetheless, other N-terminal interactions have been reported including LKB1 PAK1 and LIMK1. Both PAK1 and LIMK are oncogenes that proliferate tumour growth, however, IRAS inhibited PAK1 and LIMK1 directly preventing cell migration whilst overexpression of IRAS inhibited breast tumour growth and metastasis (Baranwal et al., 2011). These benefits were shown to be due to association with LKB1 through their combined effect on cell-cycle arrest, driven by inactivation of PAK1, and/or through the effect on actin-regulated migration proteins such as PAK1, LIMK1, and cofilin (Jain et al., 2013).

The next question was whether, in *C. elegans*, rilmenidine exhibited bioactivity characteristic of imidazoline receptor activation. Based on the genetic facileness of the ERK cascade in *C. elegans,* ERK phosphorylation was selected as an appropriate read-out for *C. elegans* imidazoline receptor activity. Increases in ERK phosphorylation are consistently observed following rilmenidine treatment in cell-lines, mediated through alterations in PKC (Li et al., 2006). Indeed, in *C. elegans*, rilmenidine increased ERK phosphorylation in a characteristic bi-phasic response (Piletz et al., 2000). However, a plethora of extracellular stimuli such as growth factors, cytokines, mitogens, hormones, and oxidative or heat stress are known to increase ERK phosphorylation (Mebratu and Tesfaigzi, 2009). Thus, it is not inconceivable that hormetic stress from rilmenidine administration may have upregulated ERK activity entirely independent from any imidazoline receptor activity. The co-incubation with the imidazoline receptor antagonist efaroxan went some way to refute this proposition in that whilst not altering basal pERK levels when administered alone, it abrogated the positive effect of rilmenidine in combination. However, efaroxan could indirectly block rilmenidine activity through interaction with a yet undiscovered signalling mechanism in worms.

However, to mitigate that possibility, it was necessary to localise the ERK response to *F13E9.1* the putative protein candidate for I1RA in *C. elegans*. As such, a mutant strain was generated by CRISPR

harbouring a homozygous deletion of *f13e9.1* exons 3-8, encoding both the leucine rich repeats and the coiled coil domain. Preserving the sequence upstream of exon 3 was necessary to negate the mutation of the overlapping *F13E9.1*1 gene in the sequence. Accordingly, this meant mutant strains could theoretically still transcribe the first 2 exons of *f13e9.1* in which a PHOX domains resided. It must be assumed that this severely truncated gene product still maintains endosomal targeting capabilities characteristic of IRAS phosphoinositide lipid binding. However, *f13e9.1* mutant strains abrogated rilmenidine induced ERK, increasing the likelihood a valid imidazoline binding site is expressed on *f13e9.1.* Moreover, this is strengthened by the observation that rescue with a full length integrated fosmid, restored rilmenidine-induced ERK phosphorylation. This provides substantial evidence that an imidazoline receptor is not encoded within the first 2 exons of *f13e9.1* and that rilmenidine activates ERK signalling through an imidazoline receptor binding site coded in the C-terminus of *F13E9.1*. It is possible that *f13e9.1* is merely necessary for rilmenidine induced ERK phosphorylation and that repression of rilmenidine-induced ERK activity in *f13e9.1* worms is due to impaired capacity to increase ERK signalling above homeostatsis. Although a positive control of starvation was used in WT samples, future work would benefit from ensuring *f13e9.1* mutants could reliably increase ERK activity when starved. Lastly, whilst *f13e9.1* showed significant structural and functional similarity to IRAS, it is again conceivable that although *f13e9.*1 may have an imidazoline binding site, it may not function downstream in a similar manner to IRAS and elicits divergent cell signalling pathway which may coincidentally converge on ERK. To clarify this, research would benefit from upstream studies in *C. elegans* including repetition of rilmenidine-induced ERK phosphorylation in the presence of inhibitors of protein kinase A (PKA) (H-89 and Rp-BrcAMPS) or protein kinase C (PKC) (calphostin C and staurosporine). Furthermore, DAG accumulation studies following rilmenidine administration to *C. elegans* would provide a clearer signalling map to implicate *f13e9.1* as a bone fide IRAS homolog. This would also provide a more targeted pathway to dissect when considering the pro-longevity or CRM effect of rilmenidine.

Notwithstanding, *f13e9.1* mutants displayed similar shortened body size to their murine counterparts suggesting the phenotypes and functionality of IRAS are conserved in *f13e9.1*. Researchers noted that size differences between WT and IRAS mutant mice were evident from embryonic development (Dong et al., 2017; Zhang et al., 2013). The Li lab likened the phenotype to Silver–Russell syndrome (SRS, OMIM: 180860), a genetic disease with disturbances of genes in insulin-like growth factor pathway, such as GRB10, IGFBPs, IGFs, and so on (Zhang et al., 2013). Indeed, IRAS has been purported to associate with insulin receptor substrate, a receptor for IGF, and knockout of IRAS may reduce signal transduction. However, this association is enacted at the carboxyl terminus, which is not present in *f13e9.1* and does not show any alignment to upstream *F13E9.1* protein sequences. Alternatively, it is possible that

shortened body size is secondary to downregulated mTOR following AMPK activation in mutant IRAS mice, which would accordingly eventuate in prenatal growth defects and the postnatal growth retardation (Dong et al., 2017). Such prenatal growth defects manifested in reduced brain mass with abnormal organization of both the cerebrum and cerebellum and absence of the oral and nasal cavities. Also, the gastrointestinal tract failed to completely form in the mutant embryos. In *C. elegans* it is demonstrated that *f13e9.1* mutants develop at a similar rate to WT, however investigation was limited to reproductive function. Whilst it could be argued that *IRAS* is only required for more complex tissue development such as the cerebellum and face in mice, more advanced phenotypical analysis of *f13e9.1* mutant development is required including body length measurement during larval stages, to clarify the cause of the small phenotype. Another necessary point is the potential role IRAS may have as a sorting nexin which drives specific developmental processes and embryogenesis in mice, drosophila and *C. elegans*. A recent paper, published after the gathering of these results demonstrates sorting nexin mutants in *C. elegans* to exhibit a mix of developmental rates which were for the most part normal to WT (Vieira et al., 2018). However, importantly *sorting nexin 3* caused significantly smaller body types in adulthood.

The association of IRAS to body size and development places an interesting agenda towards the potential involvement of rilmenidine on longevity and CRM. It has not been fully elucidated whether rilmenidine's agonism of the I1R affects IRAS and *f13e9.1* in an inhibitory or activating manner. Thus it is plausible that rilmenidine may inhibit *f13e9.1* to cause an upregulation of AMPK activity, subsequent mTOR repression, with a longevity phenotype. However, conversely whilst nishcarin LOF mutations may elicit developmental retardation, rilmenidine treatment *in-vitro* induces a gene ontology characteristic of accelerated growth including embryonic cranial skeleton morphogenesis, embryonic hemopoiesis, negative regulation of embryonic development and bronchus development (See chapter 3.2.1). In *C. elegans*, rilmenidine did not cause any significant effect on development, although this was again limited to reproductive function and the effect on body length with larval rilmenidine treatment should be recorded. This may provide additional evidence to suggest that the ageing programme which rilmenidine effects is decoupled from pathologically hyperfunctional developmental programmes hypothesised to cause ageing (Blagosklonny, 2013). More work needs to be done to decouple rilmenidine from developmental and also fecundity driven extension of lifespan, particularly through an assessment of fertility and brood size assay. This would help to further the pleiotropic mimicry rilmenidine may have on dietary restriction such that it may also reduce fecundity in adulthood – a metric which was not measured herein. With that said, toxicological studies in mice have revealed rilmenidine to not affect fertility, suggesting rilmenidine may not completely phenocopy undesirable consequences of DR (Verbeuren et al., 1990).

In summary, the *C. elegans F13E9.1* protein encodes an acidic region within a coiled coil domain that displays conservation to the imidazoline binding site on human IRAS. Furthermore, *F13E9.1* shares structural similarity and domain identity through the additional expression of a PHOX domain and leucine rich repeats. Rilmenidine was able to exert increases in ERK phosphorylation that was dependent on imidazoline receptor binding and also *f13e9.1*. Lastly, *f13e9.1* mutants displayed morphological similarities to IRAS mutants, but lacked a similar developmental phenotype. It is possible the developmental and morphological phenotypes are associated with reduced mTOR functioning, although loss of sorting nexin capacity is also possible. Given the inexorable association between ageing, calorie restriction, body size and developmental rates, it is advisable the relationship between *f13e9.1* and rilmenidine be explored further to ascertain directionality.

5. Results: Rilmenidine Improves Thermotolerance and Markers of "Healthspan" Dependent on *f13e9.1*

5.1. Introduction

Thus far this thesis has demonstrated rilmenidine to increase mean and maximum lifespan in *C. elegans* even if administered late in life. As highlighted in chapter 1.1.5 , ageing represents an accelerating vulnerability to disease and decreased adaptability to internal and external stress. The human body, model organisms and even individual organs, strive to maintain homeostasis and express the capacity to endure recurrently stressful conditions. Even in the absence of disease, one's refractory capacity will erode, culminating in mortality (Fries, 2005). Ultimately, ageing can thus be defined by survival and mortality, and determines the longevity of an organism. The probability of death increases exponentially with advancing age and is inescapable for the cohort, proving survival a popular metric of ageing.

However, lifespan is an ambiguous and loaded definition of ageing. Death rarely occurs as a result of ageing, and despite measures taken within experimental setting, incontrollable variables such as disease often eventuate death (Bronikowski, 2010; Pedro de Magalhães et al., 2018). Thus, lifespan is not sufficient in determining whether an intervention such as rilmenidine affects the ageing process or another age-independent factor. Researchers have hence looked for biomarkers that can eliminate the confounding influence of disease and measure underlying processes of ageing. As defined by Baker and Sprott, a biomarker "should reflect some basic biological process of ageing and certainly not the predisposition toward a disease state or some error in metabolism" (Moreno-Villanueva et al., 2015). That said, general patterns of age-change are common in certain tissue and moreover, such changes have been elucidated in most organ systems and serve as the source of many age-associated chronic diseases. These diseases are universal and inevitable manifestations of age that accelerate and can become symptomatic in line with functional decline, further compromising one's stress resistance and vulnerability to death (Fries, 2005). The ability to compress the period of overt, symptomatic age-associated disease, and maintain "healthy ageing" is one of the central tenants of anti-ageing research and a key target for pharmacological intervention (Stefánsson, 2005). Moreover, despite continued improvements in life expectancy, functional capacity and "healthspan" trajectories have not improved simultaneously, leading to a rapidly ageing population wherein, death is generally the result of chronic conditions, which develop over a long lifespan (Crimmins, 2015). Accordingly, intervention studies in model organisms have begun to shift the emphasis from calculating lifespan to measuring

healthspan (Keith et al., 2014). Biomarkers can be employed that can predict the general demise of ageing alongside the progressive enfeeblement characteristic of a prohibitively short healthspan including decreased adaptability to internal and external stress. This of course manifests in the phenotype of frailty, which is defined as having three or more of five physical components: unintentional weight loss, self-reported exhaustion, weakness, slow walking speed and low physical activity (Fried et al., 2001). Recent evidence has demonstrated that frailty is the best indicator of remaining health and lifespan (Pyrkov and Fedichev, 2019). Although the exact pathogenesis remains elusive, frailty is accompanied by significant sarcopenia driven by muscle mitochondrial energy dysregulation and an accumulation of oxidative damage and aggregates in muscle cells (Angulo et al., 2016). Indeed, *C. elegans* exhibit a comparable age-associated deterioration in muscle function (Glenn et al., 2004; Herndon et al., 2002). Moreover, studies have shown that the rate of age-related decline in body movement is a good predictor of lifespan and that long-lived *C. elegans* mutants tend to exhibit prolonged locomotory capacity (Herndon et al., 2002; C. Huang et al., 2004; Newell Stamper et al., 2018). Importantly, calorie restriction in *C. elegans* preserves body movement capacity with age, and putative CRMs are able to replicate this phenomena (Calvert et al., 2016; Onken and Driscoll, 2010).

Secondly, lipofuscin accumulation is a routinely utilised biomarker of healthy ageing (Son et al., 2019). Lipofuscin is a heterogeneous mixture of cross-linked biomolecules that accumulates in the lysosomes of ageing cells (Yin, 1996). This post-mitotic phenomena, oft-cited as “the ageing pigment”, is well-conserved from humans to nematodes and correlates almost linearly with age and can be used to calculate the age of crustacean (Moreno-García et al., 2018) . Furthermore, it has been implicated in the development of neurodegenerative diseases by its triggering of neuronal loss, proliferation, and activation of glial cells. In normal brain ageing, intraneuronal deposits of lipofuscin significantly expand which correlates with altered neuronal cytoskeleton and cellular trafficking (Moreno-García et al., 2018). It is thought that accumulation may be driven by age-associated lysosomal impairment degradation failure which signals a more global proteostatic collapse. Thus, interventions which may preserve proteostatic function through a deceleration of the ageing process may manifest in reduced lipofuscin accumulation (Gerstbrein et al., 2005). Indeed, dietary restriction in murine models significantly reduces the prevalence of lipofuscin in the hippocampus, frontal cortex and retinal epithelium (Idrobo et al., 1987; Katz et al., 1993).

Whilst biomarkers may serve to calculate current age states, Butler et al., (2004)have also insisted biomarkers should predict the outcome of a wide range of age-sensitive tests, for which lifespan is inclusive. Following on from the position that stress resistance and homeostatic flexibility play a central

role in preventing mortality with age, it is conceivable that basal resistance to stress could predict the depth of reserve which an organism may harness to survive, and thus represents a viable biomarker of longevity. To that end, interventions that increase tolerance to multiple stressors, should in turn attenuate the diminishments in functional reserve.

Stress resistance comprises two functions: the ability to prevent stress becoming malapropos and the resilience to recover from malapropos stress (Hamilton and Miller, 2016). Unsurprisingly, in *C. elegans*, long lived mutants exhibit high resistance to heat, oxidative, osmotic, hypoxic, ultraviolet, and heavy metal stresses, whereas progeric mutants exhibit greater sensitivity to these stressors (Dues et al., 2018; Keith et al., 2014) . In fact, when assessing the validity of a compound as a CRM beyond its inability to alter food intake, researchers propose that they must also activate stress response pathways, provide protection against stressors and lead to a preservation of healthspan (Ingram et al., 2006).

It is imperative that the longevity effects of rilmenidine are indeed correlated to an attenuation of age biomarkers. Furthermore, as demonstrated, calorie restriction may delay ageing phenotypes through a preservation of homeostatic flexibility and improved resilience. For rilmenidine to be considered as a viable geroprotector it should extend healthspan and ideally, to qualify as a CRM, improve stress resistance. It would also be helpful to clarify whether these effects are mediated through *f13e9.1* such that the pathways controlling rilmenidine-induced stress resistance are elucidated.

5.2. Results

5.2.1. Rilmenidine Attenuates Locomotory Decline But Not Autofluorescence Accrual During *C. elegans* Ageing via *f13e9.1*

Initially, continuing with lifespan as an appropriate metric to assess the geroprotective properties of rilmenidine, it was necessary to establish whether lifespan extension was conferred via interaction with an endogenous imidazoline binding site in *C. elegans*, and furthermore, whether the putative IBS carrying gene, *f13e9.1*, was necessary for lifespan extension.

Firstly, hypothesising that blockade of imidazoline binding sites in WT worms would abrogate lifespan extension by rilmenidine, WT worms were co-incubated from day 1 adulthood with rilmenidine (200μM) and efaroxan (2mM), an established selective I_1-imidazoline receptor antagonist. Importantly, efaroxan alone significantly increased mean lifespan by +12.5% in WT worms compared a 1% DMSO vehicle control (efaroxan=24.5 days ± 0.5 S.E.M vs 1% DMSO 21.77days ± 0.64 S.E.M; log rank test w/ Bonferroni correction; p=0.013) (Figure 14). Furthermore, rilmenidine alone, as repeated in previous trials, significantly extended mean lifespan by +11.25% in WT worms compared a 1% DMSO vehicle control

(rilmenidine=24.22 days ± 0.54 S.E.M vs 1% DMSO 21.77days ± 0.64 S.E.M; log rank test w/ Bonferroni correction; p=0.05). However, whilst rilmenidine and efaroxan combination therapy increased mean lifespan in WT worms compared to controls, there was no additive effects, and co-treatment with rilmenidine and efaroxan failed to impart further mean lifespan extension than worms treated with either rilmenidine or efaroxan monotherapy (Ril vs. Ril & Efar p=0.202; Efar vs. Ril & Efar p=0.391).

It was hypothesised that an IBS, through which the prolongevity effect of rilmenidine is derived, is encoded within the *f13e9.1.* Thus, lifespan assays were performed on *f13e9.1* mutants. This would establish the contributions of *f13e9.1* to a normal lifespan, the ageing process, and furthermore whether *f13e9.1* was a longevity associated gene. Lastly, and ideally, the necessity of *f13e9.1* for the prolongevity effect of rilmenidine should be assessed. As such, employing the previously described solid media NGM based lifespan assay (see chapter 2.7), adult lifespan was measured in WT (WT), *f13e9.1 (-/-)* and two nominally identical strains of rescue mutants PHX945 and PHX946 *(syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry]*. At L4/adult molt, worms that had previously developed on FUDR-free NGM and were transferred to, and remained on thereafter, NGM plates that had been supplemented with 400µM FUDR and seeded with x10 concentrated live *E.coli*. Survival analysis demonstrated that *f13e9.1* mutants had modest but significantly +6.3% increased mean lifespan in comparison to WT animals (WT =23.01 days ± 0.37 S.E.M vs *f13e9.1* 24.46days ± 0.41 S.E.M; log rank test w/ bonferroni correction; p=0.01) (Figure 15). Rescue mutants did not give a consistent lifespan phenotype. The second rescue strain PHX946(syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry) lifespan did not significantly differ to that of *f13e9.1* mutants (log rank; p=0.22), and as such was statistically more long-lived than WT (WT =23.01 days ± 0.37 S.E.M vs *PHX946* 25.50days ± 0.38 S.E.M; log rank test w/ bonferroni correction; p<0.001) (Figure 15). Conversely, PHX945(syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry) rescues seemingly restored lifespan that did not significantly differ to WT (WT) (log rank; p=1), and as such were modestly but insignificantly short-lived in comparison to *f13e9.1(sybIs62)* mutants (PHX945=23.43 days ± 0.30 S.E.M vs *f13e9.1* 24.46days ± 0.41 S.E.M; log rank test w/ bonferroni correction; p = 0.09). Experimental failures owing to a broken incubator prohibited the completion of a final set of lifespan assays verifying the ability of *f13e9.1* to mediate rilmenidine-induced lifespan extension in *C. elegans*.

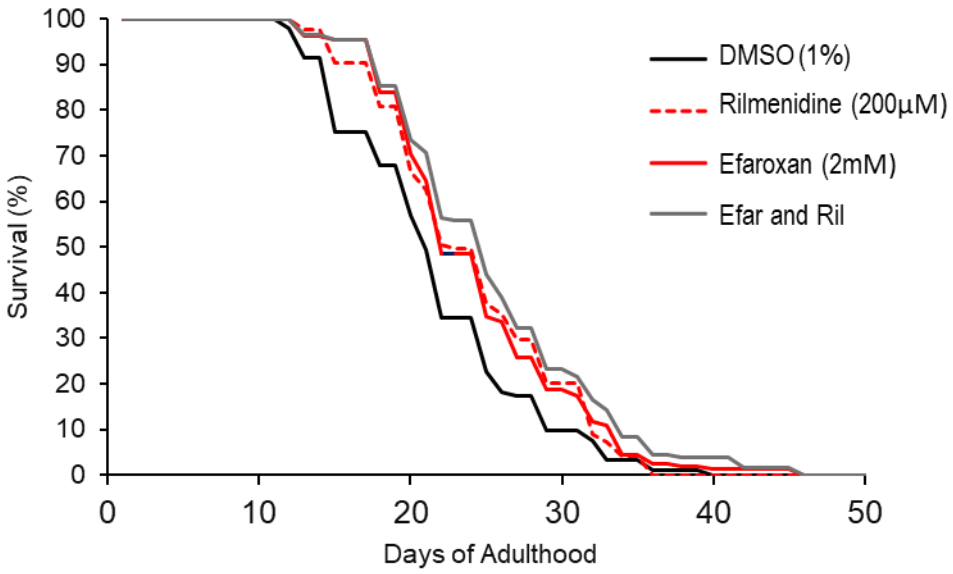


Figure 14. Rilmenidine- Induced Lifespan Extension is Mediated By an Imidazoline Receptor Binding in C. elegans. *Pooled survival curves of adult WT worms fed UV-killed OP50 also treated with rilmenidine (200µM) and efaroxan (2mM) independently, or in combination, compared to control animals treated with a 1% DMSO vehicle. Both rilmenidine and efaroxan increased lifespan independently, but these effects were not additive when tested in combination. Kaplan Meir survival analysis performed on pooled data from at least 2 independent trials in no less than 90 animals per condition. Groups tested by Log-rank with Bonferonni correction; $p<0.05$ considered significant.*

Table 9. Summary lifespan data of WT WT worms exposed to various compounds. *Differences in mean and maximum lifespan of WT C. elegans exposed to various compounds relative to the control within the same sets of trials. Mean lifespan in days is the pooled mean of treatment cohort and the standard error (± S.E.M). Maximum lifespan defined as the day at which 90% cohort mortality was reached. Lifespan assays performed at 20 °. Adjusted p-value was determined by log-rank test using the Kaplan-Meier survival analysis and subsequent bonferonni correction. Pooled n = number of deaths observed across all respective trials.*

Genotype/Perturbation	***Pooled n=***	**No. trials**	**Mean lifespan (days)**	**Std. error**	**% Chg Vs. Ctrl.**	**90% Mortality (days)**	**Adj. p-value**
WT (Live OP50 *E.coli*)							
Live OP50 *E.coli*	179	3	23.01	0.37	N/A	30	N/A
WT (UV Killed OP50 *E.coli*)							
1% DMSO	93	2	21.77	0.17	0.01	29	N/A
2mM Efaroxan	155	2	24.50	0.50	12.54	34	0.057
200µM Rilmenidine	125	2	24.22	0.54	11.25	32	0.013
Rilmenidine and Efaroxan	177	2	25.57	0.51	17.46	34	0.001
f13e9.1(sybIs62)							
Live OP50 *E.coli*	180	3	24.46	0.41	N/A	32	N/A
PHX945 (*f13e9.1* (syb767) IV; sybIs62[*f13e9.1*+])							
Live OP50 E.coli	187	3	23.43	0.39	N/A	30	N/A
PHX946 (*f13e9.1* (syb767) IV; sybIs62[*f13e9.1*+])							
Live OP50 E.coli	214	3	25.5	0.38	N/A	32	N/A

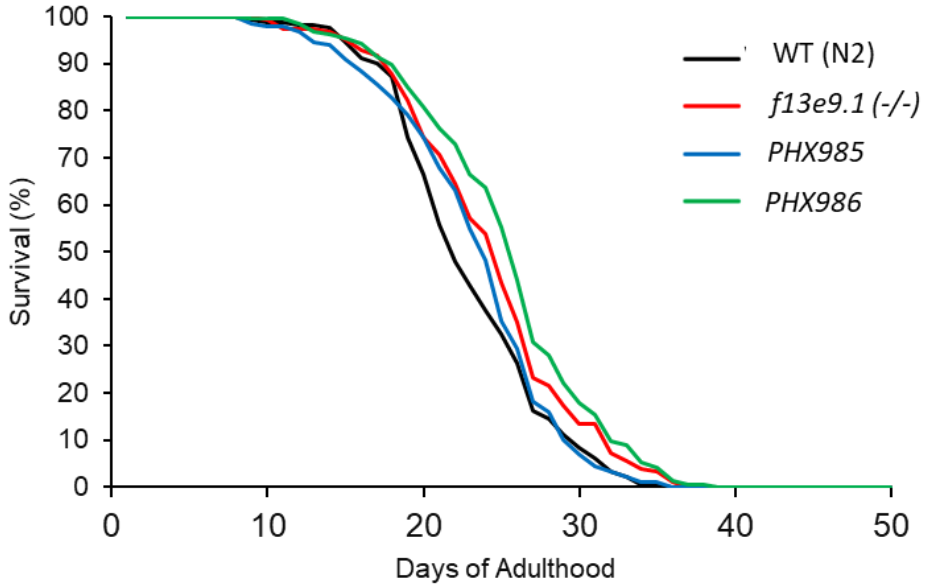


Figure 15. Effects of a f13e9.1 mutation and transgenic rescues on C. elegans lifespan. *WT , f13e9.1 mutants, and two transgenic rescue lines PHX945 and PHX946 (syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry), were bleach synchronised and arrested in L1 overnight. All strains were transferred to NGM plates seeded with live OP50 E.coli until L4/adult Molt. Day 1 commenced upon the transfer of strains onto NGM plates containing 400µM FUDR and seeded with live OP50 E.coli. Worms remained on the same plates until completion. Kaplan Meir survival analysis performed on pooled data from at least 3 independent trials in no less than 170 animals per genotype. Groups tested by Log-rank with Bonferonni correction; $p<0.05$ considered significant.*

Thus, to expand on the geroprotective and CRM properties of rilmenidine, it is essential to establish that rilmenidine can also prolong healthspan in *C. elegans* and can attenuate metrics of frailty. Using the common assay of sigmoidal motility as a surrogate marker for frailty we measured body bends in WT worms exposed to rilmenidine at 3 different concentrations (200µM, 300µM, 400µM) (see chapter 2.11.2). Worms were observed at days 5,10,15 to straddle proposed window of muscular decline that becomes significant at around day 8 in WT worms (Glenn et al., 2004). Results correlated with lifespan somewhat, in that, 200µM was the only concentration that significantly ameliorated deterioration in WT animal motility over the three time points ($p<0.05$; 2-way anova and Tukey post-hoc); however when time points were individually compared, at day-15 the difference did not reach statistical significance (WT w/1% DMSO = 10.1±1.21 bends/min vs. WT w/200µM = 13.2±1.30 bends/min; $p=0.057$) (Figure 16A). Concentrations of 300 and 400 µM did not affect motility decline in either direction over the 3 time points (Figure 16B,C)

Next it was important to assess whether preservation of healthspan by rilmenidine may be dependent on the putative IRAS homolog, *f13e9.1*. First of all, *f13e9.1* mutants, treated with only a vehicle (1% DMSO) exhibited significantly accelerated declines in motility compared to WT which is suggestive of a shortened healthspan ($p<0.05$; 2-way anova and Tukey post-hoc); this difference was significant at all time points (Figure 16D). The supplementation of *f13e9.1* mutant worms with 200µM rilmenidine from day-1 failed to improve motility (Figure 16E). Rescue of *f13e9.1* mutant using either the PHX945 or PHX946 strains failed to significantly restore age-related declines in motility to that observed in WT (Figure 13E). However, PHX945 was able to reinstate the preservation of motility into age by rilmenidine as observed in WT worms. This provides some evidence that rilmenidine may preserve healthspan in *C. elegans*, which is dependent on the gene *f13e9.1*.

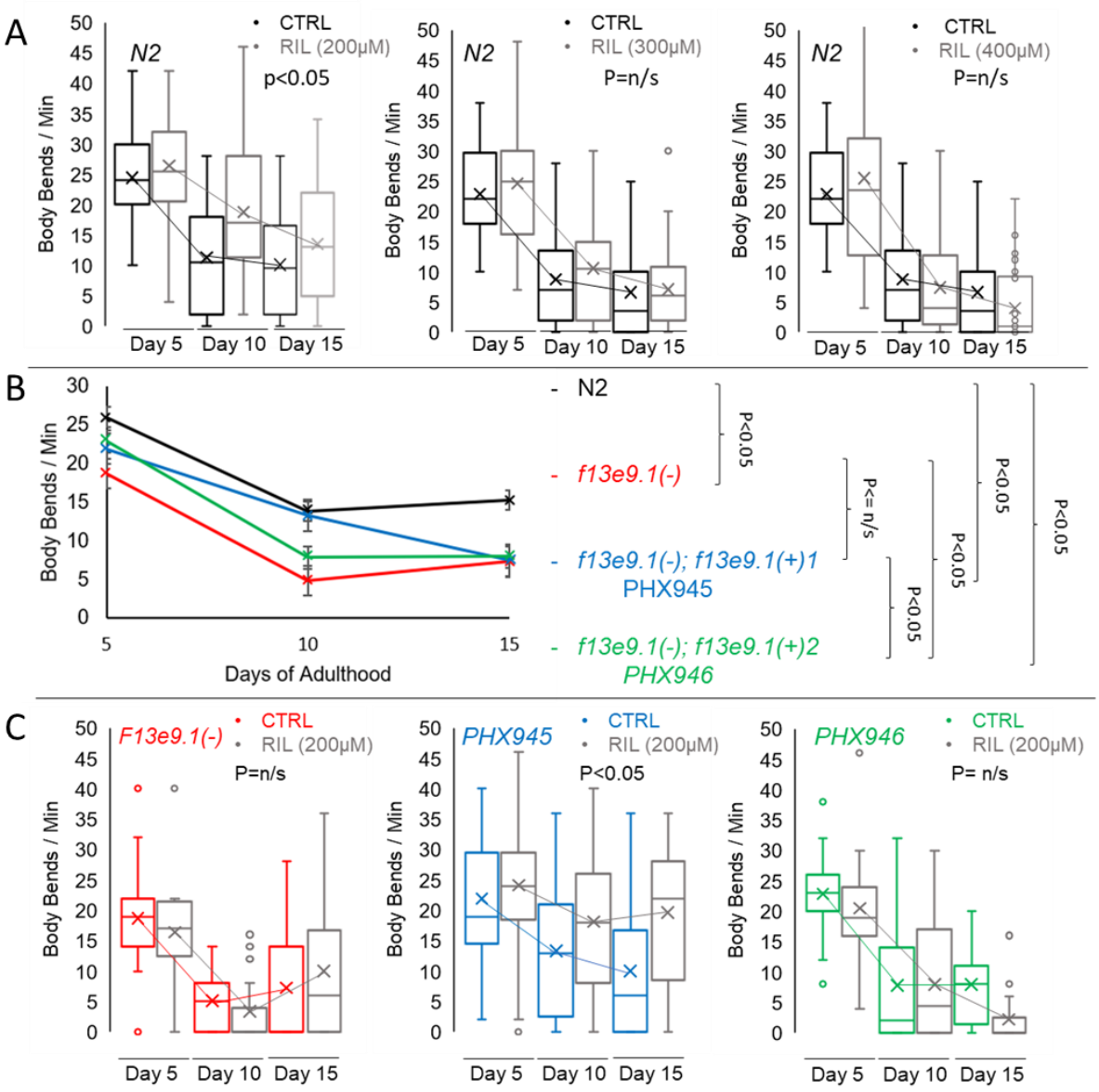


Figure 16. Quantification of motility deterioration with age in WT (WT,WT), f13e9.1 (-/-) mutants, and two f13e9.1 rescue mutants (PHX945 and PHX946) in the presence or absence of rilmenidine. *Motility was measured as number of stimulated sigmoidal body bends on solid NGM over a 1 minute period at day 5, 10 and 15. Data is represented as a pooled mean (X) of 10 animals per time-point and condition/genotype repeated over 3 independent trials (n=30) overlaid on a box plot representing quartiles. Error bars, upper:Q3 + 1.5*IQR; minimum: Q1 -1.5*IQR. A) Effect of differing concentration of rilmenidine (200, 300 and 400µM) on motility over time compared to 1% DMSO vehicle controls in WT worms. Only rilmenidine at 200µM significantly reduced age-related deterioration in animal motility in WT worms (adj. p<0.05; 2-way ANOVA and Tukey post-hoc). B) Effect of differing genotypes on motility over time on NGM agar without 1% DMSO. f13e9.1 (-/-), f13e9.1(-); f13e9.1(+)1 PHX945 and f13e9.1(-);f13e9.1(+)2 PHX946 all showed significantly different age-related deterioration in motility (adj. p<0.05; 2-way ANOVA and Tukey post-hoc). C) Quantification of body bends f13e9.1 (-/-), f13e9.1(-); f13e9.1(+)1 PHX945 and f13e9.1(-);f13e9.1(+)2 PHX946 transgenic animals fed from Day 1 of adulthood with 200µM rilmenidine or a 1% vehicle control. Rilmenidine significantly affected motility deterioration in f13e9.1(-); f13e9.1(+)1 PHX945 rescue worms but not in f13e9.1 (-/-) or f13e9.1(-);f13e9.1(+)2 PHX946 worms (adj. p<0.05; 2-way ANOVA and Tukey post-hoc).*

As worms age, it is suggested they accumulate lipofuscin in the intestinal cells from base of the pharynx to rectum. Lipofuscin generates a measurable autofluorescence which can be visualised and quantified using a DAPI channel filter (Rollins et al., 2017). This pigment increases in a time-dependent fashion in line with ageing from day 5 to day 10 (Keith et al., 2014). Undoubtedly, researchers seemingly prefer to measure age-related autofluorescence through the DAPI channel, and indeed this wavelength is advocated in guidelines (Bansal et al., 2015; Collins et al., 2008; Keith et al., 2014; Rollins et al., 2017). Work from the Gems lab has sought to overturn the commonly held belief that autoflourescence emitted through the UV spectrum signifies lipofuscin. Instead, it is argued that the DAPI signal is due to anthranilate acid esters originating from gut granules and furthermore, that this signal intensified preceding the animal's ensuant death. This likens DAPI autofluorescence to survival in that, rather than predicting the biological age of an animal, it actually demarcates the mortality risk of the cohort (Coburn et al., 2013). Nonetheless, well-cited papers, in highly ranked journals, have continued to image autofluorescence in the DAPI channel to measure an already ill-defined concept of healthspan (Bansal et al., 2015; Chen et al., 2019; Dehghan et al., 2017; Hsieh et al., 2017; Kwon et al., 2016; Lee et al., 2015; Rollins et al., 2017). To monitor this process and assess the effect of rilmenidine to attenuate lipofuscin accumulation, synchronised adult day 1 worms were cultivated on NGM agar supplemented with FUDR in the presence or absence of prolongevity dose of rilmenidine (200μM) (see chapter 2.11.1). A cross-sectional cohort of worms were imaged at day 2 to establish baseline autofluorescence and an additional separate cross-sectional cohort were measured at day 10 to establish rate of accumulation in line with previous methods (Pluskota et al., 2009). Firstly, the rate of lipofuscin accrual of no different between WT and *f13e9.1*; neither of the two groups had a significant accumulation of lipofuscin over the 10 day period Figure 17.

In the WT cohorts, median accumulation in pixel intensity between day 1 and day 10 was less in the rilmenidine treated group compared to the control group, however this did not reach statistical significance (Figure 17). This is owing to the fact that WT (WT) worms did not significantly accumulate lipofuscin over 10 days in either the control (1% DMSO) group or the rilmenidine-treated group (Mann-Whitney U; $p>0.05$ for both treatments). In the *f13e9.1* mutant cohort, rilmenidine treated worms had a more significant accrual of lipofuscin over the day 10 period compared DMSO treated worms suggesting rilmenidine worsened lipofuscin accumulation with age in *f13e9.1* mutants (*f13e9.1* DMSO Day 1 vs. DMSO Day 10, $p=0.34$; *f13e9.1* Ril Day 1 vs. Ril Day 10, $p=0.01$). Given the poor induction of phenotype by rilmenidine rescue was deemed surplus to requirements.

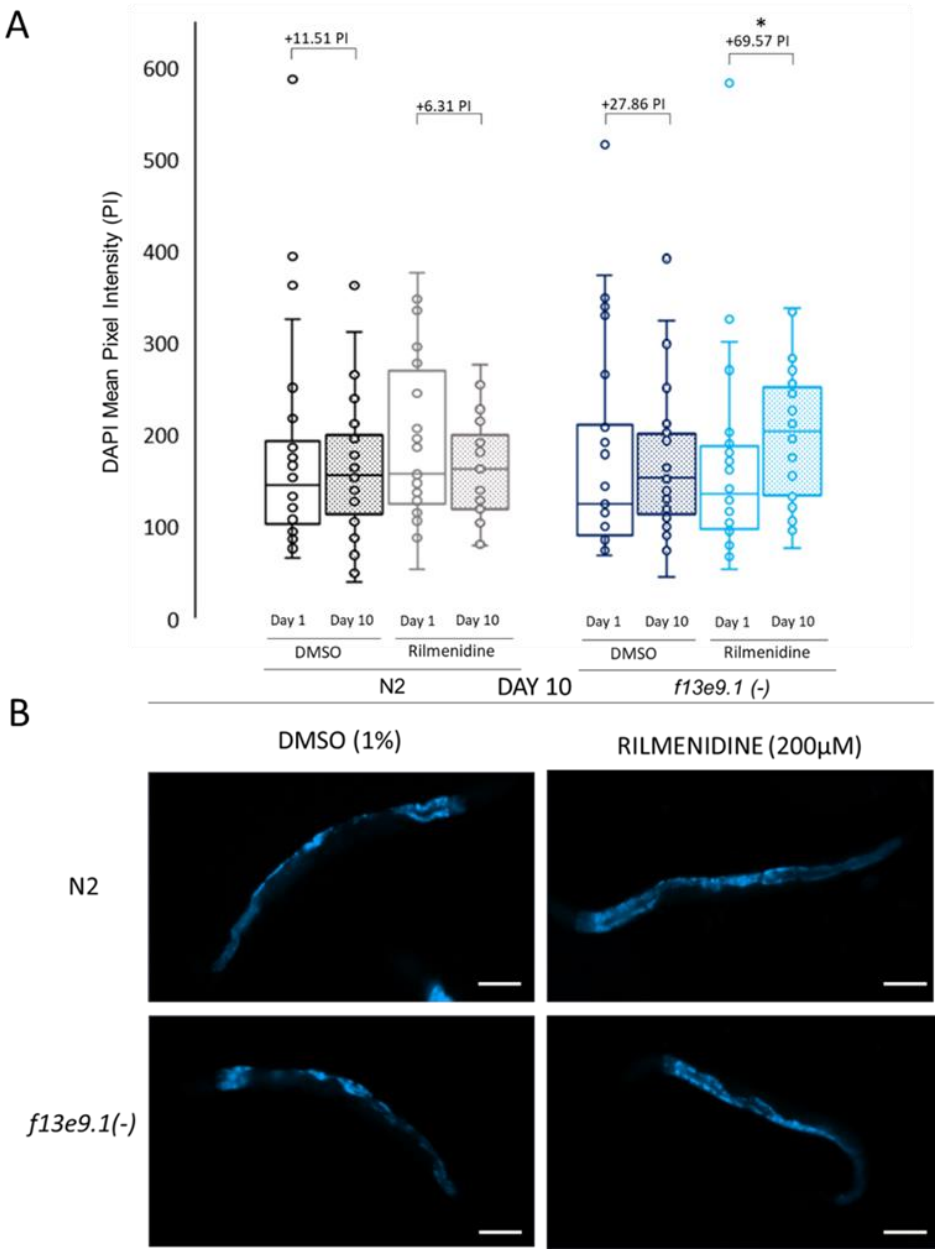


Figure 17. Effect of rilmenidine treatment on lipofuscin accumulation in WT and f13e9.1 mutants. *L4/Adult moult worms were transferred to NGM plates seeded with UV killed OP50 E.coli containing either 200µM rilmenidiine or a 1% DMSO control. On day 2 and 10 they were imaged for age-related lipofuscin accumulation. A) Lipofuscin was quantified by autofluorescence as measured by mean pixel intensity in the DAPI channel (ext on Zen Blue with background subtraction. N>28 per condition and genotype across 3 independent trials. Median Pixel intensity was calculated for each cohort and compared to respective control; *p<0.05, no indicator = not significant; two-tailed Mann-Whitney U. PI= pixel intensity through DAPI channel in Zen Blue Error bars, upper:Q3 + 1.5*IQR; minimum: Q1 -1.5*IQR B) Representative images of lipofuscin fluorescence in DMSO-treated nematodes and rilmenidine-treated nematodes are shown. Animals were immobilized and mounted onto glass slides in 20mM solution of tetramisole hydrochloride before being imaged through both brightfield and DAPI filter using a Zeiss Axio Observer (300ms exposure, x10 objective). Scale bar = 100µm.*

5.2.2. Rilmenidine Improves Thermotolerance But Not Oxidative Stress Resistance

Lastly, it was necessary to assess the hypothesis that rilmenidine may be a CRM due its capacity to improve stress resistance. Upon consultation with various stress-resistance assay guidelines, thermotolerance and oxidative stress were selected based on frequency of adoption (Keith et al., 2014; Lionaki and Tavernarakis, 2013) (Park et al., 2017). Furthermore, each would separately recapitulate an aspect of ageing. Heat-shock response is critical to proteome fidelity and the same machinery used to improve resilience is implicated in proteostatic collapse in ageing. Secondly, oxidative stress assays in which worms are exposed to high levels of ROS can aggressively recapitulate DNA damage and mutation accumulation proposed in organismal ageing.

The thermotolerance assay was started at day 1 adulthood, to avoid premature age-related proteostastic collapse (Ben-Zvi et al., 2009) and to negate the need for FUDR which has been shown to interfere with thermotolerance and proteotoxic stress (Angeli et al., 2013). After 24h of respective drug exposure, worms were added to an incubator preset to 37ºC to induce heat-shock whilst still exposed to the respective drugs (Zevian and Yanowitz, 2014) (see chapter 2.92). After 3 hours, worms were removed from the incubator and being kept on the same drug plates, shifted to 20ºC and scored for survival after a ~20h "recovery period" (Kumsta et al., 2017). We validated the assay demonstrating proline to improve survival to the above protocol, as previously reported (data not shown) (Edwards et al., 2015a). Rilmenidine at a prolongevity dose (200µM) significantly improved survival in comparison to WT worms (one way ANOVA $p<0.05$) (Figure 18B). To establish whether this effect might be mediated by the putative IRAS orthology *f13e9.1* we exposed *F13E9.1*(syb767) mutant worms to the same heat stress in the presence or absence of rilmenidine. *F13E9.1*(syb767) worms were not anymore sensitive to heat-shock than WT worms; however 24h treatment with rilmenidine significantly reduced their survival, implying that rilmenidine required *f13e9.1* to improve thermotolerance. To clarify the relationship further, I exposed PHX945 and PHX946 rescue strains to equivalent heat shock (Figure 15B). Survival was improved in comparison to WT and *F13E9.1*(syb767) worms, but this did not reach significance. Furthermore, 24h pre-incubation with rilmenidine (200µM) did improve survival by ~19% in both PHX945 and PHX946, but this failed to reach significance. Whilst this may suggest that *f13e9.1* does not mediate the thermotolerance effects of rilmenidine, it is likely the high survival observed by rescue strains masked the benefits of rilmenidine.

To assess oxidative stress response in *C. elegans,* treated with rilmenidine, we used the popular pro-oxidant hydrogen peroxide H_2O_2 in a routinely used solid media based assay (Ludewig et al., 2013; Oláhová and Veal, 2015). Again, adult day 1 worms were exposed to rilmenidine or 1% DMSO for 24h

before being transferred to freshly prepared NGM plates containing 200µM rilmenidine and 2mM H_2O_2 (see chapter 2.9.1). A subset of worms were removed from drug exposure whilst incubated on H_2O_2 plates in order to account for any confounding external reactivities of H_2O_2 and rilmenidine not related to endogenous superoxide scavenging (see supporting table). Survival was then scored every 2 hours for 10 hours as in previous assays (Jansen et al., 2002; Senchuk et al., 2017). A prolongevity dose of rilmenidine failed to affect survival in response to H_2O_2 induced oxidative stress. To establish whether this was due to an insufficient dosing, neither 300µM or 400µM significantly altered survival (see supporting table 3). To clarify that this was not a ubiquitous phenomena, 5-10mM NAC (+ve control) supplementation significantly improved survival in oxidatively stressed WT worms (see supporting table 3). Interestingly, *F13E9.1(syb767)* mutants were more sensitive to oxidative stress than WT worms and their demise was significantly more accelerated (log rank; p<1.70E-07) (Figure 18A). Moreover, treatment with rilmenidine additively worsened the response. Rilmenidine treated *f13e9.1* mutants died significantly quicker than f13e9.1 worms treated with just a 1% DMSO vehicle (log rank; p<2.40E-05) (Figure 18A). Together these results suggest rilmenidine does not improve oxidative stress response but improves thermotolerance.

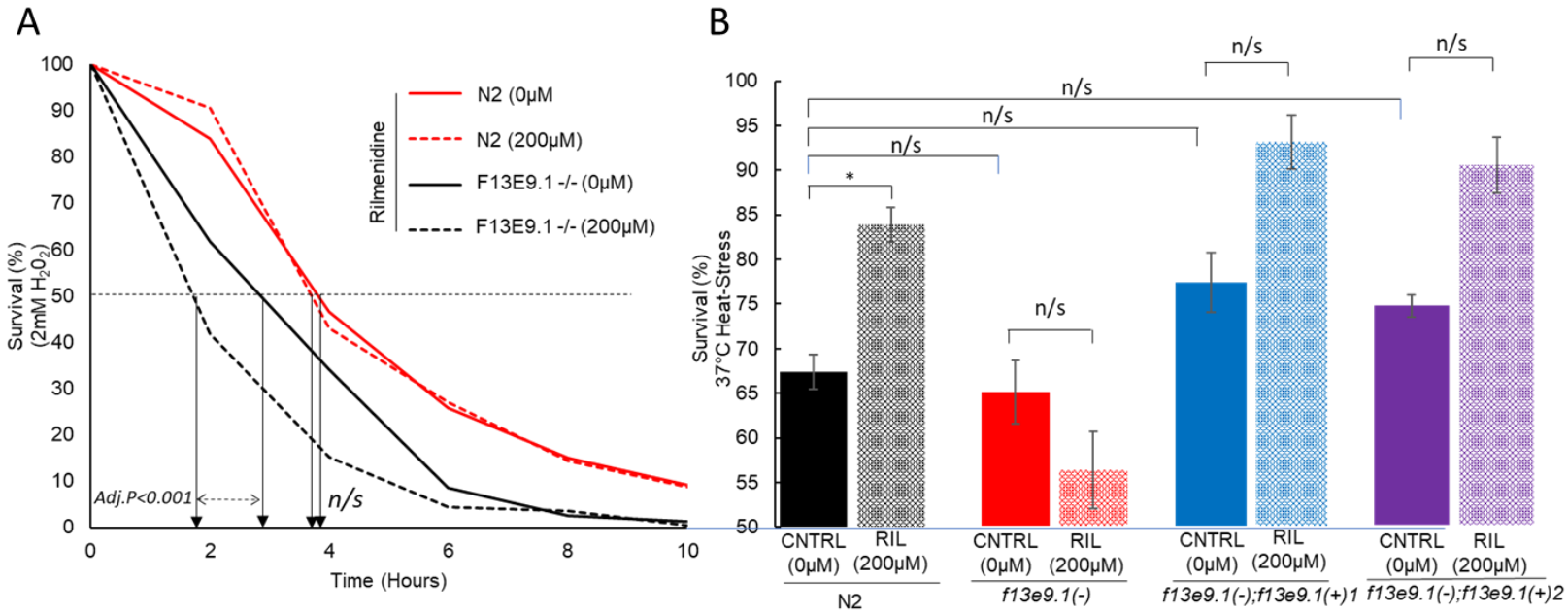


Figure 18. Rilmenidine increases thermotolerance but not oxidative stress resistance dependent on f13e9.1. *Survival of Day 1 Adult WT (WT,WT), f13e9.1 (-/-) mutants, and two f13e9.1 rescue mutants (PHX945 and PHX946) in the presence or absence of rilmenidine following exposure to either heat-stress or oxidative stress. A) Oxidative stress: survival of WT WT worms and f13e9.1 (-/-) worms exposed to 2mM H_2O_2 plates seeded with dead OP50 E.coli, following 24h treatment with either 200μM rilmenidine or a 1% DMSO vehicle. Survival curves are a representative composite of 3 experiment with a total n>170 animals per condition measured at 2, 4, 6, 8 and 10 h. Kaplan-Meier log rank analysis was used to compare survival curves; Bonferroni adjusted p-value <0.05 considered significant. Complete, raw experimental data, with replicates, statistics, and percent changes in survival time provided in Table S2. B) Thermotolerance: Survival of day 1 adult WT WT, F13e9.1 (-/-), f13e9.1(-); f13e9.1(+)1 PHX945 and f13e9.1(-);f13e9.1(+)2 PHX946 worms exposed to 37ºC for 3 hours and ~20h recovery period measured by single-time point % survival. Drug-treated animals were placed on NGM plates supplemented w/ 200μM rilmenidine 24 hours prior to heat exposure and remained on the same plates through recovery. Data are represented as mean %survival ± S.E.M from three independent trials of at least 150 animals per strain and/or condition. *p<0.05 (one-way ANOVA with Tukey post hoc comparisons). See supporting table 3 for additional data.*

5.3. Discussion

Whilst rilmenidne extends lifespan, the metric of survival is somewhat limited and does not define rilmendinie as a longevity molecule. As such it was necessary to determine if rilmenidine could improve any biomarkers of ageing alongside an improvement in stress resistance which together may confer increases in healthspan. Indeed, in worms rilmenidine improved two ageing biomarkers, a metric of frailty and the accumulation of intestinal autofluorescence, both of which were dependent on a functioning *F13E9.1*. Furthermore, rilmenidine treated worms appeared more resistant to heat stress, but were equally sensitive to oxidative stress as WT worms. Improved thermotolerance by rilmenidine in *C. elegans* was also dependent on *f13e9.1*, and strikingly, rilmenidine treatment increased sensitivity to oxidative stress in *f13e9.1* mutant worms. When examining the healthspan of *f13e9.1* worms, despite living longer than WT couterparts, they were counterintuitively frail at younger ages and accumulated lipofuscin faster than WT. Together these findings further uncover a complex and potentially dynamic relationship between rilmenidine and it's putative *C.eleagns* receptor *f13e9.1*. Moreover, it bolsters evidence that such a relationship is associated with a dietary restricted phenotype.

The data presented herein sought to establish whether the longevity effect of rilmenidine correlated to an ncrease in healthspan which may translate to longer periods of frailty-free, healthy years in clinical trials. Locomotory decline has been routinely employed in *C. elegans* ageing research as a surrogate marker of frailty owing to their shared sarcopenic aetiology (Bansal et al., 2015; Collins et al., 2008; Keith et al., 2014; Rollins et al., 2017). In particular, body bends in response to stimulation represents a popular and easily implemented metric for ascertaining locomotive potential within a nematode which correlates to lifespan and tissue deterioration (Collins et al., 2008; Herndon et al., 2002; C. Huang et al., 2004). This metric becomes more appealing upon the realisation that environmentally induced CR and also CRMs appear to attenuate reductions in body bends with age (Calvert et al., 2016; Lüersen et al., 2014; Onken and Driscoll, 2010).

In this research, only 200μM rilmenidine significantly attenuated locomotory decline in *C. elegans*, which is also the dosage that increased lifespan the most. Such correlation shows that rilmenidine may act on ageing processes rather the deferment of mortality, whilst also reinforcing the utility of locomotion analysis as a predictor of lifespan. Rilmenidine treated worms demonstrated improved stimulated motility at day 10, and day 15 when tested individually: these are both time points where significant increases in "frail" individuals are expected (Herndon et al., 2002). This is in agreement with a particularly relevant clinical trial looking to measure objective and subjective improvements in quality of life in perimenopausal women

with mild to moderate hypertension following rilmenidine monotherapy (Kawecka-Jaszcz et al., 2006). They had previously noted that rilmenidine treatment reduced weakness and fatigue in women (Polskie Towarzystwo Nadciśnienia Tętniczego et al., 1999).

Biochemical perturbations underlie the very deterioration of age driven by a lifelong accumulation of molecular damage (Engelfriet et al., 2013). Whilst behavioural changes in processes such as locomotion provide an integrated and cumulative metric for functional decline, it does not characterise any cellular pathologies driving tissue ageing. The investigation of lipofuscin accumulation in ageing *C. elegans*, provided a platform to assess whether rilmenidine may fundamentally ameliorate cellular abnormalities at the foundation of an ageing phenotype. Indeed, rilmenidine attenuated the accumulation of autofluorescence in the intestine however this did not reach statistical significance. It is tempting to speculate that differences between rilmenidine and DMSO treated groups would become significant if a later end-point was defined. This is bolstered by evidence that, whilst median intestinal autofluorescence accumulated from day 2 to day 10 in control worms, the time-points were not significantly different, suggesting 10 days was not long enough for the worms to age. In our hands, WT worms, grown on UV killed *E.coli,* have longer lifespans than WT worms from comparable studies where autofluorescence accumulation was quantified at day 10 (Dehghan et al., 2017; Mi et al., 2018). Nonetheless, lipofuscin accumulation is clearly apparent in younger animals, including day 2 animals herein, and statistically different timepoints in pharmacological intervention studies have been as early as day 4 (Asthana et al., 2015; Pant et al., 2014).

Hence it is implied these organelles may harbour lipofuscin-like garbage. Given that the lipofuscin cannot be effectively degraded or exocytosed, it is likely interventions that attenuate lipofuscin accumulation do so through prevention rather than clearance (Moreno-García et al., 2018; Terman and Brunk, 1999). Such prevention is mostly initiated through efficacious protein homeostasis, a deferment of mitochondrially-induced oxidative stress or conservation of lipid metabolism (Moreno-García et al., 2018). Lipofuscin is accepted to consist of oxidized proteins (30–70%), placing onus on effective autophagic machinery to prevent the polymerisation of oxidised protein in lipofuscin formation (Höhn and Grune, 2013). Indeed rilmenidine has been demonstrated in a number of models to improve the clearance of aggregated proteins including synuclein and polyglutamate expansions (Rose et al., 2010; Williams et al., 2008). Thus, further exploration into the appealing signal of rilmenidine attenuating lipofuscin accumulation is warranted especially given the implication that treatment may have been too short to exact statistical difference: time-points extending to day 12, 15 and 17 may clarify the current uncertainty.

Deterioration in locomotory rates correlates with *in-vivo* DAPI autofluorescence, which has been validated in long-lived mutants, suggestive of a physiological ageing rate (Gerstbrein et al., 2005). However, despite a plethora or multi-taxonomical investigations demonstrating calorie restriction to improve measures of healthspan including locomotory metrics, repeatedly it has been shown to not elicit significant changes in DAPI autofluorescence with age (Bansal et al., 2015; Gerstbrein et al., 2005). Afterall this thesis searches not to reinvent measures of healthspan but to mimic age-associated phenotypes induced by dietary restriction. And, to that end, rilmenidine continues to mimic certain trends induced by calorie restriction, by matching attenuations in both locomotion and autofluorescence and ensuring worms do not exhibit periods of protracted frailty and burden.

This chapter also set out to establish whether rilmenidine might be able to improve stress resistance as required for its qualification as a CRM (Ingram et al., 2006). Resistance to heat-stress is routinely found to correlate to increased lifespan: ~80% of genes that promote thermotolerance also increase lifespan by >15%. Thermal adaption to heat stress is conserved in all eukaryotes and serves to prevent consequential proteostatic collapse in the face of heat-induced protein destabilisation and unfolding (Higuchi-Sanabria et al., 2018). An extremely recent paper, released as I write my discussion, has showed a strong positive correlation between longevity and resistance to high thermal stress (Benedetto et al., 2019). The authors argued that severe thermal stress (>40ºC) better tested heat-shock response by denying animals the ability to adapt to the stress as would be possible in the moderate stress and recovery assay employed herein. It was demonstrated that autophagy is required to mediate this response, linearizing a relationship between increased autophagy, thermotolerance and decelerated ageing.

Another relevant finding by Benedetto et al. (2019) revealed lifespan was not correlated to oxidative stress resistance, casting further doubt on the necessity of improved oxidative stress as a determinant of lifespan (Gems and Doonan, 2009; Magalhães and Church, 2006). This a somewhat comforting reassurance when considering the inability of rilmenidine to rescue worms exposed to hydrogen peroxide. To answer why this might be the case, Benedetto et al. (2019) again provide some exceptional insight: autophagic machinery is not necessary for oxidative stress resistance and indeed it may in fact worsen sensitivities to oxidative stresses, especially at young adulthood. If rilmenidine does increase autophagy to improve thermotolerance it is unlikely to offer any great resistance to animals faced with oxidative stress. Furthermore, gene signatures from rilmenidine treated cells do not appear to be enriched for any ROS scavenger or detoxifying terms which may mitigate oxidative stress in animals. However, it must be noted that oxidative stress assays in this chapter were limited to acute stresses derived from one agent.

It is possible that rilmenidine may improve oxidative stress, as demonstrated by other CRMs, when different agents and exposure times are used (Onken and Driscoll, 2010; Robida-Stubbs et al., 2012; Schulz et al., 2007).

It was still essential to clarify how rilmenidine might signal improvements in frailty indices and thermotolerance. It was hypothesised these effects were meditated through imidazoline receptor binding. In a brief return to lifespan analysis as a confirmative prologue, rimenidine was administered to worms already being treated with, the imidazoline type 1 receptor antagonist, efaroxan. Rilmenidine was unable to further extend lifespan in comparison to efaroxan or rilmenidine monotherapy, suggesting a common binding site. Efaroxan has long been used to block imidazoline binding sites, so its prolongevity effect was somewhat surprising: if rilmenidine extends lifespan through an interactional direction with the imidazoline receptor, the opposite effect of that should at the very least fail to confer lifespan. However, efaroxan has binding affinities to other receptors which may eventuate "off-site" prolongevity effects. Efaroxan is a selective agonist for at the I_3 receptor (Clews et al., 2004). Research has shown that I_3R binding may be insulinotropic in pancreatic β-cells through intracellular increases in Ca^{2+} concentrations and blockade of K-ATP channels (Soldatov et al., 2018). Attempts to characterize the associated IBSs have been unsuccessful making speculation on how efaroxan may confer lifespan extension difficult (Nikolic and Agbaba, 2012). However, a pharmacological lifespan screen by Ye et al. (2014), implied interaction with potassium and calcium channels may lead to, an undiscovered, mechanism of pro-longevity. Thus, it is probable, rilmenidine signals through an imidazoline binding site in *C. elegans* to extend lifespan which can be effectively blocked by efaraxan; however given efaroxan itself may similarly increase lifespan through independent mechanisms, the resultant dual-therapy response is similar to that exacted by either of the imidazolines alone. Ideally, this would have been confirmed in knockout-rescue studies using the putative imidazoline receptor, *f13e9.1*, however, as discussed incubator failures precluded data acquisition. Nonetheless, initial longevity phenotype characterisation had been established in mutants.

Unexpectedly, *f13e9.1* mutants had extended lifespans in comparison to wild-types - a somewhat surprising result given the agonism of binding sites on *f13e9.1* by rilmenidine increased lifespan. Additionally, maximum lifespan was also increased suggesting a deferral of mortality was an unlikely cause. To better explain the role *f13e9.1* may play in rilmenidine-induced prolongevity, healthspan and stress resistance was re-examined. Despite, *f13e9.1* mutants living longer, they displayed accelerated signs of frailty and autofluorescence, whilst also being more sensitive to oxidative stress in comparison to WT. This paradox represents a disturbing manifestation of the Tithonian error. Furthermore, rilmenidine

treatment further worsened lipofuscin accumulation, oxidative stress resistance, thermotolerance and motility; although the latter two failed to reach significance. Whilst, an abrogation of successful ageing biomarkers was expected in *f13e9.1* mutants treated with rilmenidine, through ablation of a potential IBS, a worsening was not hypothesised. Moreover, while lipofuscin accumulation and oxidative stress resistance metrics insignificantly improved responses in rilmenidine-treated WT worms, in *f13e9.1* there was a reversed response in that they now were significantly worsened effects observed in WT.

In previous chapters, based on results from mutant murine studies, an activation of AMPK and subsequent downregulation of mTOR was hypothesised to result from a deletion of IRAS. Thus, *f13e9.1* mutations might lead to a phenocopy of IRAS mouse knockouts, which had been demonstrated in observed growth defects. Phenotypes of shorter body size and catabolic AMPK/mTOR alterations are reminiscent of CR such that one questioned whether deletion of *f13e9.1* may be a genetic CRM (Mörck and Pilon, 2006). When looking at genetic models of CR in *C. elegans*, in particular *eat-2,* a healthspan and stress resistance phenotype may also be comparable. Numerous papers have shown *eat-2* mutants to display accelerated locomotory decline in comparison to WT, alongside increased lipofuscin accumulation (Bansal et al., 2015; Hansen et al., 2008; Lüersen et al., 2014). Furthermore, *eat-2* worms are often more sensitive to heat-stress however, this is not consistent with oxidative stress (Bansal et al., 2015; Houthoofd et al., 2002a, 2002b). It has also been hypothesised that IRAS and thus potentially *f13e9.1* additionally function as sorting nexins. Mutations in sorting nexins in *C. elegans* dramatically decreased stress resistance, which may be linked to the reduced size of the amphid sensory compartment: nexins are required for the morphogenesis of sensory compartments, which if improperly developed my led to altered stimulus regulation (Oikonomou et al., 2012; Vieira et al., 2018)

When analysing this pattern in association with rilmenidine treatment, a DR phenocopy could again be argued. Metformin, a potent CRM, drastically, reduces the lifespan of *eat-2* mutants which would presumably affect healthspan parameters such as frailty and lipofuscin (Onken and Driscoll, 2010). Authors suggested that metformin might induce a starvation-like state from excessive metabolic shutdown, which toppled the delicate life-extending metabolic balance of *eat-2* mutants (Onken and Driscoll, 2010). However, how rilmenidine might still be able to elicit further “DR-states” in *f13e9.1* is difficult to answer. One possibility is that *f13e9.1* mutants maintain some functionality in the 1st and 2nd non-deleted exons which may have potential binding affinity. This may then lead to inverse agonism of a dysfunctional *f13e9.1* protein which may begin inappropriately interacting with binding partners such as LKB-1. Furthermore, *f13e9.1* mutants, maintaining PHOX domain interactivity in 5’ exons may lack regulation from a deleted coiled coil domain such that it triggers unregulated membrane binding, and

deleterious outcomes for healthspan and stress resistance, which becomes even more deleterious upon activation by rilmenidine. Or *f13e9.1* functionality is significantly downregulated by its mutagenesis, which is then completely shut down by rilmenidine treatment. Alternatively, rilmenidine may weakly bind to other receptors in the absence of *f13e9.1* leading to harmful signalling related to oxidative stress, which may cause accelerations in lipofuscin accumulation. Whilst, conclusions on adverse reactions in *f1e9.1* are difficult to make, results do suggest that rilmenidine requires *f13e9.1* to attenuate locomotory declines and heat stress and that rescue strains PHX945 preferentially restores these proclivities. Despite locomotion still declining quicker in PHX945 in comparison to WT, it did rescue the ability of rilmenidine to attenuate such deteriorations, implying that a potential healthspan benefit signals through interactions with *f13e9.1*. Furthermore, improved thermotolerance can be restored to levels observed in WT, however this did not quite reach significance. This failure to reach significance is only apparent following multiplicity correction and individual analysis assumes rilmenidine to improve heat stress in both rescue lines. The difficulty in observing statistically clear survival improvements is likely potentiated by an increase in basal survival in fosmid rescues. Fosmid derived *f13e9.1* is highly tagged which may disturb function as previously documented. This may lead to moderate downregulation of *f13e9.1* mimicking moderate *f13e9.1* inhibition by rilmenidine, leading to improved thermotolerance.

When assessing how *f13e9.1* binding interactions with rilmenidine may transduce improved thermotolerance, experimental evidence in this thesis only provides ERK signalling as an option. Fortunately, heat-shock induces two major signalling events: the transcriptional induction of HSPs and the activation of ERK (Petrocchi et al., 2010). Furthermore, recent studies reported that there was a correlation between protective HSP70 expression and activation of the MAPK pathway. Thus rilmenidine, which increases pERK via *f13e9.1,* may improve heat-shock response via ERK signalling. As example, epicatechin modulates stress-resistance in *C. elegans* through increases in ERK. It is also important to note that the ERK-MAPK pathway regulates longevity and knock-down of its function shortens lifespan (Okuyama et al., 2010). Together, this provides an interesting link between rilmenidine treatment, ERK activation, thermotolerance and lifespan. This is especially relevant since ERK activation following heat stress is also diminished as a function of age, providing opportunity for rilmenidine to improve functionality into age, which may in turn improve markers of healthspan such as locomotion. Indeed, cells derived from long-lived animals exhibit a prolonged low level ERK activation following heat-stress which is suggested to contribute to improved stress resistance and longevity (Elbourkadi et al., 2014; Sun et al., 2009). Imidazolines also induce prolonged moderate increases in ERK activity, which may be able to mimic a response to stress characteristic of long-lived animals (Edwards et al., 2008; Li et al., 2006; Piletz et al., 2003b; Sun et al., 2007).

These results suggest rilmenidine to effectively attenuate frailty indices in *C. elegans* and improve thermotolerance which is dependent on *f13e9.1*, thus continuing its growing qualification as a bone fide CRM. Conversely, *f13e9.1* mutants, whilst living longer, exhibit shorter time to frailty and diminished stress resistance. This highlights *f13e9.1* as potential Tithonian mutant similar to some long-lived strains. Furthermore, oxidative stress resistance and lipofuscin are worsened by rilmenidine treatment in *f13e9.1* mutants which is overwise unaffected in WT. This suggests a sensitising effect in *f13e9.1* mutants that is decoupled from rilmenidine's interaction with an IBS on *F13E9.1*. However, making any such claims is difficult in the face of poor signalling characterisation of *f13e9.1* or indeed it's homolog IRAS. It is hypothesised that rilmenidine may improve heat-stress through an efficacious increase in pERK although this stands to be tested using ERK knockdown models exposed to heat-stress and rilmenidine. In summary, these results show a promising potential of rilmenidine to mimic CR through the improvement of thermotolerance in worms, although potentially separately through ERK activation and autophagic responses which sets the scene for greater resistance to life stressors. Such improved capacity represents greater organ reserve characteristic of "healthspan" which may prolong this period as manifested in reduced locomotory deterioration in *C. elegans* (Neustadt and Pieczenik, 2008).

6. Results: Rilmenidine Extends Lifespan Through Dietary Restriction Associated Signalling Pathways

6.1. Introduction

This thesis has so far presented evidence demonstrating rilmenidine to improve lifespan in *C. elegans* when administered throughout adulthood or agedness. This corresponded to delayed manifestations of age associated phenotypes of lipofuscin accumulation and nematodal frailty. Furthermore, rilmenidine treated worms have improved stress resistance which corelates to extended healthspan. Indeed, these characteristics are shared with calorie restriction. Specifically, DR in worms, whether by bacterial restriction or genetically induced obstruction of food consumption, attenuates age-associated lipofuscin accumulation and motility impairments (Gerstbrein et al., 2005; Son et al., 2019). Moreover, DR in worms leads to greater resistance to thermal and oxidative stress (Houthoofd et al., 2005). Whilst such effects are mimicked by rilmenidine, transcriptional analysis revealed no similarity between DR and rilmenidine treatment. However, previously published transcriptional profiles of DR have been contradictory and gene expression effects vary in magnitude between studies (Gillespie et al., 2016). Furthermore, calorie restriction is vastly pleiotropic and heterogeneity through secondary effects likely leads to such ambiguity. This may mask more targeted gene expression changes elicited by rilmenidine, which whilst altered by DR, are diluted within “off-site” gene expression upstream by DR.

As previously highlighted, two key nutrient responsive gene products, AMPK and TOR, have been considered “master regulators” of dietary restriction. The ability of potential CRMs to affect the activity of these pathways specifically may offer a more orthogonal approach to CR mimicry without superfluously auxiliary effects. Classical genetic epistasis analysis reasons that if two interventions result in an additive phenotypic response their mechanism of induction must differ. Thus if rilmenidine acts through the mimicry of DR, animals already undergoing DR could not further respond in a synergistic manner to rilmenidine. This logic is applicable to downstream mutational targeting of DR-associated genetic pathways. Pre-set downregulation of mTOR activity would make mTOR inhibitors unable to elicit further lifespan extension, and in a similar vein, AMPK loss of function would preclude its geroprotective activation. Thus, to clarify current uncertainty surrounding the relationship of rilmenidine to CR, the interactive pathway of rilmenidine should be followed to isolate potential genetic targets which could be mediating a CRM pro-longevity function.

However, whilst, lifespan offers a most convenient and holistic metric for ageing deceleration, it is an insufficient, indirect and resultant read-out for the activation of numerous longevity mechanisms within a cell. Any overlapping processes between dietary restriction and rilmenidine ideally should also be defined. It is demonstrated hereafter that rilmenidine likely signals through both dietary restriction and additionally through AMPK and TOR signalling. Dietary restriction, AMPK activation and TOR inhibition, unanimously converge one distinct process: autophagy.

CR is routinely cited as the most robust physiological inducer of autophagy and nutrient depletion is the gold-standard for autophagic induction in culture (Kroemer et al., 2010). Thus, autophagy is a vital component of CR-induced longevity. Multiple models have demonstrated that the longevity benefits of dietary restriction are abrogated following the knockdown of autophagic machinery (Hansen et al., 2008; Jia and Levine, 2007; Rubinsztein et al., 2011). As such, researchers often seek to demonstrate that their CRM of interest upregulates autophagy and that this upregulation mediates its lifespan extension (Eisenberg et al., 2009; Pietrocola et al., 2018; Shintani et al., 2010). In fact, the relationship between calorie restriction and autophagy is so inexorable that researchers have proposed a CRM criteria solely pertaining to how a compound upregulates autophagy (Madeo et al., 2019).

In 2008, an *in-vitro* screen for autophagy enhancers returned rilmenidine as a hit (Williams et al., 2008). Rilmenidine was able to clear A30P α-synuclein, a known autophagy substrate, in stable inducible PC12 cells, and furthermore, increased LC3-II levels (autophagic protein markers) in a dose-dependent manner (Williams et al., 2008). Subsequent exploration within the same paper revealed rilmenidine to upregulate autophagy via an mTOR independent cAMP mediated manner. Importantly, clonidine, another imidazoline receptor agonist, upregulated autophagy in a similar manner (Williams et al., 2008). Rilmenidine has since been administered in a number of disease models to assess its capacity to ameliorate neurodegeneration. Firstly, in a Huntington's murine model, rilmenidine decreased levels of mutant huntingtin and improved motor phenotypes, yet failed to improve survival (Rose et al., 2010). More recently, in a murine model of ALS, generated through transgenic expression of a G93A mutant form of human SOD1, rilmenidine elicited significantly upregulated *in-vivo* autophagy and clearance of soluble mutant SOD1 (Perera et al., 2018). However, the autophagic potential for rilmenidine has never been explored in *C. elegans* and more importantly, no efforts have been made in any model to associated this to ageing or calorie restriction. Thus this chapter serves to explore the pathways by which rilmenidine might mimic CR, whether any mimicry culminates in a change in autophagy, and finally whether that is mediated through cAMP signalling.

6.2. Results

6.2.1. Rilmenidine Extends Lifespan Mediated By Established Calorie Restriction Nexuses

Starting with an upstream evocation of CR, the *eat-2 (ad1116)* strain was employed. *eat-2* mutants lack the encoded nicotinic acetyl-choline receptor which renders the pharyngeal muscle unresponsive(Avery, 1993). Animals are unable to efficiently grind the bacteria, providing a bona fide genetic surrogate for DR on regular media plates. e*at-2* live around 30% longer than their WT counterparts, comparable to other long-lived mutants and represent the most long-lived of the eat gene family (Lakowski and Hekimi, 1998). This lifespan extension is similar to that observed in mammals and both exhibit a correspondingly pale and thin morphology. As such, this has popularised the use of *eat-2* and specifically the *ad1116* allele in renowned ageing labs (Crawford et al., 2007; Hansen et al., 2008). Lifespan assays were performed in *eat-2 (ad1116)* worms treated from adulthood with either a 1% DMSO control or rilmenidine a 200μM pro-longevity dose. Confirmatively, *eat-2 (ad1116)* worms grown on 1% DMSO NGM plates lived 14.63% longer than condition matched WT controls (Figure 19F). Although, *eat-2* worms are the most consistently long-lived lifespan variability is still apparent, potentially explaining the more modest results observed in this thesis (Lakowski and Hekimi, 1998). The administration of rilmenidine to *eat-2* worms not only failed to additively increase lifespan, it significantly reduced lifespan in a toxic manner (log rank test; p=2.90E-08) (Figure 19*F)*. Rilmenidine treated *eat-2* worms lived 16.94% shorter lives than those treated with only a 1% DMSO vehicle (*Figure 19*F). This is strikingly reminiscent of the effect of metformin on *eat-2* mutants, wherein pro-longevity doses of metformin are reproducibly detrimental to the viability of *eat-2* mutants (Onken and Driscoll, 2010). Thus, given the similarity in response it was imperative to assess the necessity of AMPK to mediate the prolongevity effect of rilmenidine. Of note, the Lifespan Machine at the Ewald lab was utilised to verify these findings; they found rilmenidine to not significantly affect lifespan of *eat-2* worms in either direction.

Onken and Driscoll (2010), demonstrated that metformin, the most renowned putative CRM, was unable to additively extend lifespan in an AMPK mutants (*aak-2*) at prolongevity doses. *C. elegans aak-2* encodes one of two homologs of the catalytic α subunit of the AMPK heterotrimeric complex, and nematode *AAK-2* is activated by AMP, as occurs for mammalian AMPK (Onken and Driscoll, 2010). *Aak-2* is the preferred catalytic subunit to mutagenize owing to ability to elicit specific longevity phenotypes unlike *aak-1* (Apfeld et al., 2004). Three mutant allele strains are available for *aak-2*; allele *gt33* harbours the largest deletion of the alleles available (606bp) (Cordero and Viollet, 2016). Hence, lifespan assays were performed in *akk-2 (gt33)* worms treated from adulthood with either a 1% DMSO control or

rilmenidine a 200μM pro-longevity dose. Accordingly, and as reported similarly elsewhere, *aak-2* mutants lived 11.33% longer than condition matched WT controls (WT 23.84 ± 0.17 mean days; *aak-2* 26.54 ± 0.33 mean days S.E.M, log rank test: $p<0.05$) (Figure 19E). Whilst rilmenidine treatment did result in significant mean lifespan extension in *aak-2* mutants, the effect was far more tempered and modest to that elicited in WT: rilmenidine increases mean lifespan in WT wildtype by 19% whereas, in *aak-2* mutants, this effect was only 5.43% *(*Figure 19E). Seemingly, some prolongevity benefit from rilmenidine may be in some way derived from interaction with AMPK. It is thus likely, rilmenidine interacts with other longevity associated pathways as well.

As highlighted in chapter 1.3.1, mTOR inhibition is one the proposed mechanisms of DR induced lifespan extension and geroprotection; and key downstream transcription factors of TOR: S6K, eIF2 and eIF4G, all increase lifespan in *C. elegans* when suppressed (Hansen et al., 2007). Furthermore, Hansen et al. (2008) showed CR and TOR inhibition in combination does not further extend lifespan in *C. elegans*, thus highlighting an overlap in their pro-longevity signalling. Downstream targets of nematode DR are similar to that of TOR inhibition and include FOXO types, SKN-1 and HIF-1 (Yanase et al., 2019). CeTOR exists in two distinct complexes: TORC1 containing the principle TOR component, the coactivator DAF-15/Raptor and TORC2 including RICT-1/Rictor. TOR in worms is coded for by *let-363*, and its homozygous deletion causes dauer-like larval arrest, limiting it's use for translational lifespan assays (Jia et al., 2004). Additionally, *daf-15* homozygous mutants show larval arrest (Jia et al., 2004). Heterozygous strains have been developed to lessen the developmental penetrance, resulting in a gain in lifespan. Thus researchers routinely use this model, which is often crossed with a phenotypical marker gene, when assessing if an anti-ageing interventions signal via the TOR pathway (Han et al., 2017; Honda et al., 2015; Sanchis et al., 2019). The strain *daf-15(m81)/unc-24(e138)* IV, developed in the Riddle lab, was utilised owing to its natural selection pressure to heterozygotes given homozygous lethality of the *unc-24* gene (Jia et al., 2004). Confirmatively, *daf-15(m81)/unc-24(e138)* IV worms grown on 1% DMSO NGM plates lived 11.28% longer than condition matched WT controls (WT 23.84 ± 0.17 mean days; *daf-15* 24.56 ± 0.95 mean days S.E.M, log rank test: $p<0.05$) Figure 19B. I further validated the model by demonstrating that, at concentrations, previously shown to extend lifespan in *C. elegans*, rapamycin (100μM) failed to significantly affect the basal mean lifespan of *daf-15(m81)/unc-24(e138)* heterozygotes (log rank test: $p>0.05$) (Robida-Stubbs et al., 2012) (Figure 19*B*). Importantly rilmenidine also elicited an insignificant change in lifespan, suggesting that lifespan extension by rilmenidine is dependent on TOR functionality (log rank test: $p>0.05$) (Figure 19*A*).

Due to a poor sample size (n<50 per condition), precipitated by difficulty in identifying penetrant heterozygotes for hand-picking onto small drug plate, an alternative approach was sought after to confirm epistatic observations. Accordingly, WT worms were co-incubated from day 1 adulthood with rilmenidine (200µM) and rapamycin (100µM), an established CeTOR inhibitor (Robida-Stubbs et al., 2012). Importantly, rapamycin alone significantly increased mean lifespan by +23.92% in WT worms compared to a 1% DMSO vehicle control (1% DMSO=22.07 days ± 0.5 S.E.M vs rapa 27.35days ± 0.49 S.E.M; log rank test w/ Bonferroni correction; p=0.000) (Figure 19A). Likewise, rilmenidine alone, as repeated in previous trials, significantly extended mean lifespan by +36.16% in WT worms compared a 1% DMSO vehicle control (rilmenidine=30.05 days ± 0.61 S.E.M vs 1% DMSO=22.07 days ± 0.5 S.E.M; log rank test w/ Bonferroni correction; p = 0.05) (Figure 19A).. However, whilst rilmenidine and rapamycin combination therapy increased mean lifespan in WT worms compared to controls, there was no additive effects, and co-treatment with rilmenidine and rapamycin failed to impart further mean lifespan extension than worms treated with either rilmenidine or rapamycin monotherapy (Ril vs. Ril & Rapa p=0.703; Rapa vs. Ril & Rapa p=1) (Figure 19A).

It is also well established in worms, that AMPK activation and TOR inhibition by genetic or pharmacological intervention, like in mammals leads to activation of key longevity transcription factors such as *skn-1* and *daf-16* (Robida-Stubbs et al., 2012; Templeman and Murphy, 2018). Moreover, on solid media, *daf-16* is indispensable for CR-induced longevity. To explore whether rilmenidine induced some of its longevity effects, a *daf-16* mutant model was employed, specifically the commonly used *daf-16(mgDf50)* null allele due to the presence of a large deletion within the gene. It has been routinely used when assessing the signalling pathways of potential longevity compounds (Hunt et al., 2011; Wilson et al., 2006). Lifespan assays on *daf-16* were employed using the lifespan machine by the Ewald lab, and found rilmenidine did not significantly affect lifespan in either direction (*daf-16* w/DMSO 19.37± 0.21 days; *daf-16* w/ rilmenidine 18.52 ± 0.22 days S.E.M, log rank test: p<0.15) (Figure 19G).

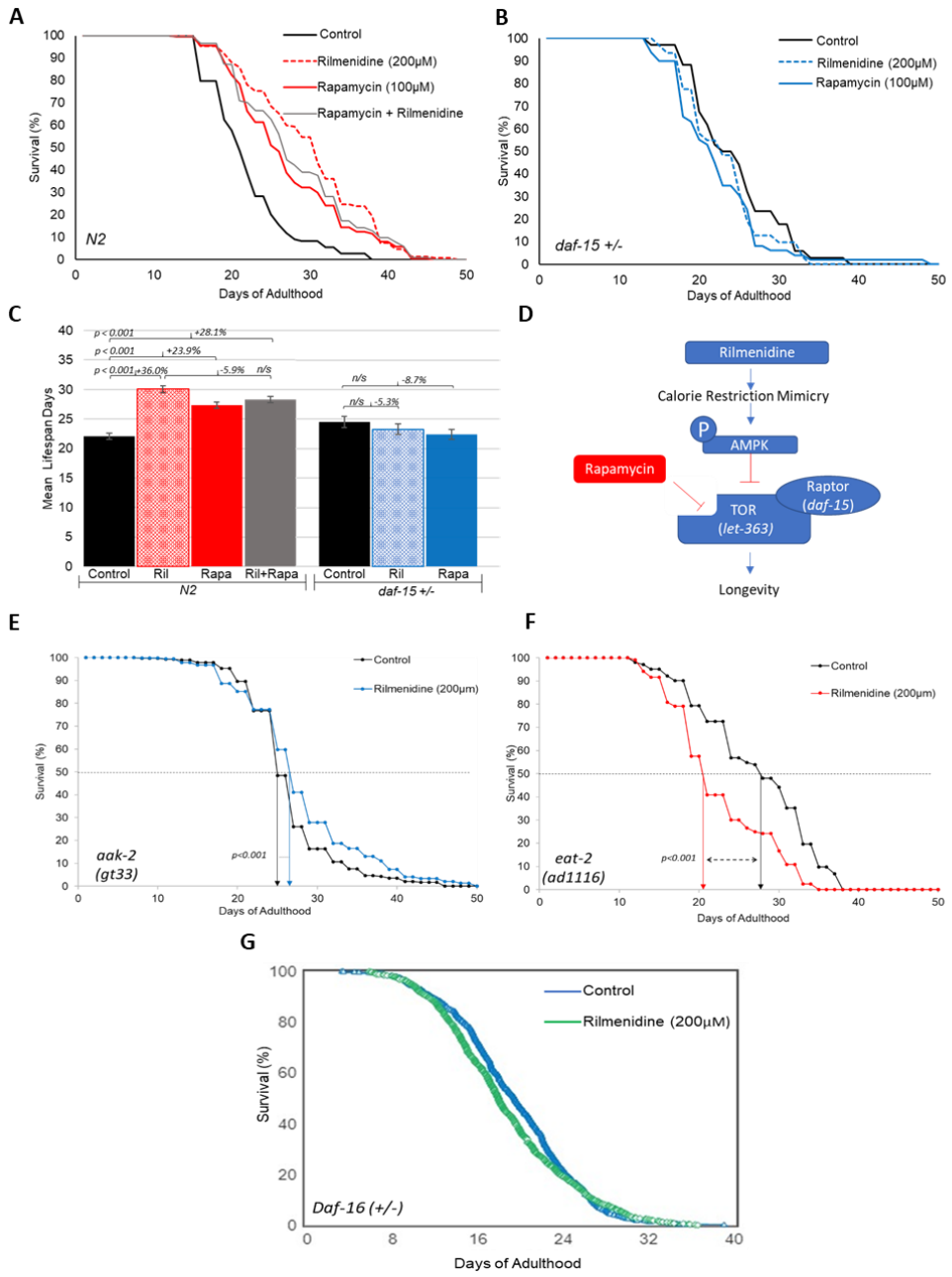


Figure 19. Effects of rilmenidine treatment on survival of CR associated mutants. *Pooled survival curves of f) eat-2 e) aak-2 b) daf 15 +/- g) daf-16 worms fed UV-killed OP50 also treated either a prolongevity dose of rilmenidine (200μM) or a 1% DMSO vehicle. Statistical data analysis of survival curves between treatments and not genotype was performed using log-rank testing and bonferonni correction; statistical data available in table. C) Pooled survival curves of adult WT worms fed UV-killed OP50 also treated with Rilmenidine (200μM) and rapamycin (100μM) independently, or in combination, compared to control animals treated with a 1% DMSO vehicle. Statistical data analysis of survival curves between treatments and not genotype was performed using log-rank testing and Bonferroni correction; statistical data available in table. D) mean lifespan (days) of pooled trials measuring the survival curves of panel C. Error bars represent SEM; adjusted p-value derived from log-rank test and Bonferroni correction. F) Artistic representation of possible prolongevity signalling of rilmenidine. Rilmenidine may act in a CRM manner through the activation of AMPK and subsequent downstream inhibition of ceTOR.*

6.2.2. Rilmenidine Induced Lifespan Extension is Mediated by Autophagic Upregulation

To assess the capacity of rilmenidine to increase autophagy, reference was made to published guidelines (Chen et al., 2017; Papandreou and Tavernarakis, 2017; Zhang et al., 2015). Therein, authors recommend monitoring the appearance of distinct autophagic puncta in response to interventions. In *C. elegans*, autophagy is a highly conserved process, and autophagosomes are formed by the proteins LGG-1 and LGG-2, homologous to Atg8/mammalian LC3B (cytosolic microtubule-associated protein 1 light chain 3-MAP1LC3), however their mechanism remains largely elusive (Papandreou and Tavernarakis, 2017). Nonetheless, it is known that LGG-1/LGG-2 are conjugated to a phosphatidylethanolamine (PE) lipid during autophagosome formation and these lipoproteins decorate the outside of the membrane (Wu et al., 2012). When an autophagosome has engulfed its requisite target, it subsequently fuses with a late endosome or lysosome to form an autophagolysosome (or autolysome) for degradation (Meléndez et al., 2008). The congregation and lipidation of LGG-1 during autophagosome causes the diffuse cellular distribution pattern to become distinctively punctate (Papandreou and Tavernarakis, 2017). Researchers have utilised this phenomena by generating a number of transgenic reporter strains carrying fluorescent tags of LGG-1, which can visualise and quantify autophagic induction to assess response to environmental conditions and potential anti-ageing compounds (Chen et al., 2017). The transgenic worm (vkEx1241) expresses an extrachromosomal N-terminally fused mCherry::LGG-1 construct driven off an intestinal *nhx-2* promoter and is recommended for implementation in guidelines (Palmisano and Meléndez, 2016; Zhang et al., 2015). It has been routinely used to identify compounds which upregulate autophagy (Gosai et al., 2010; Li et al., 2014). Furthermore, the pro-longevity effect of DR is primarily regulated by intestinal autophagy (Gelino et al., 2016).

Autophagosome formation was observed in the transgenic autophagy reporter strain *vkEx1241* by fluorescence microscopy. Autophagic activity was quantified by the number of LGG-1:mCherry puncta cross-sectionally present per mm^2 of fluorescent posterior intestine (See chapter 2.8.1). 24h incubation with rilmenidine in 2 day adults significantly increased mCherry::LGG-1 puncta formation in a dose-dependent manner (Figure 20B). Specifically, the pro-longevity dose of rilmenidine produced the most modest, but still significant, increases in puncta formation (one-way ANOVA; adj. $p<0.01$). 300µM incubation further increased median puncta number by a magnitude of 2-fold, and 400µM incubation increased median puncta number by 2.5 fold in comparison to a 1% DMSO vehicle (300µM vs DMSO $p=0.001$; 400µM vs DMSO $p=0.001$) (Figure20B). Increases in autophagy in LGG-1 positive punctae can be attributed to either activation of autophagy or decreased autophagosome clearance due to a block in autophagosome-lysosome fusion. Given the hypothesis that rilmenidine increases autophagic induction, it is essential to infer the turnover of autophagosomes in the presence and absence of lysosomal

degradation. Confirmatively, rilmenidine continued to increase LGG-1 positive puncta in adult day 2 worms co-incubated with chloroquine, at previously utilised doses (20mM) to stall autophagic clearance (Figure 10) (Burkewitz et al., 2012; Choe and Strange, 2008).

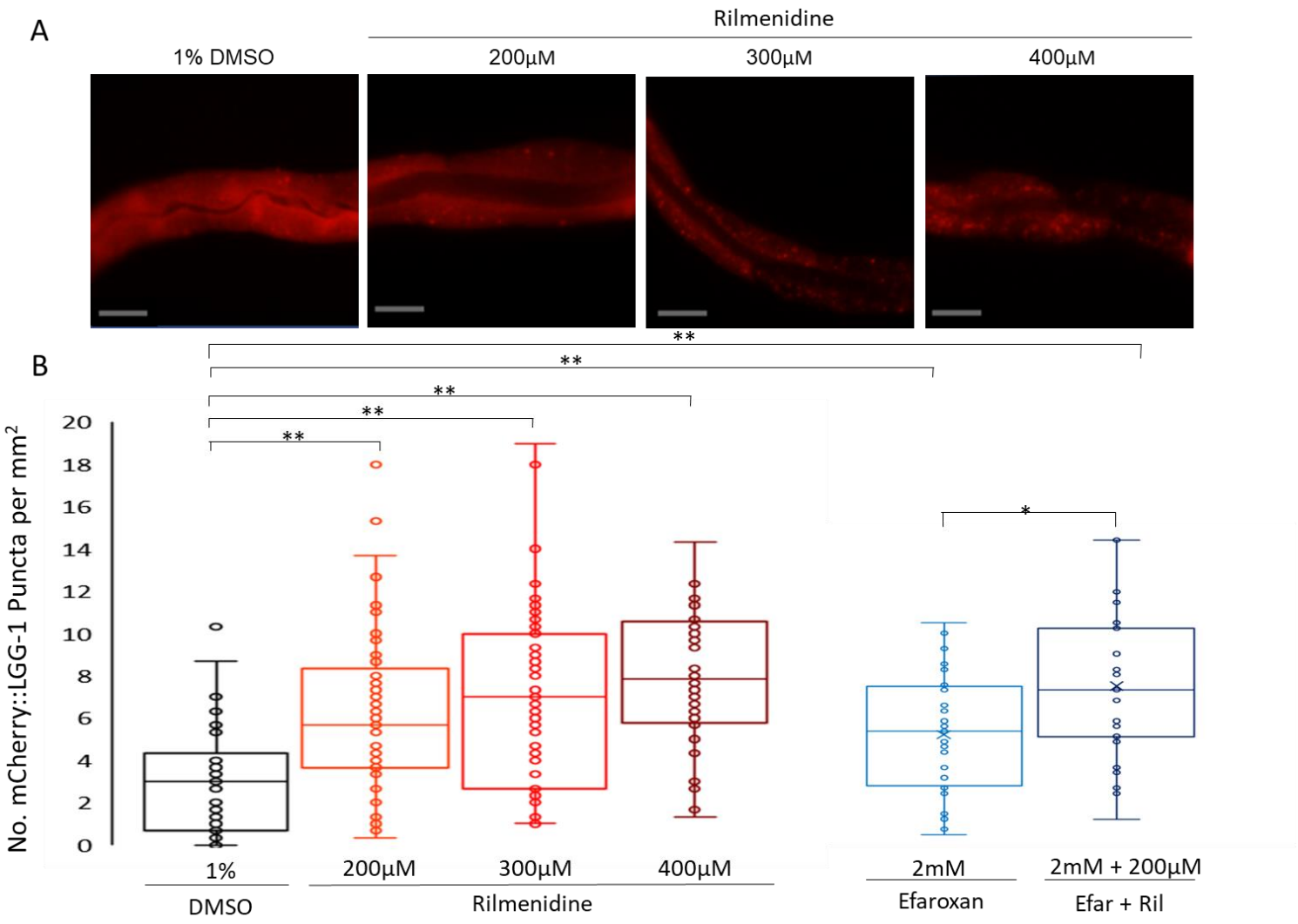


Figure 20. Induction of autophagy by rilmenidine and efaroxan in a synergistic manner. *A) Representative Images of well-fed day 2 adult transgenic animals, expressing the intestinal specific autophagy reporter gene Pnhx-2mCherry::lgg-1 and exposed to varying concentrations of rilmenidine or a 1% DMSO vehicle for 24h. Worms were immobilized and mounted onto glass slides in 20mM tetramisole prior to imaging at x10 objective on a Zeiss Axio Observer with a 150ms exposure. Grey triangular arrows highlight characteristic puncta to guide Scale bar, 20 µm. B) Graph shows the interquartile distribution of mean number of mCherry::LGG-1 puncta in the posterior intestine of the animals in each condition. Error bars, upper:Q3 + 1.5*IQR; minimum: Q1 -1.5*IQR. mCherry::LGG-1 puncta were counted in each of three rectangular boxes of 1mm2; they were placed in tandem beginning with the most posterior region of the intestinal and extended toward the vulva; mean number per mm^2 was calculated for each animal. N>25 animals analysed per treatment in at least 3 independent trials . **P<0.01, *P<0.05; one-way ANOVA followed by a Tukey's post hoc test.*

Having previously demonstrated that rilmenidine-induced ERK activation is abrogated by the blockade or deletion of the imidazoline binding site, it was hypothesised that autophagy may be upregulated via interaction with this binding site. Conversely, blockade of the imidazoline binding site by efaroxan increased median autophagosome formation in VkEx1241 strains 2-fold (adj. p=0.002) (Figure 20B). Moreover, co-incubation with rilmenidine synergistically and significantly increased autophagosome formation in comparison to worms treated with either rilmenidine or efaroxan monotherapy (Ril vs. Ril & Efar p=0.001; Efar vs. Ril & Efar adj. p=0.026).

Next, it was prudent to determine whether upregulated autophagy was required for the prolongevity effect of rilmenidine. Autophagy is an essential component of development and autophagy mutants exhibit divergent lifespans. Furthermore, the effects of rilmenidine on lifespan are measured from adulthood, so thus the inhibition of autophagic machinery is best done via RNAi using a feeding protocol (see chapter2.8.2) that allows knockdown commencement from adulthood (Meléndez et al., 2008). Selection of autophagy genes to knockdown is also important to ensure complete coverage of the pathway. Using the Ahringer library, *E.coli* strains transformed with either the plasmids CUUkp3301P034Q (*lgg-1)* or CUUkp3303WT 02Q (*bec-1)* were used to knockdown different stages of the autophagy pathway as performed in previous research (Schmeisser and Parker, 2018; Seo et al., 2018). Bec-1 is involved in the vesicle nucleation, whereas, *lgg-1* participates in vesicle elongation (Meléndez and Levine, 2009). Knockdown by dsRNA was validated by measuring autophagy inhibition of vKEx1093(*nhx-2*::*mCherry*::*lgg-1)* worms in the presence of rapamycin rather than RT-PCR; a decision based on previous methods (Figure 21D) (Miedel et al., 2012; Pietrocola et al., 2018; Schipanski et al., 2013) . Knockdown of *bec-1* RNAi modestly decreased the lifespan of WT worms as previously demonstrated (Figure 21A) (Jia and Levine, 2007). Given the necessity for live *E.coli* to be used in RNAi experiments, concerns were raised that live bacteria may metabolise rilmenidine and reduce its bioavailability. Nevertheless, worms treated with a prolongevtiy dose (200µM) of rilmenidine from adulthood on plates seeded with live HT115 vector *E.coli* continued to demonstrate a significant 8% lifespan extension (log rank; adj. p=1.40E-05). It is likely the lessened prolongevity effect is due to some degree of bacterial metabolization as referenced in other pharmacological intervention studies (Zheng et al., 2013). Importantly, *bec-1* RNAi prohibited lifespan extension in WT worms treated with a prolongevity dose (200µM) of rilmenidine, and treated worms exhibited similar lifespans to *bec-1* RNAi WT worms (log rank; p=1).

Lgg-1 knockdown alone significantly reduced lifespan in WT worms as previously reported (Hashimoto et al., 2009b; Schmeisser and Parker, 2018). As demonstrated in *bec-1* knockdown, *lgg-1* RNAi

prohibited lifespan extension in WT worms treated with a prolongevity dose (200μM) of rilmenidine, and treated worms exhibited similar lifespans to *lgg-1* RNAi WT worms (log rank; adj. p=0.482) (Figure 21B). Taken together these results suggest that the longevity effect of rilmenidine requires autophagy.

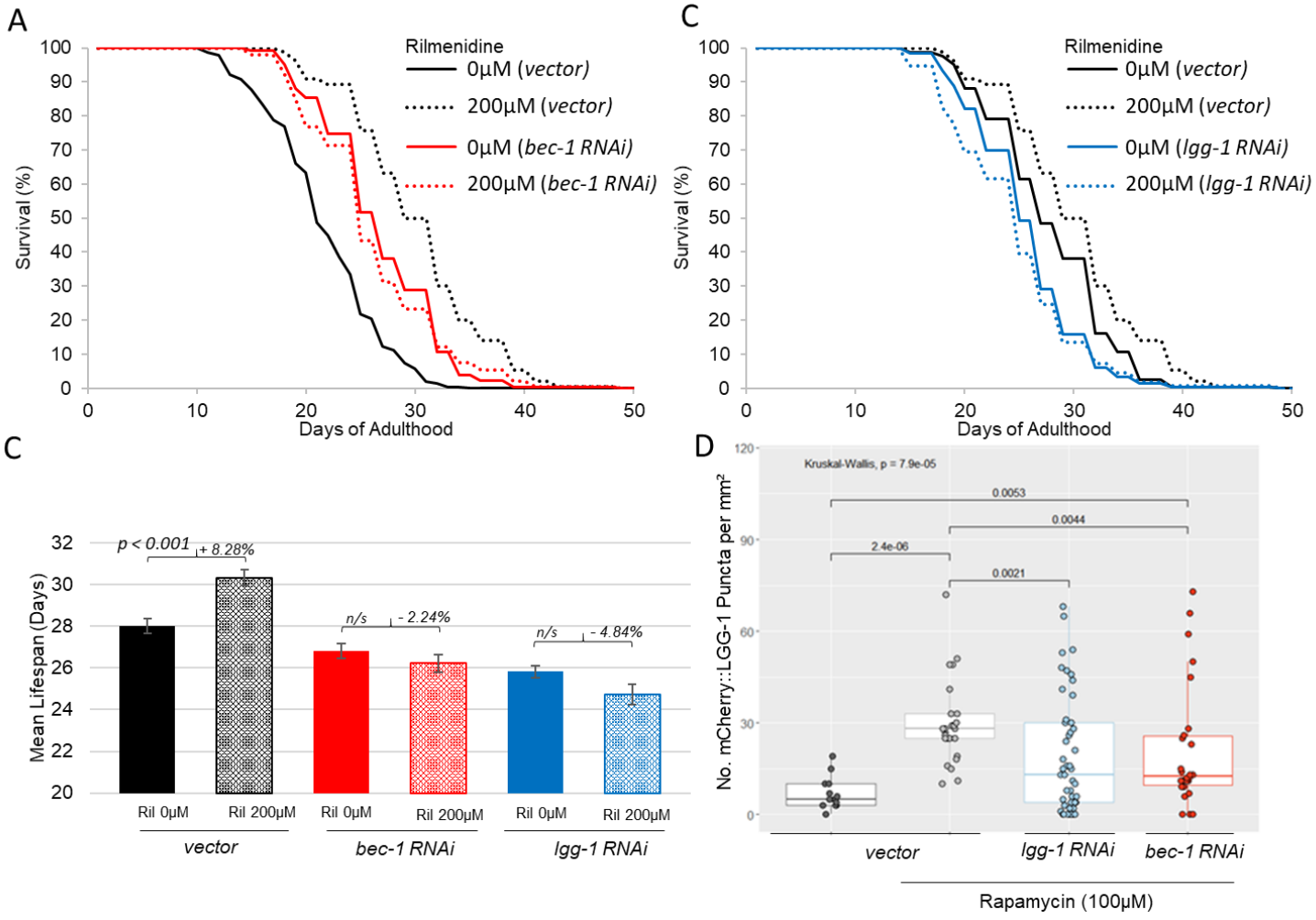


Figure 21. Inhibition of autophagy abrogates the prolongevity effect of rilmenidine. *Survival curves of WT animals fed either HT115 E.coli expressing an empty vector or HT115 E.coli expressing (A) bec-1 dsRNA (B) lgg-1 dsRNA from day 1 adulthood in the presence or absence of 200µM rilmenidine. C) Quantification of mean lifespan (days) and % change in WT worms under different RNAi and rilmenidine treatment. Statistical analyses performed using the log-rank test and Bonferroni correction against pooled data of 3 independent trials. N>150 worms per pooled condition. Rilmenidine significantly increased lifespan by 8.29% in WT animals fed HT115 E.coli expressing an empty vector (p<0.001), however failed to significantly affect lifespan in worms fed HT115 E.coli expressing bec-1 or lgg-1 dsRNA; n/s = p>0.05). D) quantification of RNAi knockdown validation. Adult vKEx1093(nhx-2::mCherry::lgg-1) worms were fed either HT115 E.coli expressing an empty vector or HT115 E.coli expressing bec-1 dsRNA lgg-1 dsRNA from day 1 adulthood in the presence or absence of an autophagy inducing dosage of rapamycin (100µM). After 3 days the no. of visible lgg-1::mcherry puncta were counted in at least 12 worms across 3 independent trials. Bec-1 and lgg-1 RNAi significantly reduced the number of lgg-1::mcherry puncta in rapamycin-treated worms (Kruskal-Wallis; lgg-1 vs. vector p=0.021, bec-1 vs. vector p=0.0044).*

6.2.3. Rilmenidine Attenuates PolyQ Aggregation

One important hallmark of neurodegenerative diseases is the accumulation of protein aggregates, which should typically be degraded via autophagy. Age-related protein aggregation and misfolding is evident in routine *C. elegans* ageing and precipitates a pronounced, widespread decline in proteostasis, which some argue is a programmed event (Labbadia and Morimoto, 2014). Neurodegenerative diseases are therefore often symptomatic of an accelerated functional decline during ageing. As such, researchers have been continually using models of neurodegeneration in *C. elegans* to elucidate molecular aspects of disease. The inexorable tie between proteostatic collapse and ageing means many interventions that extend lifespan in worms also alleviate phenotypes of aggregative transgenic worms (Devagi et al., 2018; Keowkase et al., 2018; Kim et al., 2019; Marcellino et al., 2018; Negi et al., 2017; Zhang et al., 2012). Moreover, researchers often demonstrate that potential pro-longevity compounds upregulate autophagy alongside a conferred amelioration of aggregation phenotypes in worms, whilst also increasing lifespan in wild-type worms (Boasquívis et al., 2018; Denzel et al., 2014).

As introduced in this chapter, rilmenidine has already been shown to alleviate neurodegenerative phenotypes in a mouse model of Huntington's disease through a autophagy driven attenuation of polyglutamine expansions (Rose et al., 2010). It thus seemed logical to utilise a similar model in *C. elegans* to observe *in-vivo* attenuation of PolyQ aggregations which could demonstrate the autophagy upregulated by rilmenidine was indeed productive and not an idiosyncratic off-site artefact. Differing dosages of rilmenidine (200,300,400µM) were administered to L1 transgenic polyQ model AM141, which expresses polyQ40::YFP fusion proteins in body wall muscle cells and shows a discrete fluorescent aggregate phenotype when reaching adulthood (See chapter 2.9.5). Rilmenidine, at all treatments significantly delayed the accumulation of YFP aggregates, but by 96h, aggregates matched that observed in WT (all treatments $p<0.05$; 2-way repeated measure ANOVA with Tukey correction) (Figure 22).

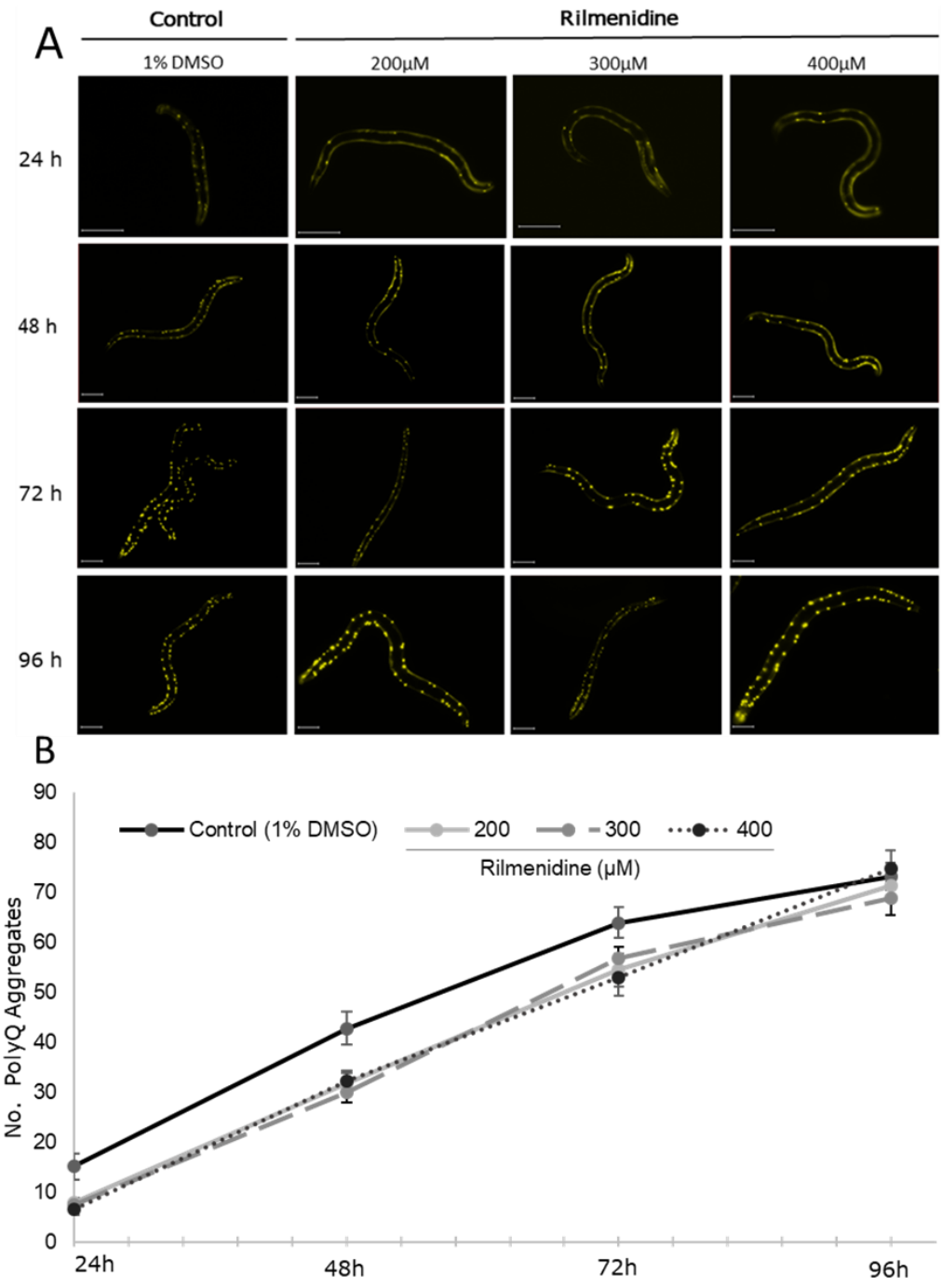


Figure 22. Reduction of polyQ40 aggregation by differing rilmenidine concentrations in C. elegans AM141. *(a) Fluorescent micrographs of AM141 nematodes expressing polyQ40::YFP in body-wall muscle cells at the indicated times and treatment for L1. Scale bars = 100µm. B) quantification of Q40::YFP aggregates per entire nematode after treatment with differing rilmenidine concentrations for the indicated times from L1. At least 30 worms were quantified for each treatment and time-point across 3 independent trials. Data represented as the pooled mean no. aggregates per worm. Error bars are ±S.E.M. Significance derived from 2-way repeated measures ANOVA. All concentrations of rilmenidine significantly attenuated aggregation of Q40::YFP; $p<0.05$.*

Table 10. Differences in mean and maximum lifespan of different C. elegans genotypes exposed to various perturbations relative to the control within the same sets of trials. *Mean lifespan in days is the pooled mean of treatment cohort and the standard error (± S.E.M). Maximum lifespan defined as the day at which 90% cohort mortality was reached. Lifespan assays performed at 20 °C. Adjusted p-value was determined by log-rank test using the Kaplan-Meier survival analysis and subsequent bonferonni correction. Pooled n = number of deaths observed across all respective trials.*

Genotype	*Pooled n=*	No. trials	Mean lifespan (days)	Std. error	% Chg Vs. Mean Ctrl.	90% Mortality (days)	Adj. p-value
WT							
1% DMSO	109	3	22.07	0.5	N/A	28	N/A
Rilmenidine (200µM)	150	3	30.05	0.61	36.16	39	0.00E+00
Rapamycin (100µM)	223	3	27.35	0.49	23.92	39	0.00E+00
Rilmenidine and Rapamycin	231	3	28.28	0.5	28.14	39	0.00E+00
WT							
1% DMSO	268	4	23.67	0.32	n/a	32	
Rilmenidine (200µM)	119	3	27.37	0.53	16.6	36	5.40E-08
Forskolin (100µM)	272	3	20.18	0.43	-14.74	32	0.0014
cAMP (5mM)	165	2	21.19	0.36	-10.48	27	5.20E-06
DDE (1mM)	81	1	26.73	0.65	12.9	36	2.60E-05
Rilmenidine and Forskolin	275	3	22.38	0.40	-5.45	32	1
Rilmenidine and cAMP	154	2	23.12	0.37	-2.32	29	1
DDE and Forskolin	123	1	18.76	0.38	-20.74	25	0.00E+00
DDE and cAMP	156	1	19.09	0.22	19.35	22	0.00E+00
eat-2							
1% DMSO	102	2	27.33	0.71	N/A	35	N/A
Rilmenidine (200µM)	120	2	22.57	0.56	-16.94	33	2.90E-08
aak-2							
1% DMSO	281	2	26.54	0.33	N/A	34	N/A
Rilmenidine (200µM)	465	2	27.98	0.34	5.43	39	0.0001
daf-15 (+/-)							
1% DMSO	34	2	24.56	0.95	N/A	32	N/A
Rilmenidine (200µM)	31	2	23.26	0.88	-5.29	30	1
Rapamycin (100µM)	49	2	22.43	0.86	8.67	27	0.9
daf- 16							
1% DMSO	766	4	19.37	0.21	N/A	27	N/A
Rilmenidine (200µM)	716	4	18.59	0.22	-4.03	27	0.15
RNAi w/WT							
Empty Vector	244	3	28.01	0.35	N/A	36	
Empty Vector and Rilmenidine (200µM)	240	3	30.33	0.39	8.28	39	4.80E-06
bec-1 and 1% DMSO	225	3	26.82	0.35	-4.25	34	0.0325
bec-1 and Rilmenidine (200µM)	189	3	26.22	0.43	-6.39	34	0.0252
lgg-1 and 1% DMSO	298	3	25.82	0.28	-7.82	32	6.60E-07
lgg-1 and Rilmenidine (200µM)	154	3	24.74	0.46	-22.67	32	4.30E-07

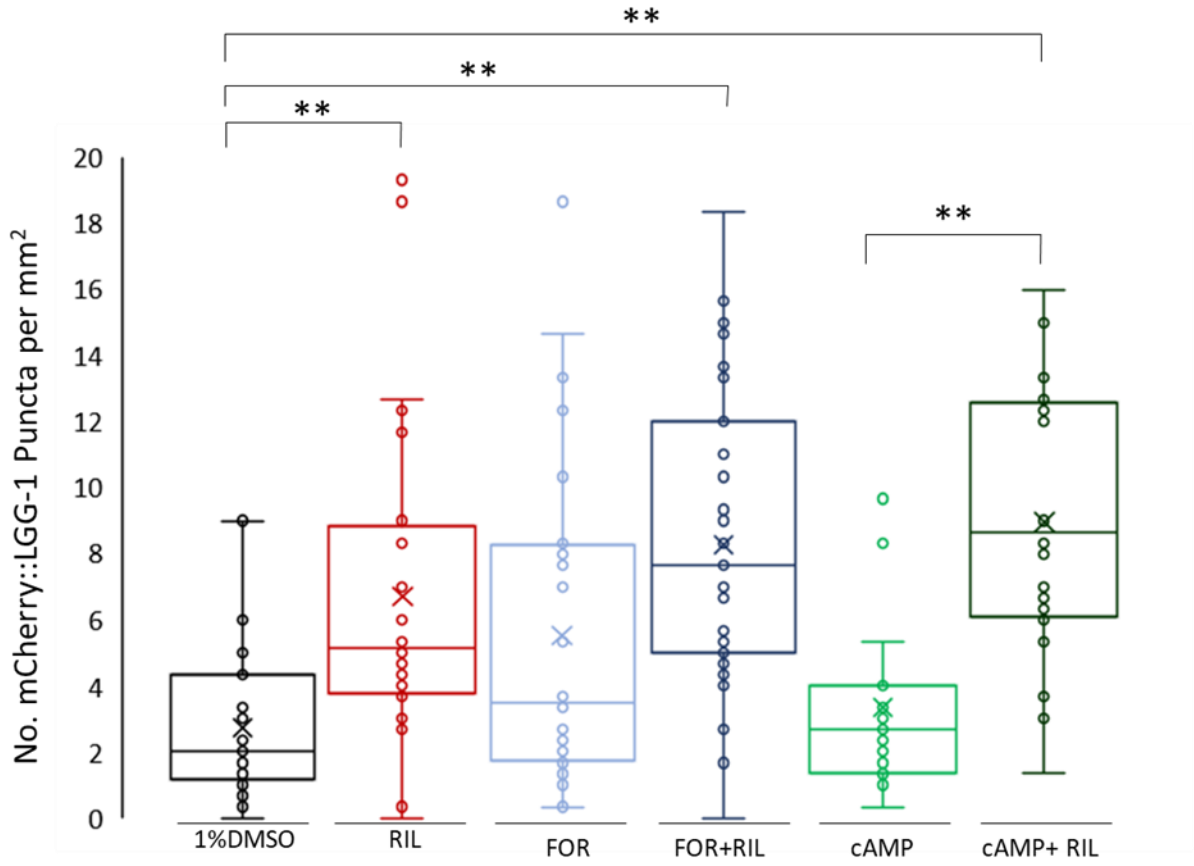


Figure 23. Induction of autophagy by rilmenidine is independent of cAMP signalling. *Well-fed day 2 adult transgenic animals, expressing the intestinal specific autophagy reporter gene (Pnhx-2mCherry::lgg-1), were exposed to rilmenidine or a 1% DMSO vehicle for 24h in the presence or absence of cAMP accumulation by forskolin (500µM) or exogenous cAMP (5mM). Worms were immobilized and mounted onto glass slides in 20mM tetramisole prior to imaging at x10 objective on a Zeiss Axio Observer with a 150ms exposure. Graph shows the interquartile distribution of mean number of mCherry::LGG-1 puncta in the posterior intestine of the animals in each condition. Error bars, upper:Q3 + 1.5*IQR; minimum: Q1 -1.5*IQR. mCherry::LGG-1 puncta were counted in each of three rectangular boxes of 1mm^2; they were placed in tandem beginning with the most posterior region of the intestinal and extended toward the vulva; mean number per mm^2 was calculated for each animal. N>25 animals analysed per treatment in at least 2 independent trials. **P<0.01, *P<0.05; one-way ANOVA followed by a Tukey's post hoc test.*

6.2.4. Rilmenidine Increases Autophagy Independent of cAMP Signalling

Williams et al. have previously posited that rilmenidine upregulates autophagy *in-vitro* through the reduction of cAMP (Williams et al., 2008). This furthers preceding evidence that rilmenidine and other I(1) receptor-selective imidazolines decrease forskolin-stimulated cAMP level in PC12 cells (Greney et al., 2000). Forskolin is a naturally occurring diterpene compound that has been shown to directly activate adenylyl cyclase (AC): an enzyme responsible for the conversion of adenosine triphosphate into cAMP (reviewed in Insel and Ostrom, 2003). As such, forskolin is frequently used in-vitro and in-vivo to increase intracellular cAMP levels (Greney et al., 2000; Urosevic et al., 2004). In 2008, Williams et al., posited that if rilmenidine cleared synuclein, and forskolin worsened clearance, rilmenidine must increase autophagy via cAMP reductions. Furthermore, exogenous cAMP supplementation produced a comparable worsening of synuclein aggregation to forskolin (Williams et al., 2008). This was validated by the observation that 2',5'-Dideoxyadenosine (DDA), an adenyl cyclase inhibitor, could effectively clear synuclein and increase autophagy proportional to rilmenidine (Williams et al., 2008). Downstream, it is hypothesised that decreased cAMP prohibits increased IP3 generation by PLCε-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2): this reverses the homeostatic IP3 mediated mobilisation of ER Ca^{2+} stores that inhibit autophagy (Renna et al., 2010).

To assess whether rilmenidine-induced autophagy was mediated by cAMP levels, an extension of William's reductionist approach was employed. In theory, rilmenidine should increase *C. elegans* autophagy whilst forskolin should not increase autophagy. Moreover, if rilmenidine signals through the downregulation cAMP levels, the enzymatic increase of cAMP production by forskolin should block autophagy signalling by rilmenidine - and this effect should be mirrored by exogenous cAMP supplementation. Throughout this thesis, drugs have been administered by dissolving them in NGM agar. To continue that approach, it was necessary to find an appropriate dosage using an NGM medium, to effectively and non-toxically increase cAMP levels by forskolin or exogenous cAMP. Fortunately, this method has been employed elsewhere. Liu et al., (2017) added 500µM of forskolin to NGM or 5mM exogenous cAMP to rescue cAMP reduction caused by cold stress in *C. elegans*. Accordingly, that dosage seemed appropriate and was followed hereafter.

vKEx1093*(nhx-2::mCherry::lgg-1)* worms were assessed for puncta accrual in response to alteration in cAMP levels as previously described (see chapter 2.8.1). First, it was re-established that rilmenidine at a prolongevity dose (200µM) increases autophagy in *vKEx1093* worms (One-way ANOVA adj. $p<0.008$) (Figure 23). Forskolin and cAMP both modestly increased median punta accrual, 1.22 fold and 2 fold respectively, however this failed to reach significance when corrected for multiplicity (One-way ANOVA;

FOR adj. p=0.12, cAMP adj. p=0.90). Strikingly, co-administration of rilmenidine with forksolin, failed to abrogate the autophagy inducing effects of rilmenidine, and FOR+RIL increased no. of mCherry::LGG-1 positive puncta by 3 fold (Figure 23) (One way ANOVA; 1%DMSO vs. FOR+RIL p=0.01). This effect was replicated through cAMP treatment. Transgenic vKEx1093(*nhx-2::mCherry::lgg-1*) worms treated with exogenous cAMP did not demonstrate any statistical difference in no. of mCherry::LGG-1 positive puncta, whereas rilmenidine and cAMP combination significantly increased no. of mCherry::LGG-1 positive puncta (One way ANOVA; 1%DMSO vs. cAMP p=0.9; 1%DMSO vs. cAMP+RIL p=0.001) (Figure 23). Together these results provide evidence that rilmenidine increases autophagy independent of cAMP, contrary to previous hypotheses.

A wealth of previous evidence has inexorably tied rilmenidine to downregulation of cAMP: Rilmenidine and other imidaozline receptor agonists have been shown to reduce cAMP levels alone, to reduce forskolin-stimulated cAMP levels in cell culture and inhibit isoproterenol stimulated cAMP accumulation in rabbit iris-ciliary bodies (Chu et al., 1996; Edwards et al., 2012; Urosevic et al., 2004) . However as demonstrated in this chapter, this does not necessitate its involvement in rilmenidine induced autophagy. Nevertheless, the downregulation of cAMP may still be essential to the pro-longevity effect of lifespan, independent of autophagy. Thus, if homeostatic cAMP levels normally seen in OP50 fed WT worms were disrupted to abnormal levels, it should cause significant difficulty for rilmenidine to increase lifespan if mediated through cAMP. In replication of the autophagy experiments, cAMP levels were increased by 500µM forskolin administration to lifespan assay plates on which adult WT worms were assayed for survival. It would be reasonable to assume that forskolin integrity and concentration within the agar would diminish throughout the assay. However, forskolin addition produced a crystallization effect in the NGM indicative of its presence which could be monitored for bioavailability throughout the experiment. Forskolin addition to plates, significantly reduced WT lifespan by 15% (log rank; adj. p=0.001) (Figure 24A). Interestingly, co-treatment with rilmenidine was able to rescue the forskolin induced lifespan phenotype which corresponded to statistically indifferent lifespan to WT worms fed a 1% DMSO vehicle (log rank; adj. p=1). To assess whether this rescuing was due to a refraction of forksolin induced cAMP levels by rilmenidine and not an off-site toxicity mediated by forskolin, we exogenously increased cAMP levels using 5mM cAMP. As expected, results were reflected and cAMP significantly reduced WT lifespan which was rescued by rilmenidine combination (log rank test; DMSO vs cAMP p<0.001; DMSO vs. cAMP+RIL adj. p=1) (Figure 24B). This presented an interpretive dilemma: was rilmenidine actually normalising cAMP levels or merely extending lifespan independently of cAMP signalling? To answer this question, the adenyl cyclase inhibitor DDE was co-administered to forskolin and cAMP treated worms to definitely normalise cAMP, using dosages previously used to do so (Müller et al., 2015). DDE failed to

rescue both forskolin and cAMP lifespan phenotypes, and in fact shortened lifespan further than just forskolin or cAMP by -7 and -10% respectively (log rank; FOR vs. FOR+DDE p=0.02; cAMP vs. cAMP+DDE p=1.00E-07) (Figure 24C,D). Lastly, to confirm that this was not due to inappropriate dosing of DDE and subsequent toxicity, WT WT worms were treated with DDE. WT WT lifespan was increased 12.9% by DDE (log rank; adj. p<0.001), confirming no toxicity and assisting in the conclusion that rilmenidine increases lifespan independently of cAMP signalling (Figure 24E).

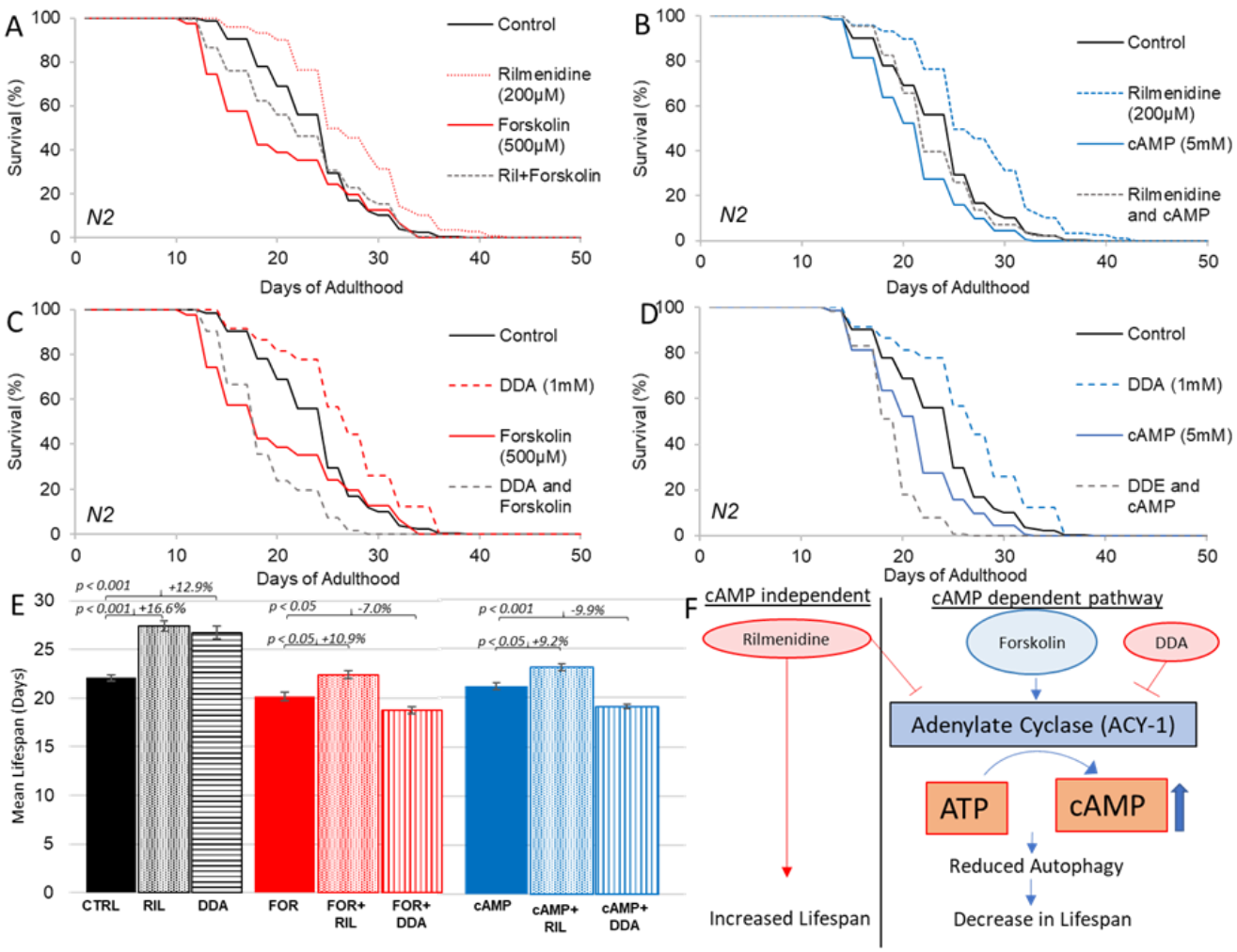


Figure 24. Rilmenidine- Induced Lifespan Extension is Independent of cAMP Signalling. *A+B) Pooled survival curves of adult WT worms fed UV-killed OP50 also treated with Rilmenidine (200μM) and Forskolin (500μM)/cAMP (5mM) independently, or in combination, compared to control animals treated with a 1% DMSO vehicle. C + D) Survival curves of WT adult worms treated with 1mM DDE in the absence or presence of cAMP or Forskolin.Kaplan Meir survival analysis performed on pooled data from 3 independent trials in no less than 100 animals per condition. Groups tested by Log-rank with Bonferonni correction; p<0.05 considered significant; detailed analysis available in table. E) mean lifespan (days\ of cohorts of WT animals derived from survival curves represented in A,B,C and D. Error bars represent SEM; adjusted p-value derived from log-rank test and Bonferroni correction. F Schematic diagram of rilmenidine increasing lifespan independently of cAMP signalling. On the right panel, forskolin activates ACY-1 to increase cAMP levels which inhibits autophagy and shortens lifespan.*

6.3. Discussion

This thesis thus far has unravelled an intriguing relationship between dietary restriction and rilmenidine that appears associative yet unmasked. Rilmenidine can indeed increase lifespan and induce gene expression changes in functions also affected by CR. Similarly, protection against environmental stresses by CR are mirrored in rilmenidine treatment that be involved in the observed improvement in a loosely defined metric of "healthspan". Therefore, the candidacy of rilmenidine as a CRM required definitive testing.

Epistatic interactions between rilmenidine and CR in the form of reduced swallowing capacity (*eat-2*) demonstrated the inability of rilmenidine to extend lifespan in an additive manner; instead, rilmenidine doses that normally benefit wild-type were actually deleterious to *eat-2*, possibly through a hyper-induction of CR signalling. Downstream genetic signalling pathways of CR, AMPK, *DAF-16* and TOR, contributed to the ability of rilmenidine to extend lifespan, although AMPK was not critical to the pro-longevity effect. Given that both AMPK and TOR converge on autophagic pathways, it seemed sensible to test autophagy in worms treated with rilmenidine, especially given previous evidence showing mice treated with rilmenidine have increased autophagy (Rose et al., 2010). Reassuringly, rilmenidine not only increased autophagy in a dose-dependent manner, but it demonstrated functionality by improving the clearance of polyglutamine expansions. Furthermore, functioning autophagic machinery was essential to rilmenidine lifespan increases, suggesting the vast majority of rilmenidine's geroprotective properties are derived from increased autophagy, similar to that observed by CR. Lastly, a previously proposed cAMP dependent mechanism was tested for its necessity for rilmenidine induced autophagy and longevity. Indeed, rilmenidine was able to increase lifespan and autophagy in spite of severe cAMP aberrations casting new doubt on how rilmenidine might increase autophagy.

This chapter started by administering a prolongevity dose of rilmenidine to *eat-2* worms. It was hypothesised that worms already undergoing geroprotective CR could not experience further longevity gains by rilmenidine; a hypothesis tested in myriad publications qualifying the CRM candidacy of their own compounds (Calvert et al., 2016; Kim et al., 2018; Onken and Driscoll, 2010; Shintani et al., 2017 - to reference a pertinient few). In a similar response to that elicited by metformin, rilmenidine actually significantly shortened *eat-2* lifespan (Onken and Driscoll, 2010). This is an intriguing result which suggests rilmenidine may mimic CR so directly, that it sends CR signals already upregulated in *eat-2* into overdrive, eliciting a deleterious "extreme CR response" (Gillespie et al., 2016). Indeed, extreme calorie restriction, as seen in the Minnesotta Starvation Experiment as well as in Anorexia Nervosa patients, increases mortality risk as well as causing chronic weakness and reduced aerobic capacity (Most et al.,

2017). However, humans cannot delve into a Dauer response which should have theoretically formed, but did not, following metabolic shutdown from both rilmenidine and metformin.

When rilmenidine pro-longevity was assessed in key genetic pathway mutations of DR, a more modest response was found. Rilmenidine did significantly increase lifespan in *aak-2* mutants however this was far more nuanced than that seen in WT, suggesting that AMPK activation, as in CR, likely contributed to the prolongevity effect of rilmenidine – although this should be clarified by protein phosphorylation studies. Research suggests that several CR regimes, including *eat-2* models, similarly don't require *aak-2* for their pro-longevity effect (Greer et al., 2007). However, rilmenidine lifespan extension was entirely dependent on normalised TOR function (whether that be pharmaceutically or genetically downregulated), implying that rilmenidine signalling likely converged on TOR to elicit most of its geroprotective properties. How rilmenidine might interact with TOR remains to be seen. Given that rilmenidine also required *daf-16* to elicit lifespan extension it is possible that rilmenidine causes TOR inhibition leading to *daf-16* activation and transcription of longevity and importantly heat-shock genes (Robida-Stubbs et al., 2012). This provides a potential mechanism of rilmenidine-induced geroprotection and thermotolerance. This would differentiate rilmenidine from rapamycin in its mechanism, which is *daf-16* independent, owing to its interaction with TORC2 (Robida-Stubbs et al., 2012). Chronic administration of rapamycin is associated with activation of mTORC2 which supposedly causes detrimental effects on metabolism, including hyperglycaemia, hyperlipidaemia, and insulin resistance in mice, and thus if rilmenidine can precisely downregulate mTORC1 signalling, without affecting mTORC2 it may present a more tolerable and durable CRM than rapamycin (Lamming et al., 2012; Robida-Stubbs et al., 2012). Future work would greatly benefit from immunoprecipitation analyses of the changes in mTOR signalling elicited by rilmenidine. Nonetheless, these lifespan analyses provide more evidence supporting the hypothesis that rilmenidine is an inverse agonist to IRAS which causes an upregulation of AMPK activity and TOR inhibition, in line with murine IRAS-/- models (Dong et al., 2017). However, this does not explain the ability for rilmenidine to increase lifespan despite *aak-2* mutations. Hence, it is also reasoned that rilmenidine may have dual roles, and it may also interact with IRAS or *f13e9.1* to increase tumour suppressive interaction with LKB1 and reduce TOR activity (Jain et al., 2013; Shaw et al., 2004).

Given that rilmenidine seemingly signalled through dietary restriction pathways, it was rationed that it might increase autophagy. *In-vitro* and murine work has confirmed rilmenidine can induce autophagy, however this has never been conceived in *C. elegans* or in the ageing arena (Perera et al., 2018; Rose et al., 2010). Accordingly, rilmenidine increased autophagy in a dose-dependent manner. This is important because prolongevity doses of rilmenidine did not increase autophagy as much as larger toxic

and potentially progeroid doses. Whilst plenty of evidence has demonstrated autophagy induction to increase a multitude of ageing markers, it represents a sharp double-edged sword, whereby overactive autophagy may actually precipitate cellular pathology (Benedetto et al., 2019; Benedetto and Gems, 2019; Kubli and Gustafsson, 2014; Qiu et al., 2018; Rubinsztein et al., 2011). Hence dosages around 200μM provides a beneficial upregulation, but dosages exceeding 300μM may induce pathological amounts of autophagy. Dosages of 200μM rilmenidine were tested for autophagic induction in the presence of the imidazoline receptor blocker efaroxan. Again, mirroring lifespan experiments, efaroxan increased autophagy in *C. elegans*, but critically differed in the fact rilmenidine and efaroxan synergistically increased autophagy to levels higher than rilmenidine or efaroxan alone. This result defied expectation and previous lifespan and imidazoline binding site mechanisms elucidated in this study. Unfortunately, it is seemingly assumed amongst the scientific community that rilmenidine increases autophagy dependent on an imidazoline receptor, and as such no literature exists testing this hypothesis. Given that rilmenidine is a highly specific imidazoline receptor agonist it is highly unlikely an ulterior binding would elicit such marked cell changes. A plausible explanation lies in the duplicity of efaroxan being a ligand for both imidazoline and α2-adrenergic receptors (Lehner et al., 2012). Efaroxan and its analogue, idazoxan (also with the same duplicity) were recently shown to increase autophagy in murine macrophage cell-lines (Nakagawa et al., 2018). The authors suggested this autophagic induction was independent of the imidazoline receptor, albeit based on a single read out of downstream Jun activity. Activation of the α2-adrenergic receptors also been associated with increased autophagy and it is reasonable to inductively deduce that efaroxan may increase autophagy via α2-adrenergic binding rather than imidazoline receptor binding (Han et al., 2018; Oh et al., 2019). Furthermore, whilst efaroxan significantly attenuated rilmenidine induced ERK activation in chapter 4, signalling remained more elevated with combination rather than efaroxan mono-treatment, suggesting efaroxan may have only partially blocked the IBS. Together, efarxoan may increase autophagy via α2-adrenergic binding but only partially block the IBS such that rilmenidine might still modestly upregulate autophagy, leading to an additive autophagic effect. Nonetheless, establishing the necessity of an IBS for autophagy induction by rilmenidine is critical. It would perhaps be advisable to cross *f13e9.1* mutants with vkEx1241 transgenic mCherry::LGG-1 strains to identify possible abrogation of autophagy induction by rilmenidine in *f13e9.1 -/-* reporter strains.

In assessing the functional relevance of autophagic induction, it was shown rilmenidine attenuated the build-up of polyglutamine expansions in a transgenic overexpression *C. elegans* model. This reconfirms evidence from Huntington's disease mouse models showing rilmenidine to attenuate *in-vivo* polyglutamine expansions (Rose et al., 2010). Furthermore, this adds to a catalogue of evidence

demonstrating rilmenidine to clear age-associated pathological protein aggregates including synuclein, misfolded forms of SOD1, TARDBP, *tau*, as well as poly-q aggregates and implicates rilmenidine as a robust autophagy inducer with a capacity to mitigate a number of age-associated proteinopathies (Moreau et al., 2014; Perera et al., 2018; Williams et al., 2008). Loss of proteostatsis, represents a conserved hallmark of ageing across species including *C. elegans* (Ben-Zvi et al., 2009; López-Otín et al., 2013). Additionally, loss of proteostasis underlies the onset of many diseases of ageing, including neurodegenerative diseases, meaning the deferment of its collapse is critical for maintaining disease-free living into later life. As such, when evaluating potential compounds for "healthspan" benefits, researchers are keen to utilise the genetic tractability of *C. elegans* to assess resistance to acute perturbation of proteostasis (Boasquívis et al., 2018; Denzel et al., 2014; Rollins et al., 2017). Thus, the clearance of toxic aggregates by rilmenidine serves not only as a demonstration of autophagic efficiency, but as an additional marker of health-span preservation alongside the attenuation of locomotory decline and improved thermotolerance. More recently, however, researchers have begun querying the phenotypical consequences of rilmenidine-induced autophagy. Whilst, Rose et al. (2010) found an attenuation of symptoms in their murine Huntington's model, other researchers have found rilmenidine to exacerbate neurodegenerative phenotypes in *SOD1* mutant mice (Perera et al., 2018). Furthermore, when under proteostatic challenge by either chloroquine or aggregative protein models, lysosomal efficiency following autophagy induction by rilmenidine appears dysfunctional (Button et al., 2017; Civiletto et al., 2018). Thus, particularly in geriatric populations, whom are likely burdened with sub-clinical or overt aggregative phenotypes, the potential contraindications could limit the translative potential of rilmenidine (Serrano-Pozo et al., 2011).

The results demonstrate not only the presence, but the necessity of functional autophagy for rilmenidine to extend lifespan. This study followed a similar schema to that implemented by Hansen et al. (2008) when assessing the same dependency in dietary restriction. Utilising the practical RNAi feeding method (see chapter 2.8.2), Hansen et al. (2008) knocked down autophagic function using dsRNA against *bec-1* and *vps-34*. Given the role *vps-34* has in endocytic cargo transport, a function shared by IRAS, it was decided the autophagy-specific gene *lgg-1* should be used, as in other studies (Jia et al., 2009; Samokhvalov et al., 2008). Knockdown of these genes prevented rilmenidine from extending lifespan supporting the hypothesis that rilmenidine mimics dietary restriction in the geroprotective upregulation of autophagy. Disrupting autophagy genes from adulthood does not perturb other phenotypes normally associated with dietary restriction, including morphological, spectrofluorimetric or reproductive changes, and whilst it is likely this is replicated in rilmenidine-treated worms, this cannot be ruled out (Hansen et

al., 2008). Nonetheless, it is likely autophagy is specifically required for the prolongevity benefits of rilmenidine, and upregulated autophagy defers the proteostatic collapse in *C. elegans* ageing.

Finally, this chapter sought to establish a mechanism elicited by rilmenidine to signal an increase in autophagy in *C. elegans*. It has been tentatively proposed that *in-vitro*, rilmenidine upregulates autophagy by reducing intracellular cAMP levels, prohibiting IP3 mediated Ca^{2+} release from the ER (Renna et al., 2010). Indeed, it is mechanistically proven that *in-vitro*, rilmenidine can reduce both basal and forskolin-stimulated cAMP levels (Greney et al., 2000). Thus it is logical to hypothesise, rilmenidine may increase autophagy via downregulation of cAMP signalling in *C. elegans* as well. However, a direct mechanistic link between cAMP modulation and autophagy induction by rilmenidine has never been proven. In this thesis, pharmacologically increasing levels of cAMP with either exogenous cAMP or forskolin, did not prohibit the ability of rilmenidine to increase autophagy levels indicating that rilmenidine acts independently of the cAMP pathway to upregulate autophagy. There is a possibility that the remissive properties of rilmenidine outweighed the augmentation by either cAMP or forskolin. However, these experiments were performed at the lowest autophagy inducing concentrations of rilmenidine and furthermore, the degree of autophagic induction was proportional in cAMP/forskolin treated worms to control worms. Of course, confirmative quantification of cAMP levels would be preferential, however, this is precluded in the notoriously difficult and noisy *C. elegans* (Kashyap et al., 2014).

Given that autophagy is critical to lifespan extension by rilmenidine, prohibition of the requisite signalling by forskolin or cAMP supplementation, should abrogate any lifespan extension. However, it was also found that rilmenidine increased lifespan independently of cAMP levels. This corroborates our autophagy results to support the likelihood that rilmenidine increases autophagy through a cAMP independent mechanism. As rilmenidine and forskolin (or cAMP) co-administration did not significantly affect WT lifespan, it can be assumed any increases in autophagy observed during cotreatment were legitimately beneficial and not responses to toxicity manifesting in autophagy mediated survival and apoptotic response.

However, the explicit confirmation of mTOR independent autophagy induction by rilmenidine is lacking specifically in *C. elegans*. Further work is needed to establish whether rilmenidine inhibits *Ce.*TOR leading to autophagy induction. This would provide a sophisticated pathway demonstrating rilmenidine to inhibit *ceTOR* leading to autophagy induction and lifespan extension. Conversely, it is possible that *ceTOR* is required for alternative pathways necessary for longevity that depend of autophagy. For example, rilmenidine may not only clear cellular waste, but help in the generation of new raw material for protein synthesis that requires functioning *ceTOR.* Taken together, it can be concluded that rilmenidine

mimics genetic dietary restriction and the key CR nexuses, *ceTOR*, AMPK and *daf-16* function in the prolongevity effect of rilmenidine. Furthermore rilmenidine increases autophagy in *C. elegans* independent of cAMP, which is necessary for its prolongevity effect. However it is not possible to conclude, at present, which pathway rilmenidine signals through to induce autophagy, but it is likely the beneficial effect of autophagy is mTOR dependent.

7. Conclusions

7.1. Rilmenidine Is Likely A Calorie Restriction Mimetic

Ageing is, by definition, a time-dependent increase in disease susceptibility and mortality. However, despite its universality, and clear perceptibility, it remains the most elusive and least well-understood aspect of human biology (Kirkwood, 2005). I have argued herein that ageing should be considered a disease which routinely imparts dramatic physiological and psychological suffering. The ageing process imposes limitations and reductions in liberty through enfeeblement, frailty and pathology and thus, a failure to advocate and fund scientific research into ageing not only violates one's right to health but prevents the translation of immediate strategies urgently needed to curtail growing healthcare costs and late-life suffering. However, the deceleration of ageing that provides protection against the full spectrum of diseases of ageing thereby assuring late-life health extension, unfortunately comes with an incidental corollary of increased lifespan. With that said, the propensity of moderate lifespan extension strategies to outspread health through a suppression of age-related pathology already exists and the pursuit of its viable translation fascinates many leading scientists in the field.

At present, calorie restriction offers the most feasible and robust intervention to decelerate ageing. However, as highlighted, several concerns surrounding the applicability, tolerability and safety of calorie restriction currently precludes its adoption. Thus, the search for a viable alternative that could recapitulate the phenotype of calorie restriction without dietary implementation has greatly accelerated in the last decade. Calorie restriction mimetics have led the way in easily translatable alternatives with two compounds at the forefront. Both rapamycin and metformin have shown promise and at least demonstrated potential in mimicking the geroprotective properties of CR. However, they both come with substantial criticism: metformin may provide a still unsubstantiated pleotropic effect whilst rapamycin appears to aggressively downregulate one pathway associated with CR. Thus, much work is still required to identify compounds which provide a safe and tolerable profile that extends lifespan and healthspan in a manner similar to CR.

I set out to identify and conduct an introductory exploration into the repurposing of a clinically approved drug for the deceleration of ageing and compression of morbidity mediated through a mimicry of calorie restriction. It should bypass the pleiotropy of CR to modulate specific downstream pathways of CR in a hope it might provide a more precise and directed evocation of the beneficial aspects of CR without any adverse associations. Furthermore, when assessing the validity of a compound as a CRM it must also

activate stress response pathways, provide protection against stressors and lead to a preservation of healthspan (Ingram et al., 2006).

In the knowledge that allantoin, a weak imidazoline receptor agonist, could increase lifespan in WT worms but not in *eat-2*, it was hypothesised that more specific imidazoline receptor agonists such as rilmenidine might possess prolongevity qualities. Transcriptomic analysis revealed rilmenidine gene expression to share significant connectedness to a number of age-related gene signatures. Moreover, rilmenidine gene expression significantly opposed ageing signatures, such that rilmenidine treatment seemingly rescued age-associated hyperfunctional over-expression that was enriched for immune response, inflammation and extra-cellular matrix function. Whilst the transcriptome of rilmenidine treatment lacked direct overlap with any assessed CR signatures, gene lists from both CR and rilmenidine were both functionally enriched for inflammation and extra-cellular matrix integrity. The American Academy of Anti-Ageing medicine, as written by Holt (2011), defines a calorie restriction mimetic as "a pharmaceutical…which has the ability to reproduce one or more of the principal biological effects of calorie restriction" and the reduction of inflammation and modulation of metabolism expression represents two of those effects. To that end alone, rilmenidine mimics calorie restriction, however, this thesis seeks to repurpose rilmenidine as an intervention to decelerate ageing and compress morbidity.

The most comprehensive and well-cited definition and criteria for a calorie restriction mimetic was established in 2006 by Donald Ingram et al. (Ingram et al., 2006; Ribarič, 2012; Testa et al., 2014). Therein, he requested all CRMs should fulfil the following: 1) extend life span and reduce or delay the onset of age-related diseases, 2) activate stress response pathways observed in CR and provide protection against a variety of stressors similar to CR 3) elicit metabolic, hormonal and physiological effects similar to CR. 4) not require a significant reduction in long-term food intake. Lastly, researchers have called for autophagy induction capacity to be an essential component of CR mimicry and thus it should be assessed when analysing the candidacy of rilmenidine (Madeo et al., 2019).

Firstly, rilmenidine extended lifespan in *C. elegans* not only when administered from early adulthood but also when the ageing process was already considerably advanced. Lifespan extension, when commenced from early adulthood was comparable to that observed through the treatment of *C. elegans* with metformin or rapamycin (Onken and Driscoll, 2010; Robida-Stubbs et al., 2012). Furthermore, rilmenidine delayed the onset of frailty in *C. elegans* as measured by the well-established surrogate marker, locomotory decline. Additionally, rilmenidine was able to delay the rate of poly-q aggregation in a transgenic worm model. Indeed, both the locomotory assay and the transgenic polyQ model used

herein have been assessed previously in response to CR and found to be ameliorated as a result (C. Huang et al., 2004; Lüersen et al., 2014; Moroz et al., 2014; Raynes et al., 2012).

Secondly, rilmenidine was able to improve stress resistance, specifically acute thermotolerance. Survival from stress is one of the key suggestions by Ingram to infer CR candidacy (Ingram et al., 2006). Whilst rilmenidine failed to improve oxidative stress, novel research has suggested that oxidative stress resistance may be dispensable for increased lifespan and is uncorrelated to longevity in *C. elegans* (Benedetto et al., 2019). Moreover, debate still exists in literature regarding the way in which CR may modulate oxidative stress in conferring lifespan extension. Although diet-restricted worms show increased oxidative stress resistance, knockout of antioxidant capacity does not reduce lifespan in two different types of DR. This suggests that enhanced antioxidant defence may not be essential for DR-induced longevity. Conversely, in *eat-2* models of CR, a functioning heat-shock response is indispensable for longevity effects (Hsu et al., 2003). It must be noted that activation of key stress response pathways was not assessed in this thesis, and thus, at present, rilmenidine cannot be deemed to activate key stress response pathways and does not entirely meet the second clause of the CRM criteria. Nonetheless, this research highlights the indispensability of *daf-16,* a key stress pathway modulator for lifespan, which increases the likelihood that rilmenidine induces *daf-16* nuclear localisation. Future work should assess nuclear localisation using the well-cited *daf-16::GFP* reporter strain.

Thirdly, I sought to assess whether rilmenidine signalled through key CR pathways that may affect conserved metabolic and physiological effects seen in CR. Whilst *C. elegans* is continuing to emerge as a useful model to assess physiological metabolism, the primary utility of *C. elegans* lies in the genetic dissection and assessment of nutritional and metabolic signalling pathways rather than the physiological consequences thereafter. AMPK and TOR are two key metabolic pathways critical to DR-induced metabolic changes. Rilmenidine required complete functionality of both AMPK and TOR to elicit the extensiveness of its longevity effect, suggesting rilmenidine converged on key signalling pathways critical to metabolic regulation. Moreover, in the setting of global CR, rilmenidine failed to additively extend lifespan, highlighting a converging prolongevity mechanism between CR and rilmenidine. It was not attempted in this thesis, but the exploration of fat metabolism that could be expeditiously assessed using Nile-red straining of fat deposits. Ingram et al (2006) also highlights the importance of modifying body temperature and insulin control when assessing CRM candidacy. Whilst these metrics were not assessed in *C. elegans*, mammalian evidence supports this line of enquiry. Imidazoline receptor agonists have been shown to reduce body temperature in mice, rats, cats, sheep and goats (Bhalla et al., 2011; Lomax and Foster, 1969; Maskrey et al., 1970; Thorn et al., 2012). The advocacy of imidazoline receptor

agonists for metabolic normalisation is decades old, yet has received little attention (Krentz and Evans, 1998). This was based on observations that in animal models of insulin resistance, such as the fructose-fed rat and the obese spontaneously hypertensive rat, moxonidine might also have favourable effects on other features of the metabolic syndrome (Krentz and Evans, 1998). Although amelioration of vasoconstriction is a plausible mechanism, improvements in skeletal-muscle glucose transport and insulin-receptor autophosphorylation with increased expression of insulin-receptor substrate-1 suggest direct effects on insulin action at cellular level (Krentz and Evans, 1998). This research led to more modernised clinical studies into the effects of common I1R agonists. Controlled double blind study over a 6 month period demonstrated that rilmenidine could significantly lower plasma fasting and 2-h glucose levels and insulin concentration after an oral glucose tolerance test in hypertensive patients with metabolic syndrome (Luca et al., 2000). Similarly, moxonidine improved insulin resistance as indicated by an increase in the glucose infusion rate and the insulin sensitivity index (Haenni and Lithell, 1999). More recently, 37 hypertensive non-diabetic patients were treated with rilmenidine to access potential metabolic improvements (Konrady et al., 2006). A sub group were treated with atenolol. The two groups were treated for 26 weeks. Whilst both drugs produced similar BP reductions, the atenolol group showed deterioration in oral glucose tolerance whilst rilmenidine maintained healthy oral glucose tolerance through reducing hyperinsulinemia and hyperglycaemia.

Fourthly, I sought to explore the propensity of rilmenidine to increase autophagy. Not only did rilmenidine increase autophagic induction in *C. elegans*, but the prolongevity benefit of rilmenidine was dependent on autophagy. This pattern is mirrored in *eat-2* worms who require intact autophagy to extend lifespan (Hansen et al., 2008). However, Madeo and colleagues suggest that the criterion of mere autophagic induction is insufficient, and instead, CRM candidates should specifically induce autophagy via a reduction in protein acetylation and the inhibition of EP300 acetyltransferase activity(Madeo et al., 2019). Thus, further work should explore whether rilmenidine may induce such effects.

According to Ingram and Roth, CRMs by definition, must not exert their benefit through a reduction in food intake and any doing so would constitute an induction of CR rather than a *de facto* mimicry (Ingram and Roth, 2015). However, this is unlikely the case as numerous reports have concluded absolutely no difference in body weight or food intake in animals treated with imidazoline receptor agonists including rilmenidine (Aubertin et al., 2019; Mukaddam-Daher et al., 2008; Parkin et al., 2016; Rose et al., 2010).

Beyond elucidating the similarity of rilmenidine to CR to affirm its CRM candidacy, it was essential that rilmenidine could present an orthogonal alternative to the pleotropism of CR. The oft-cited unsuitability of CR in many human subpopulations is in part driven by the pleiotropic nature of CR that induces a

catalogue of physiological effects. The numerous and largely undefined pathways potentiated by CR, do of course, also involve themselves in the regulation of immunity, tissue repair, thermoregulation and reproduction (Le Couteur et al., 2012). Furthermore, the restriction of energy in elderly or immunocompromised individuals presents a significant risk for morbidity, frailty and hospitalisation. Whilst CRMs have sought to overcome these limitations, it is well established that, metformin and rapamycin both elicit adverse side-effects themselves which rilmenidine should avoid.

Principal concerns surrounding the applicability of calorie restriction derive from observations that CR may lead to frailty in that unintentional weight loss is part of the frailty syndrome and also represents an important susceptibility trait (Fried et al., 2001). Ageing, of course, is associated with an increase in sarcopenia, reserve capacity diminishment, malnutrition, and hence, the addition of caloric restriction is likely to rapidly accelerate that process. As such, both mice and rats initiated on CR in late-life often demonstrate negligible effects or indeed reductions in longevity (Forster et al., 2003; Lipman et al., 1998, 1995; Ross, 2009). Conversely, this thesis found rilmenidine treatment to elicit lifespan extension even when initiated in later-life after the development of age-associated phenotypes. Granted, assessments of "healthspan" were not made during late-life administration trials, however, lifelong treatment with rilmenidine significantly attenuated locomotory decline in *C.elegan;* a valuable surrogate frailty assessment. Thus, given that late-life and whole-life rilmenidine treatment yields similar lifespan gains, it is reasonable to assume metrics of frailty may be comparable. As evidence in this thesis suggests autophagy to be the driving force behind the longevity effect of rilmenidine, there is a strong likelihood that autophagic inductive capacity by rilmenidine is preserved during ageing. Furthermore, CR has been recently found to attenuate the age-related sarcopenic impairment of autophagy in rodent skeletal muscle, and therefore presents a possible mechanism by which rilmenidine may be able to preserve autophagic induction capacity into old-age and defer the development of frailty in *C. elegans.* This is supported by transcription data in this thesis showing rimenidine to promote muscle tissue regeneration. Clinical evidence proves imidazoline agents including rilmenidine to be tolerable and safe in elderly populations whilst also ineffective at changing body weight, supporting its potential implementation later in life and in murine models (Kirkendall, 1986; Martin et al., 2005; Nowak et al., 2005; Rose et al., 2010). Notably, rilmenidine did not precipitate musculoskeletal decline in old age.

In addition, concerns have been raised regarding the immunological function of animals under CR. Gardner et al. (2005; 2011), in a series of papers, first provided experimental evidence that CR mice exhibited CR impaired responses to the influenza virus resulting in a much higher risk of mortality. These concerns were compounded by increased colonisation of various nematode infections following

inoculation in CR mouse strains, which additionally proliferated an aberrant t-cell response (Kristan, 2007; Shi et al., 1998). In *C. elegans*, harmane, an endogenous imidazoline receptor agonist, improves immune response and survival in pathogenic environments (Jakobsen et al., 2013). Moreover, recently, rilmenidine and other imidazoline receptor agonists were demonstrated to have an inhibitory effect on the replication of various influenza virus strains, which translated to improved survival in mice inoculated with lethal influenza (Matsui et al., 2018). Gene expression data derived from this thesis supports a general downregulation in inflammatory pathways which contributes to infection pathophysiology and may contribute to the improved immune function in rilmenidine-treated animals. Adjunctly, CR is associated with poor wound healing. In contrast, research in this thesis shows rilmenidine to upregulate genes associated with wound healing and regenerative processes.

Lastly, it would be beneficial to further explore the extent that rilmenidine mimics CR pleotropism, and specifically whether rilmenidine may also reduce fecundity in adulthood – a metric which was not measured herein. With that said, toxicological studies in mice have revealed rilmenidine to not affect fertility, suggesting rilmenidine may not completely phenocopy the undesirable consequences of DR (Verbeuren et al., 1990).

Whilst, these results may suggest rilmenidine to elicit more orthogonal prolongevity effects in comparison to CR, the lifespan results were far more modest than commonly observed in CR animals. Thus, in an attempt to target other longevity pathways potentially missed by rilmenidine, it is possible additive effects by compound/treatment combinations may be beneficial. Interestingly, it is suggested in chapter 5 that rilmenidine may function as an inverse agonist to IRAS and hence may mimic effects induced by IRAS-/- mutants. Dong et al. (2017) demonstrated that IRAS -/- mutants had increased phosphorylation of AMPK, which is similar to the increase in phosphorylation observed by imidazoline receptor agonist treatment in WT mice (Aubertin et al., 2019; Dong et al., 2017). Importantly, IRAS LOF enhanced sensitivity to AMPK activators including metformin which suggests rilmenidine may be able to synergise with metformin to provide even greater CR mimicry (Aubertin et al., 2019; Dong et al., 2017). Allantoin, the CR mimetic with established imidazoline receptor affinity and the stimulus for this thesis, was recently demonstrated to fail in synergising with metformin and did not induce additive lifespan effects (Admasu et al., 2018) . However, allantoin is a far weaker imidazoline receptor agonist and elicits no significant gene expression changes which may not truly represent the synergistic potential of metformin and more specific imidazoline receptor agonists (Admasu et al., 2018; Nikolic and Agbaba, 2012). Rilmenidine and metformin combination therapy is not clinically contraindicated and may provide synergistic antihypertensive and antihyperglycemic roles which could be invaluable in the treatment of metabolic

syndrome highly prevalent in ageing populations whilst also decelerating the ageing process. Admansu et al. (2018) also demonstrated allantoin to lack synergy with rapamycin for lifespan extension, which is in agreement with research conducted in chapter 5 of this thesis, that showed rilmenidine to not synergise with rapamycin. However, despite the high likelihood of rilmenidine converging on TOR in competition with rapamycin, rilmenidine may do so in a more nuanced way making it a more preferential CRM to rapamycin. Chronic administration of rapamycin is associated with activation of mTORC2 which supposedly causes detrimental effects on metabolism, including hyperglycaemia, hyperlipidaemia, and insulin resistance in mice (Lamming et al., 2012; Robida-Stubbs et al., 2012). In *C. elegans,* TORC1 inhibition is *daf-16* dependent whereas TORC2 inhibition is *daf-16* independent (Robida-Stubbs et al., 2012). Given that rapamycin extends lifespan independently of *daf-16* and rilmenidine extends life dependent on *daf-16*, although both signal through the TOR complex, it is likely that rilmenidine bypasses any TORC2 inhibition and thus is unlikely to elicit the aforementioned mTORC2 side-effects (Robida-Stubbs et al., 2012). Conversely, clinical evidence seemed to demonstrate an improvement in glycaemic and lipemic control following rilmenidine treatment (Nowak et al., 2005)

In sum, this research presents a strong novel case for rilmenidine to be considered a calorie restriction mimetic through its prolongevity and health preserving effects, increased stress resistance and increased autophagy. However, whilst the profound effect on ageing and associated pathologies by DR are still being elucidated, it is extremely difficult to target molecular study beyond pathways known to be affected by CR. The problem is further compounded by a great heterogeneity in CR definitions which hinders the development of consensus protocols. This is no better exemplified in *C. elegans* research, whereby different dietary restriction regimes elicit varying effects on lifespan, age-associated biomarkers, and even signal via different mechanisms (see for review Zhang and Mair, 2017). Future work should assess the prolongevity effect of rilmenidine in epistatic lifespan assays under different DR regimes.

7.2. Prolongevity Signalling by Rilmenidine is Mediated Through F13E9.1

The aim of the thesis was to identify a drug which decelerated ageing through the mimicry of calorie restriction without necessarily recapitulating the entirety of CR induced effects. To entertain that possibility, it was essential to begin unravelling the signalling mechanisms of rilmenidine which co-ordinated the favourable phenotype of increased lifespan, improved “healthspan” and stress resistance. Indeed, this thesis provided a number of pieces in the jigsaw. Firstly, rilmenidine is bioactive in *C. elegans* and increases ERK activity via an imidazoline binding site encoded on a novel and uncharacterised IRAS homolog *f13e9.1.* Thereafter it was confirmed that the phenotypes of improved healthy longevity,

including thermotolerance and attenuated frailty were modulated via *f13e9.1.* Further work confirmed that rilmenidine increased lifespan via AMPK, *Ce*TOR and *DAF-16* signalling. Downstream, rilmenidine increased autophagy, which was essential for lifespan extension, and was induced independently of cAMP signalling. In association with a number of congruent findings published elsewhere in the field, the potential geroprotective signalling mechanisms of rilmenidine can be rationally proposed herewith.

If rilmenidine elicits improved markers of longevity dependent on F13E9.1, a protein with significant homology to that of rilmenidine's characterised mammalian receptor IRAS, it is highly likely that rilmenidine signals via *f13e9.1* to elicit geroprotective effects. This is bolstered by the characterisation of *f13e9.1* as a modulator of rilmenidine induced ERK activity, a canonical signal elicited in cell lines upon IRAS binding with rilmenidine (Li et al., 2006). That alone may be sufficient to extend lifespan in *C. elegans.* Work from Okuyama et al. (2010) identified MPK-1 as a key longevity factor and knockdown of *mpk-1* significantly shortened WT lifespan. More recently, researchers established that the prolongevity effect of resveratrol was modulated via MPK-1 activity (Yoon et al., 2019). Furthermore, resveratrol increased constitutive MPK-1 phosphorylation and was able to defer declining MPK-1 activity during ageing (Yoon et al., 2019). Given that rilmenidine increased MPK-1 activity in young adult nematodes, it suggests increased activity may mediate the prolongevity effect of the drug. However, the lifespan effects of resveratrol are mediated through MPK-1 and SKN-1, a pathway not examined in this thesis. Nonetheless, regulation of lifespan by MPK-1 is dependent on *daf-16,* a gene which controls rilmenidine-induced lifespan extension (Okuyama et al., 2010). Thus, it could be suggested that rilmenidine increases lifespan through activating MPK-1 which leads to increased *DAF-16* activity: however, there is not the experimental evidence to confirm or refute this. Nonetheless, this pathway could legitimately programme the upregulation of autophagy. Infection with *P.aeruginosa* PA14 triggers the phosphorylation of MPK-1 which is essential for the consequent autophagic induction, and that this response is dependent on *daf-16* (Zou et al., 2014).

Therefore, rilmenidine may be geroprotective through the upregulation and preservation of longevity-associated MPK-1 signalling in ageing that activates *daf-16* to increase autophagy and stress resistance. However, such linear upregulation of *DAF-16* activity would normally confer a more wholesale stress-resistance that would include improved protection against hydrogen peroxide (Lin et al., 2018b; Weinkove et al., 2006). Specific thermotolerance is primarily regulated by HSF-1 in metazoans, working in concert with *Daf-16* - even in permissive temperature HSF-1 appears to regulate *DAF-16* activity to extend lifespan (Douglas et al., 2015). *f13e9.1* expression appears highly regulated by HSF-1 and rilmenidine increases autophagy, thermotolerance and delays the formation of polyQ aggregations, processes

controlled by HSF-1. Therefore there could be an alternative signalling route, whereby MPK-1 activation is an unrelated signalling process to rilmenidine induced-longevity (Hesp et al., 2015).

Rilmenidine induced prolongevity was also partially dependent on *aak-2*, implying rilmenidine may increase longevity through increasing AMPK signalling. Indeed, as this thesis was being completed, experimental murine work demonstrated a rilmenidine-like, highly specific, imidazoline receptor agonist, to increase AMPK phosphorylation and improve metabolic markers of increased AMPK activity (Aubertin et al., 2019). These effects were strikingly similar to models of I1R IRAS -/- murine models. Specifically, IRAS knockouts displayed a phenocopy of imidazoline receptor agonism including improved glucose tolerance, HOMA-index, and lipid metabolism (Dong et al., 2017). Thus, this heightens the likelihood that rilmenidine may function as an inverse agonist to *f13e9.1/IRAS* to repress IRAS binding and inhibition of AMPK. Consequent increased AMPK activity leads to inhibition of mTOR which is reflected by *ceTOR* dependent rilmenidine-induced lifespan extension. Both Reduced *ceTOR* activity alongside AMPK activation would independently lead to *DAF-16* activation, upregulation of autophagy and improved stress resistance and longevity (Tullet et al., 2014). However, rilmenidine has been demonstrated to increase autophagy independent of mTOR, therefore, it may be possible that AMPK alone can increase autophagy in response to rilmenidine through mTOR-independent mechanisms such as ULK1 (Perera et al., 2018; Roach, 2011; Rose et al., 2010). The necessity of *ce*TOR for rilmenidine induced longevity is then reconciled with the requirement of alternative pathways that depends of autophagy. For example, rilmenidine may not only clear cellular waste, but may also help in the generation of new raw material for protein synthesis that requires functioning ceTOR. Such alternative autophagic induction pathways are necessary in the new light that, at least in *C. elegans*, rilmenidine does not increase autophagy or lifespan via an TOR independent cAMP pathway including activation of the Ca++/calmodulin-dependent kinase CaMKKβ and AMPK phosphorylation, but rather through an, as yet, undiscovered pathway (Aubertin et al., 2019).

However, this model Is challenged by the characteristics unravelled in the *f13e9.1* mutant model. If rilmenidine inversely agonised *f13e9.1* to induce its inhibition and ensuant AMPK activation, a knockout of *f13e9.1* should theoretically manifest in a mimicry of prolongevity models such as *aak-2* overexpression or metformin treatment (Greer et al., 2007). Although lifespan extension was observed, and stunted growth was similar to *IRAS -/-* mice, mutant worms were rendered more sensitive to oxidative stress and reached frailty quicker; both phenotypes were further exacerbated by rilmenidine. Indeed, this may represent a hyperactivation of longevity pathways that become detrimental as seen in metformin treated *eat-2* worms (Onken and Driscoll, 2010). Additionally, such a Tithonian phenotype is not uncommon of

DR worms as models often display poor stress resistance and accelerated markers of ageing (Bansal et al., 2015; Hansen et al., 2008; Lüersen et al., 2014).

Increased autophagy may also explain or at least correlate with transcriptional signature of ECM preservation by rilmenidine. Of course, diverse genetic, nutritional, and pharmacological pro-longevity interventions delay an age-related decline in collagen expression (Ewald et al., 2015). Furthermore, autophagy inhibition accelerates age-dependent loss of total collagen in worms, and thus its upregulation may serve to preserve ECM into ageing (Chen et al., 2019). But, the modulation of this effect is currently obscure. Heat shock also upregulates genes associated with the extracellular matrix and HSF also appears to regulate genes associated with collagen biogenesis (Brunquell et al., 2016). This implicates a possible mechanism by which rilmenidine may preserve extracellular matrix in the face of heat-shock to mimic an HSR, providing a convergent mechanism shared by both HIF-1 and also rilmenidine.

In summary, it is hypothesised that rilmenidine is geroprotective through the inverse agonism of *f13e9.1* such that it leads to MPK-1 and AMPK activation, both of which converge on autophagy, likely through *DAF-16*. Such signalling can recapitulate many of the beneficial hallmarks of CR and may provide a more targeted and orthogonal effect.

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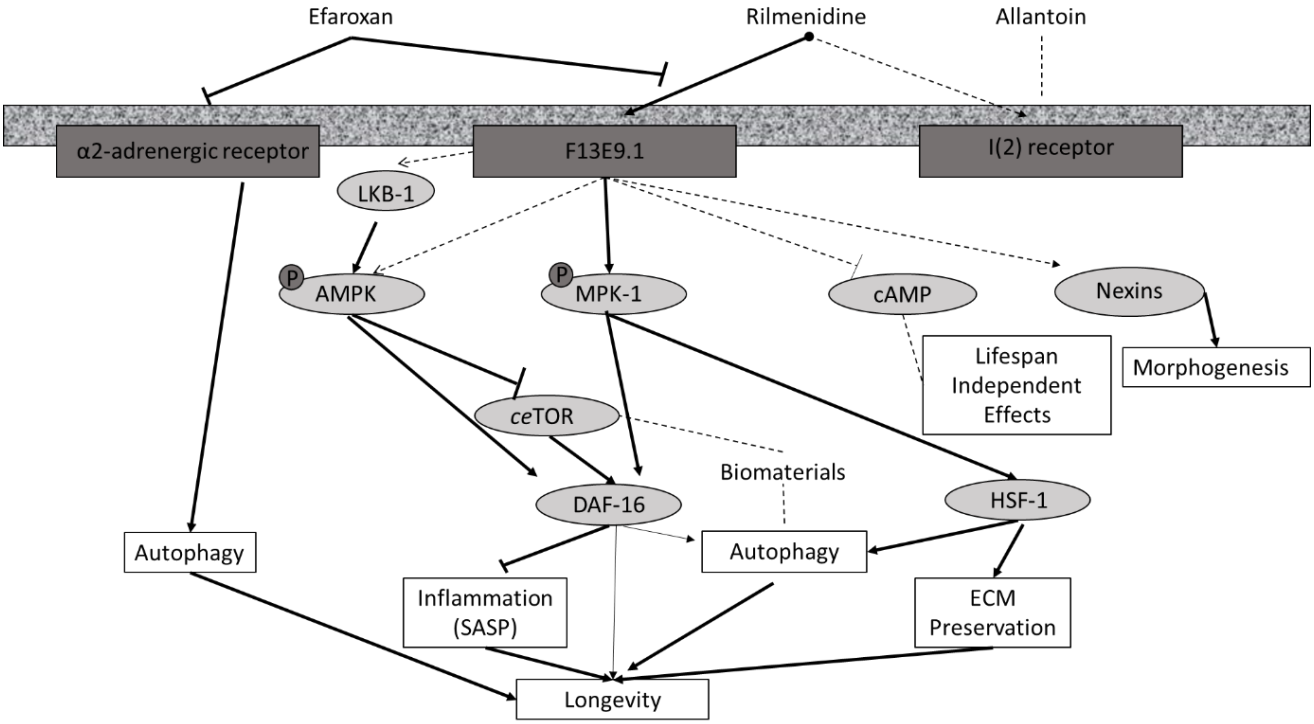

Figure 25. Schematic illustration of potential signalling cascades elicited following rilmenidine binding to f13e9.1. *Rilmenidine may function as an inverse agonist of f13e9.1 such that the inhibition of AMPK is abrogated and MPK-1 is activated. This leads to downstream activation of DAF-16 and subsequent autophagy induction, preservation of the ECM and longevity.*

7.3. Future Work:

C. elegans ageing research serves as a reasonable and low-cost platform to screen potential interventions to expedite their translation into clinical use. Following the establishment of rilmenidine as a legitimate calorie restriction mimetic with geroprotective properties, it is prudent to assess the translatability of this drug. Rilmenidine, and its analog moxonidine, are in the fortunate position of already being clinically approved drugs, widely prescribed for the treatment of resistant hypertension. In the UK, between Jul '18—Jun '19, 393,356 prescriptions were dispensed for imidazoline receptor agonists to treat hypertension or heart failure, at a cost of £657,940 (Curtis and Goldacre, 2018).

7.3.1. The Translational Potential of Rilmenidine as an Anti-Ageing Intervention

NICE recommends treatment of hypertension should involve the prescription of an Angiotensin Converting Enzyme (ACE) inhibitors (<55 years) and calcium channel blockers (CCB) (>55 years)(McCormack et al., 2013). However, In referral for specialist treatment for resistant hypertension, NICE suggests the initiation of centrally acting hypertensive drugs such as rilmenidine or moxonidine: their efficacy is well established and they appear as equally effective in treating mild-moderate hypertension as diuretics, CCBs and ACEs (Materson et al., 1995; Reid, 2000).

Additionally, moxonidine and rilmenidine are well tolerated through their selective binding to I1RAs as has been shown in ample clinical studies (reviewed in Reid, 2000). Specifically, double blind studies found no discernible adversity between rilmenidine vs placebo and moxonidine vs placebo (Ostermann et al., 1988; Scemama et al., 1995). Furthermore, moxonidine appeared to produce less adverse effects than other commonly prescribed antihypertensives drugs (Prichard, 1994; Schachter et al., 1998). These drugs must be tolerated for long periods of time. Indeed, in a study of over 18,00 patients prescribed rilmenidine, only 3.6% of participants withdrew following adverse effects (Luccioni, 1995). In studies of moxonidine, no significant adverse side effects were noted (Gaponova et al., 2011).

Moreover, rilmenidine and moxonidine may also have auxiliary clinical benefits beyond their antihypertensive properties. Imidazoline receptor agonists suppresses the sympathetic activation in ischemic heart disease and chronic heart failurehas proven effective in reducing SNA in chronic heart failure patients (Graham et al., 2004; Swedberg et al., 2002). Furthermore, although evidence in normo- and prehypertensives is absent, rilmenidine is able to reduce LVH, improve arterial distensibility, and prevent arrhythmia in hypertensives (Koldas et al., 2003; Roegel et al., 1998; Trimarco et al., 1995)

The sympatholytic mechanisms of these drugs may also alleviate the detrimental effects of sympathetic hyperactivity in ageing. At base resting levels of sympathetic activity change, a generalised sympathetic

tone increase can be observed during ageing measured by plasma noradrenaline and burst discharge rate on the muscle sympathetic nerve fibres (Iwase et al., 1991; Wallin et al., 1981). Importantly, sympathetic hyperactivity is not just an artefact of ageing, rather an active determinant of mortality and ympathetic hyperactivity is associated with poor prognosis in community dwelling elderly individuals (Reuben et al., 2000). Furthermore, whilst most neuromodulator receptors decline in ageing, it is particularly important that imidazoline receptor numbers increase with age (García-Sevilla et al., 1995). This puts a special emphasis on the increasing role that inverse agonism of imidazoline receptors may have in defining age-related changes in the brain and the body. Rilmenidine produces no increase in adverse effects in the elderly (Galley et al., 1988). One clinical trial looked to test the effect of rilmenidine on cognitive function in elderly hypertensive patients. Nedogoda et al., (2006), treated 30 patients with 24 weeks of rilmenidine monotherapy. Blood pressure was reduced by 25.3% alongside significant improvements in Wechsler Adult Intelligence Scale scores by 15%. At the same time significant increase in vocal activity of patients is noted: number of words at free associations tests, verbs and plants denomination increased by 5.8%, 5.1% and 6.3% respectively ($p<0.05$). Moxonidine administration on 10 elderly hypertensive patients for 2 weeks significantly reduced BP and significantly improved memory and thinking (Ostroumova et al., 2001).

Hypertension is a substantial and growing epidemic that burdens healthcare systems, society and the economy. High blood pressure affects more than 1 in 4 adults in England, around 12.5 million people in 2015 (Public Health England, 2017). This is compounded by the ever-increasing proportion of elderly people who have an almost intractable predeterminancy to the development of hypertension defined by vascular calcification, sympathetic hyperactivity and left ventricular hypertrophy. 63.7% of an English cohort had hypertension between the ages of 60-80, and estimates have suggested by the age of 75, 73% of English adults will suffer from hypertension (Joffres et al., 2013; MacDonald and Morant, 2008). Thus, the sheer prevalence of such a condition provides a significant platform for the preferential consideration of imidazoline receptor agonists such as rilmenidine, for hypertensive populations which would provide a safe, tolerable and cheaper[2] method to improve cardiovascular risk whilst also presenting a method of CR mimicry to over 25% of the population. Furthermore, it warrants substantial clinical investigation as to the auxiliary benefits which may underly their overt purpose as anti-hypertensives.

[2] Cost based on (Total Cost/Total Prescriptions) in 2019 NHS OpenPrescribing data between calcium channel blockers (estimated £2.58) and moxonidine (estimated £1.67)

7.3.2. Translatability of Rilmenidine in Healthy Normotensive Populations

One of the greatest issues with the analysis of antihypertensive drugs, is that the vast majority of all large-scale clinical trials, have studied the drug in hypertensive or morbid individuals. Very few studies have tested the effects of antihypertensive drugs in a normotensive and healthy populations. If antihypertensive drugs were to be considered for mass-market, far larger and inclusive studies would be needed to be conducted across all demographics. However, the availability, established supplier manufacture and clinical tolerability of rilmenidine makes the task of mass distribution more appealing than other purported geroprotectors. Rilmenidine has a good clinical profile and chronic administration is rarely a problem in hypertensives. But very few studies have assessed the effects of rilmenidine on adult populations to address concerns that sympatholytic anti-hypertensive treatment in normotensives may drive a hypotensive crisis. Upon its clinical introduction, rilmenidine was tested in a small normotensive healthy cohort and it did not induce orthostatic hypotension, a significant decrease in heart rate or a significant dryness of mouth (Dollery et al., 1988). However, given the substitutive use of moxonidine in US and UK, far more data is available pertaining to healthy subjects, which will likely mirror that seen in rilmenidine. Moxonidine given to otherwise healthy overweight young adults decreased clinic systolic BP, but did not significantly affect ambulatory BP, as assessed over the 24-hour period, nor did it improve the average BP over the night or the day (Lambert et al., 2017). Furthermore, moxonidine treated groups reported no adverse side effects or abnormal metabolic effects (Lambert et al., 2017). Similarly, otherwise healthy and normotensive poly-cystic ovary syndrome patients were treated with moxonidine and experienced no significant changes to BP, heart rate or metabolism (Shorakae et al., 2018).

In normotensive older populations, the evidence of long-term IRA treatment is severely lacking. Nonetheless, following the promising results of rilmenidine in a mouse model of Huntington's, an open label clinical trial was initiated to assess the feasibility and tolerability of rilmenidine for the treatment of Huntington's disease (Underwood et al., 2017). 16 patients received 1 mg rilmenidine daily for 6 months and then 2mg daily for the next 18 months of the study. The study reported no drug-related serious adverse events or withdrawals, and, there was no significant effect on Huntington's clinical or metabolomic outcomes (Underwood et al., 2017). However, promisingly, mean age was 53 (range 37–69) and may suggest a potential suitability for later life rilmenidine geroprotective treatment initiation.

These results must be weighed against some concerning findings in murine models. Perera et al., (2018) noted that in a murine model of ALS, generated through transgenic expression of a G93A mutant form of human SOD1, rilmenidine significantly exacerbated neurodegeneration. Furthermore, when under proteostatic challenge by either chloroquine or aggregative protein models, lysosomal efficiency following

autophagy induction by rilmenidine, appears dysfunctional (Button et al., 2017; Civiletto et al., 2018). The results in this thesis suggest that rilmenidine might continue to improve lifespan even when administered following age-associated autophagic dysfunction, however murine models suggest rilmenidine continues to increase autophagy despite post-inductive malfunction. Thus, in normal populations, as age-associated protein aggregates naturally accumulate, rilmenidine treatment may lead to exacerbation, yet to be unmasked in clinical trials, limiting its translative potential (Serrano-Pozo et al., 2011). However, it must be noted that these pathological models serve to overwhelm the autophagic machinery far quicker and more aggressively than would be experienced in ageing. Furthermore, SOD1 aggregation is not typical of normal human ageing (Paré et al., 2018). Accumulation of *tau* and amyloid dominate the aggregative landscape of normal brain ageing, and mammalian intervention studies with rilmenidine are necessary to clarify the effect on these aggregates (Reas, 2017).

In conclusion, rilmenidine is a safe, efficacious and tolerable anti-hypertensive which may have beneficial effects on fasting plasma glucose, insulin sensitivity, atherosclerosis and cardiac remodelling. However, these studies have been almost exclusively performed in hypertensive populations. Whilst this provides a large potential cohort to trial the CR mimicking effects of rilmenidine, the safety profile of rilmenidine is not sufficiently substantiated in healthy populations. In addition to concerns regarding the exacerbation of neurodegeneration alongside potential autophagic aberrancies, further murine work is required before considering clinical trials in healthy older patients.

7.4. Future Directions

Although specific follow-up work has been recommended throughout this thesis in relevant chapters, it is important to identify the themes of future study to direct the progress of this project. There are three themes that require continued exploration and tackle three pertinent models: 1) the mechanistic unravelling of rilmenidine-induced prolongevity signalling in *C. elegans* 2) ascertaining whether rilmenidine ameliorates biomarkers of ageing in mice and improves survival 3) computationally led clinical assessment of retrospective survival outcomes and biomarkers of longevity in hypertensive patients treated with rilmenidine.

7.4.1. Mechanistic Unravelling of Rilmenidine-induced Prolongevity Signalling in *C. elegans*

The extension of *C. elegans* led mechanistic studies is beneficial and can continue to profit from the genetic tractability and short life-cycle of the model organism. Several questions were left unanswered and centred around the signalling pathway induced by rilmenidine that drives autophagic upregulation

and how this might affect lifespan and its control by *f13e9.1*. Thus, the continuation of study in *vkEx1093 [nhx-2p::mCherry::lgg-1] is* advisable if paired with genetic crossing of key signalling pathways. Firstly, vkEX1093 worms should be crossed with *f13e9.1* mutants or knocked down with *f13e9.1* RNAi. Replication of autophagic induction methods as in (see chapter 2.8.1) of this thesis is necessary to decipher the dependency of *f13e9.1* to mediate rilmenidine induced autophagy. Furthermore, lifespan analysis in *f13e9.1* mutants, and respective rescues, in response to rilmenidine treatment is critical to target *f13e9.1* as the key mediator of rilmenidine-induced lifespan extension and autophagic induction. Additionally, ERK pathway RNAi as in Okuyama et al., (2010) would help to elucidate the relative necessity of this pathway for the induction of autophagy by rilmenidine in the vkEX1093 worms, and for lifespan extension and thermotolerance in WT worms. Lastly, in *C. elegans,* although this thesis exposed the dependency of *aak-2* and *daf-16* for rilmenidine-induced lifespan extension, this does not insinuate their activation. Immunoblotting for increased AMPK phosphorylation following rilmenidine treatment in both WT and *f13e9.1* strains would help replicate work observed in mammalian models which may provide a more nuanced hypothesis for rilmenidine-induced longevity. Moreover, the observation of *DAF-16* nuclear localisation following rilmenidine treatment in WT , *f13e9.1* and *aak-2* mutants would be invaluable for providing an elegant mechanistic longevity pathway whereby rilmenidine signals through *f13e9.1,* the putative IRAS homolog, leading to AMPK activation, and *DAF-16* mediated longevity.

Whilst evidence in this thesis strongly implicates *f13e9.1* as the nematodal ortholog of IRAS, confirmative work is necessary. If possible, IRAS knockout lines should be generated in PC12 cells, although IRAS -/- lines can be readily purchased in HAP1 lineage. PC12 is preferred owing to an inherent lack of α-2 adrenergic receptor expression which would negate an off-site interactivity with rilmenidine despite its low affinity for the receptor (Edwards et al., 2012). The functional homology of *f13e9.1* could then be explored following its transfection into *IRAS* knockout PC12 cell lines and assessing the subsequent restoration of rilmenidine-mediated ERK activation or DAG accumulation (Li et al., 2006).

Rilmenidine may recapitulate many of the effects of CR, but epistatic interaction was only observed in *eat-2* mutants. CR in worms varies depending on the method of induction and therefore, rilmenidine should be tested in the presence of other regimes to establish whether its mimicry is specific to only certain regimes. Furthermore, it was unconfirmed whether rilmenidine induced a CR state through the downstream regulation of CR-associated pathway or whether it merely reduced food consumption in *C. elegans*. Whilst rilmenidine does not affect food intake in mammals, it would be beneficial to confirm this in *C. elegans* using a food clearance assay.

7.4.2. Ascertaining the Effects of Rilmenidine Treatment on Ageing in Mouse Models

The examination of the physiological effects induced by calorie restriction is limited in *C. elegans*. Thus, the exploration of rilmenidine-induced phenotypes is better suited in mammalian models. Specifically, in endeavouring to fulfil Ingram's CRM criteria, rilmenidine should ideally mimic the metabolic, hormonal, and physiological effects of CR. Using UM-HET3 mice, to replicate the ITP protocols, intraperitoneal rilmenidine treatment (10 mg/kg body weight) four times a week, as in Rose et al. (2010) should be assessed to confirm the effect on CR markers including fasting adiponectin, growth hormone, SHBG, insulin levels and plasma levels of glucose after oral glucose load. Furthermore, given the intriguing anti-inflammatory gene expression profile of rilmenidine which rescued the ageing signature, it would be beneficial to establish whether rilmenidine can reduce markers of inflammation in mice through the measurement of cytokine profiles as well as pNF-κB and pJNK. Researchers must be aware of any potential sexual dimorphism in response to rilmenidine treatment which may mimic that seen in *IRAS* mutant mice and also CR (Dong et al., 2019; Ingram and de Cabo, 2017).

Ideally, large longitudinal lifespan assays with rilmenidine should be developed whereby sub-populations of mice are cross-sectionally scarified for histopathological, and gene expression data. However, even murine lifelong intervention testing programmes would take over 2 years to complete. Thus, it might be expedient to utilise a "pre-aged" C57BL/6J cohort sold by the Jackson Laboratory to initiate a more translational rilmenidine administration regime started in later life; a strategy which was shown in this thesis to increase lifespan in *C. elegans*. Alternatively, the utilisation of a progeroid mouse model may also accelerate the accrual of insightful data. Specifically, the Hutchison Gilford progeria mouse models may be an avenue to explore. ZMPSTE24-null mice, mimic clinical phenotypes of HGPS including metabolic abnormalities, bone and joint abnormalities, subcutaneous fat loss, growth defects and severe atherosclerosis and cardiomyopathy (Gonzalo et al., 2017; Piekarowicz et al., 2019). Some of the aforementioned phenotypes are likely driven by high expression of ECM proteins and low expression of ECM remodelling enzymes, which can result in aberrant ECM deposition (Gonzalo et al., 2017). It could be hypothesised that this segmental ageing disease model may exhibit improved survival following treatment by rilmenidine, especially considering the transcriptional signature of ECM preservation elicited by rilmenidine.

7.4.3. Computationally Led Clinical Assessment of Retrospective Survival Outcomes and Biomarkers of Longevity in Hypertensive Patients Treated with Rilmenidine

During this thesis I had begun to explore the clinical effect of rilmenidine on ageing. However, with no access to my own cohort, I sought to repurpose current clinical databases. I gained access to the epigenetic data of a large cohort of patients from TwinsUK, many of whom had hypertension (Moayyeri

et al., 2013). I hypothesised that hypertensive patients, taking centrally-acting anti-hypertensives may exhibit a lesser age acceleration residue as measured by Hovarth's clock that would indicate an anti-ageing effect by rilmenidine or moxonidine (Horvath, 2013). However, within the cohort, only 3 patients were taking centrally-acting antihypertensives, thus precluding any further study. Therefore, in continuation of this project, I would suggest collating data from other cohorts to increase numbers of patients taking rilmenidine or moxonidine; whole-genome methylation 450k datasets from LifeLines-deep 167,000 individual cohort or the Lothian Birth Cohorts would be an advisable starting point (Horvath, 2013; Tigchelaar et al., 2015) .

Likewise, a routinely documented metric such as mortality is valuable to assess the effect of rilmenidine on retrospective survival outcomes in longitudinal datasets. Using a repository such as the Clinical Practice Research Datalink (CPRD), hypertensives taking I1RAs could be compared to hypertensives taking other antihypertensive medications while controlling for the effects of the different therapies on blood pressure. Therefore, any difference in outcomes could be reasonably to be attributed to rilmenidine/moxonidine therapy beyond hypertensive control.

Whilst highly generalisable and vulnerable to residual confounding factors which could obscure any true effects, both epigenetic clock analysis and retrospective cohort survival provide convenient, accessible and cheap means to quickly assess the clinical potential of rilmenidine that will help in the justification of larger clinical trials into the efficacy of rilmenidine as a CRM and anti-ageing intervention.

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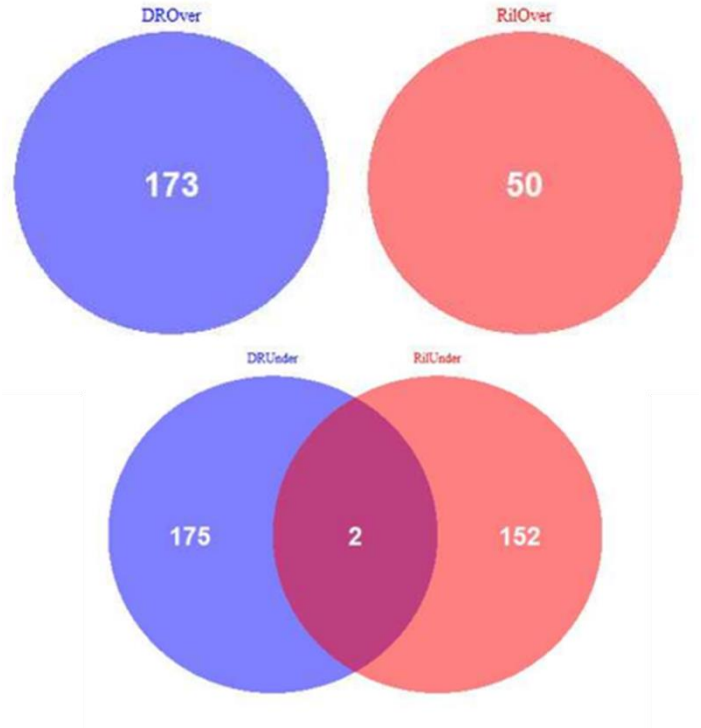
Supporting Material

Supporting Table 1. Revigo recategorised GO terms derived from TOPGO gene enrichment of DEGS that intersect both combined imidazoline receptor agonist-induced underexpression and age-induced overexpression. *Log10 -200 considered significant.*

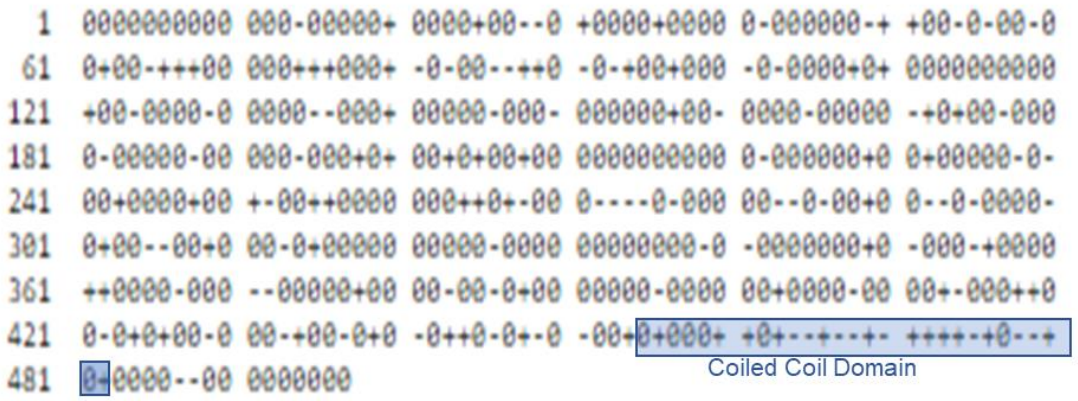
Underexpressed in Combined I1R Agonists + Underexpressed in Ageing		
GO Term	**Description**	**REVIGO Log10 P-value**
GO:1903543	positive regulation of exosomal secretion	-3.80
GO:0033209	tumor necrosis factor-mediated signalling pathway	-3.27
GO:0098609	cell-cell adhesion	-3.09
GO:0042060	wound healing	-3.07
GO:0007160	cell-matrix adhesion	-3.01
GO:0043312	neutrophil degranulation	-2.82
GO:0002931	response to ischemia	-2.77
GO:0022617	extracellular matrix disassembly	-2.77
GO:0009611	response to wounding	-2.71
GO:0043062	extracellular structure organization	-2.66
GO:0048146	positive regulation of fibroblast proliferation	-2.62
GO:0070486	leukocyte aggregation	-2.46
GO:0097284	hepatocyte apoptotic process	-2.44
GO:1902117	positive regulation of organelle assembly	-2.38
GO:0060712	spongiotrophoblast layer development	-2.35
GO:0030214	hyaluronan catabolic process	-2.28
GO:0008360	regulation of cell shape	-2.24
GO:0072574	hepatocyte proliferation	-2.24
GO:0016050	vesicle organization	-2.22
GO:0002479	antigen presentation via MHC class I, TAP-dependent	-2.20

Supporting Table 2. Statistical overlap of perturbagen DEGs and Ageing DEGs. *P values from Fischer's exact test and Bonferroni correction.*

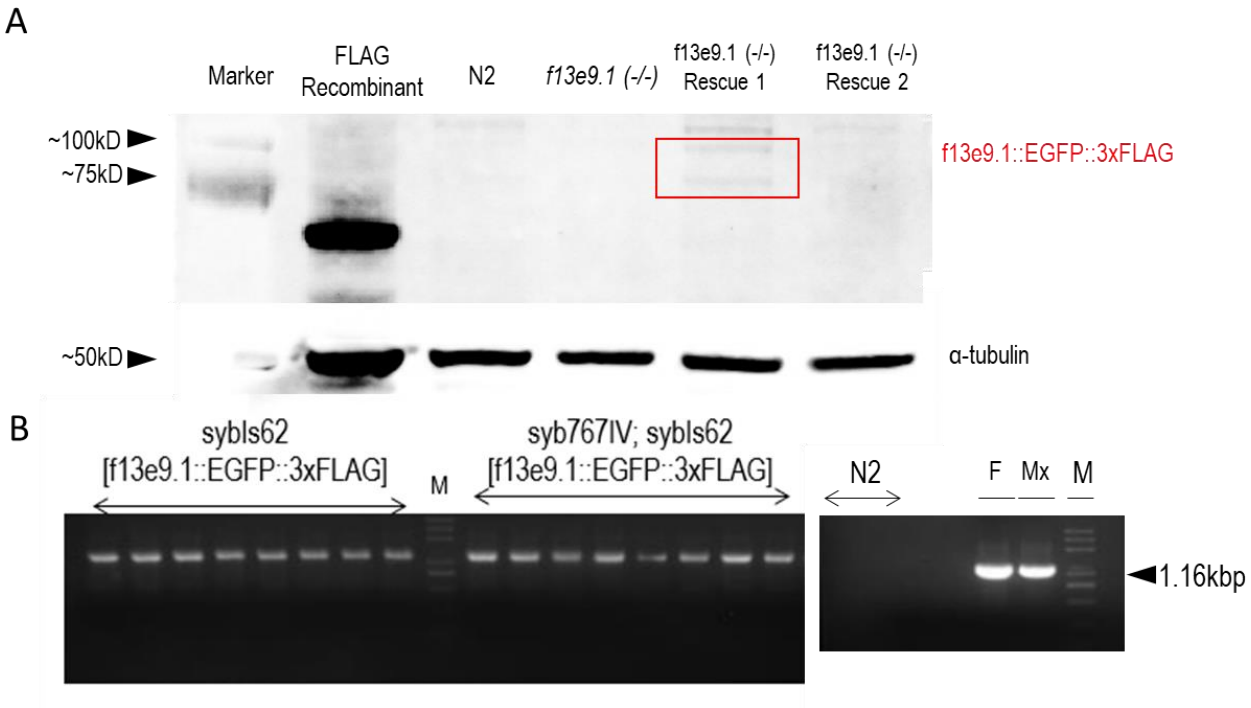
Perturbagen DEGs	Ageing DEGs	p value	adjusted p value (Bonferroni)
Rilmenidine Over	Age Under	0.492	1.000
Rilmenidine Under	Age Over	0.000	0.000
Combined Over	Age Under	1.000	1.000
Combined Under	Age Over	0.000	0.000



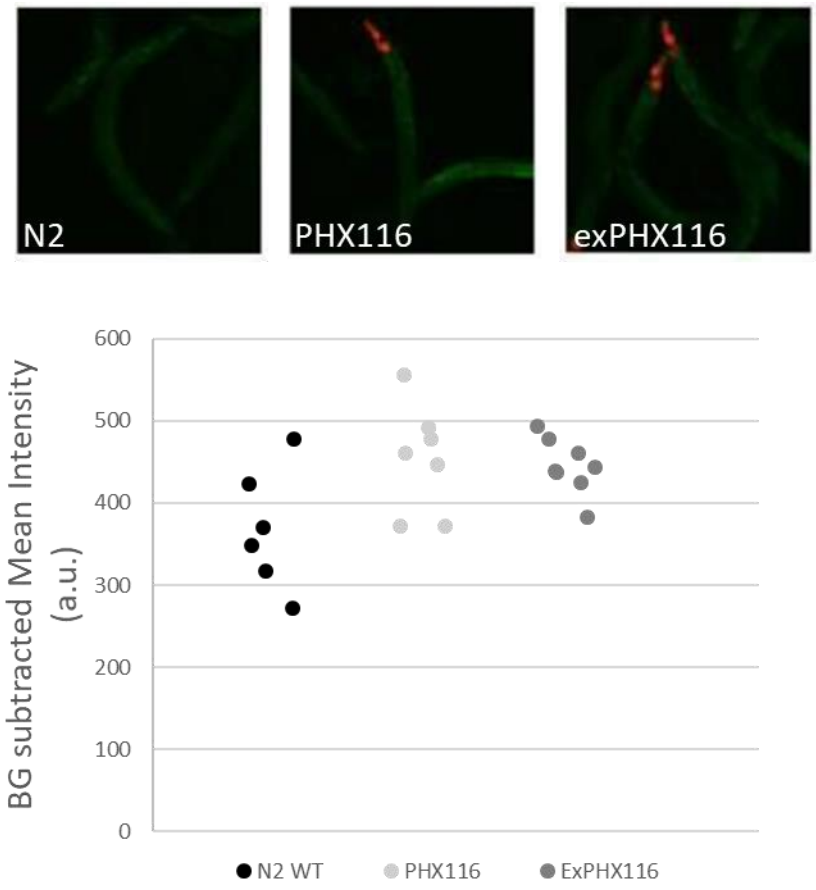
Supporting Figure 1. Pairwise comparison of genes differentially expressed in cell treated with rilmenidine or the serum of CR individuals.



Supporting Figure 2. Charge distribution analysis of F13E9.1 protein. *The protein sequence of F13E9.1 was inputted into the Statistical Analysis of Protein Sequences, SAPS, to compute the charge distribution of amino acids. The coiled coil domain of F13E9.1 is highlighted in blue, which contains an acidic residue to that denoted in hIRAS.*



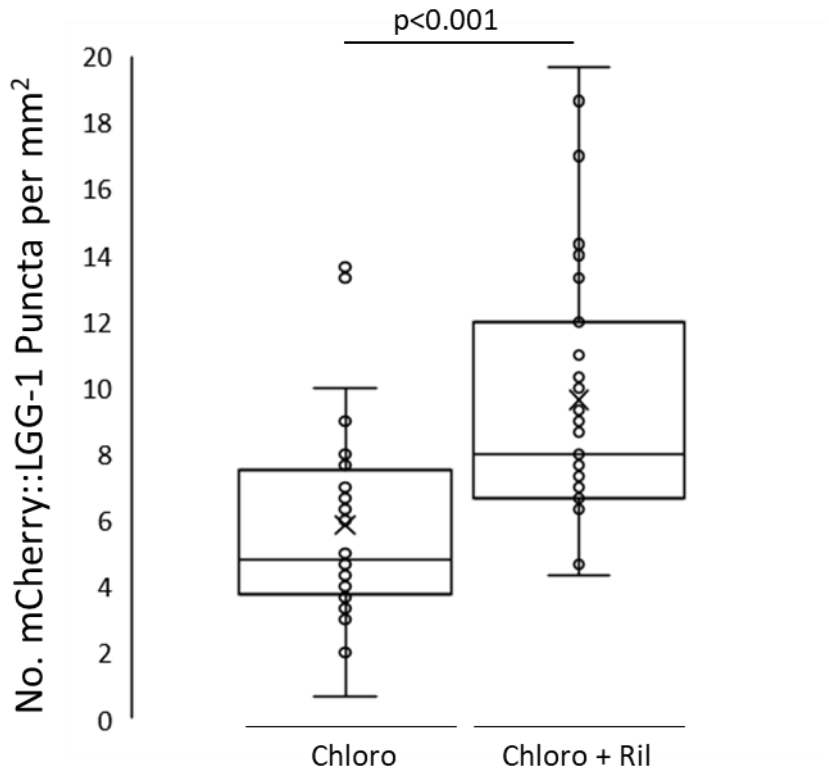
Supporting Figure 3. Verification of 2 transgenic worm rescue lines of PHX945 and PHX946 (syb767) IV; sybIs62[f13e9.1::EGFP::3xFLAG, unc-119(+) + myo-2p::mCherry. *A) Representative western blot of the FLAG-tagged F13E9.1 protein in C. elegans lysates derived as in material and methods section. Lanes from left to right are marker, recombinant FLAG vector from E.coli, lysate from WT worms, f13e9.1 mutant worms, and two rescue strains carrying the WRM0616C_F02 (f13e9.1) clone which should theoretically express a c-fused ::EGFP::3xFLAG sequence.3 Immunofluorescent bands can be seen at ~90kD for rescue one and may represent isoforms. F13E9.1 is a 57kD protein + the tag part including the EGFP, FLAG and the linker peptides accounts for a molecular mass of 35 kD = 92kD B) End point PCR representing amplification of 1.16kbp amplicon within the target fosmid. F= fosmid prep, Mx = injection mixture, M = marker.*



Supporting Figure 4. Expression of f139.1::GFP in transgenic strains under homeostatic conditions. *WT WT strains, PHX116 and ExPHX116 worms were paralysed in 10mM tetramisole solution and imaged under a Zeiss 710 confocal microscope at 20 °C with a Fluar 20x/0.75 lens. Images were acquired as in Teuscher and Ewald, (2018) which recommends a super-thin bandpass green filter (520 +/- 20nm) to overcome inherent autofluorescence within worms. No significant difference was observed between groups (one-way ANOVA p>0.05).*

Supporting Table 3. Survival of WT worms exposed to 2mM H2O2 plates seeded with dead OP50 E.coli, following 24h treatment with either 200µM rilmenidine or a 1% DMSO vehicle. *Kaplan-Meier log rank analysis was used to compare survival; Bonferroni adjusted p-value <0.05 considered significant.*

Genotype/Ril Con.	No. Trials	n=	Mean Survival Hours	Std Err	p vs. Control
24h Treatment With Different Conentrations of Rilmenidine (Rilmenidine Includ. During 10h H2O2 Exposure)					
N2 0µM	2	118	7.68	0.16	N/A
N2 200µM	2	70	7.94	0.27	0.10
N2 300µM	2	89	7.10	0.27	1.00
N2 400µM	2	99	7.72	0.21	0.50
N2 NAC 5mM	2	128	8.39	0.19	0.00
24h Treatment With Different Conentrations of Rilmenidine (Rilmenidine Omitted During 10h H2O2 Exposure)					
N2 0µM	1	32	6.56	0.45	N/A
N2 200µM	1	51	8.20	0.29	0.01
N2 300µM	1	23	9.65	0.20	0.00
N2 400µM	1	43	8.09	0.36	0.01
N2 NAC 5mM	1	62	9.19	0.24	0.00
24h Treatment With Different Conentrations of Rilmenidine (Rilmenidine Includ. During 10h H2O2 Exposure)					
N2 1% DMSO	3	174	5.43	0.19	N/A
N2 Ril 200µM	3	181	5.50	0.18	1.00
f13e9.1	3	246	4.14	0.13	0.00
f13e9.1 Ril 200µM	3	249	3.30	0.12	0.00



Supporting Figure 5. Graph shows the interquartile distribution of mCherry::LGG-1 puncta in the posterior intestine of the animals in grown on NGM agar supplemented with 20mM chloroquine in the presence or absence of 200µM rilmenidine. *Error bars, upper:Q3 + 1.5*IQR; minimum: Q1 -1.5*IQR. mCherry::LGG-1 puncta were counted in each of three rectangular boxes of 1mm2; they were placed in tandem beginning with the most posterior region of the intestinal and extended toward the vulva; mean number per mm2 was calculated for each animal. N>24 animals analysed per treatment in at least 3 independent trials.*