# Identification of novel regulators of late-life longevity in the worm *C. elegans*

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# Summary

Ageing is a ubiquitous, detrimental phenomenon experienced by almost every known uni- and multi-cellular species. The hallmarks of ageing are conserved from worm to man and suggest an origin deep in evolutionary history. However, how such an obviously detrimental process could be maintained, despite the obvious negative selection it would be subjected to, has been a much-debated topic for over a century. The most widely accepted explanation is that ageing is a consequence of evolution. It arises from the preferential selection of alleles with positive fitness effects early in life even if those alleles lead to degeneration later in life. This antagonistic pleiotropy (AP) theory of ageing has received some experimental support but to date, no investigation has found genes with detrimental effects specific to the later life period.

To address this insufficiency I developed a novel age-synchronous liquid culture technique for the worm *C. elegans* that allowed for the reliable production of large quantities of old worms absent of the use of sterile strains or drugs to inhibit reproduction. With this technique, I screened an RNAi library of nearly 800 genes involved in gene or chromatin regulation in the post-reproductive worm. This screen identified 31 novel candidate AP longevity genes including *pha-4*. The transcription factor PHA-4 (FOXA) is essential to survival and longevity in young worms. Remarkably, however, its inhibition post-reproduction extended lifespan by 33%. PHA-4 transcriptionally regulates the expression of two essential autophagy genes *bec-1* and *unc-51.* Late-life inhibition of these genes extended lifespan by an even greater degree than *pha-4*, up to 63%. Both *bec-1* and *pha-4* display AP characteristics including a switch from pro- to anti-longevity as the worm ages and a delay in the onset of ageing hallmarks only when inhibited late in life. The mechanism of this lifespan extension is independent of the major canonical life extension pathways and is likely routed in the autophagic function of these genes.

The results described in this study demonstrate a simple novel protocol for the generation of aged *C. elegans* samples that will greatly facilitate large-scale analysis of the late age period. The efficiency of the RNAi screen suggests that many more AP genes remain to be discovered. This could open a new path to the treatment of ageing and age-related disease through the targeting of detrimental AP factors outside of the development window. Furthermore, autophagy is generally considered to be a beneficial and required process throughout life. The identification of autophagy genes as a detrimental force in late-life could radically redefine our understanding of this process.

# Zuzammenfassung

Altern ist ein allgegenwärtiges Phänomen in fast allen bekannten ein- oder multizellulären Spezies. Die Merkmale des Alterns zeigen sich in ähnlicher Weise vom Fadenwurm bis hin zum Menschen und suggerieren, dass die Herkunft des Alterns tief in der evolutionären Geschichte verankert ist. Wie solch ein offensichtlich schädlicher Prozess evolutionär bewahrt wurde, ist eine viel diskutierte Problematik der letzten Jahrhunderte. Eine Theorie besagt, dass es eine Bevorzugung in der Selektion von Allelen gibt, welche ein positiven Effekt auf die Lebensfähigkeit in den ersten Lebensstadien eines Individuum haben, auch wenn dies zu Lasten der Lebensfähigkeit im Alter geht. Diese Theorie der antagonistische Pleiotropie (AP) wurde bisher durch verschiedene Experimente gestützt, aber bis heute wurden noch keine Gene nachgewiesen, welche nur in den späten Lebensstadien einen negativen Effekt auf die Lebenserwartung haben.

Um diese Problematik zu ergründen, habe ich eine neue Flüssigkulturmethode für den Fadenwurm *C. elegans* entwickelt, welche die verlässliche Produktion von großen Mengen an alters-synchronisierten Würmern in späten Lebensphasen erlaubt, ohne die Nutzung von sterilen Fadenwurmstämmen oder reproduktions-inhibierenden Substanzen. Mit Hilfe dieser Methode wurde in einem Screening eine RNAi Bibliothek von fast 800 Genen untersucht, die an der Gen- oder Chromatin-Regulation von postreproduktiven Fadenwürmen beteiligt sind. Unter den 31 gefundenen Kandidaten ging das *pha-4* Gen als aussichtsreichster Anwärter auf AP hervor. Der Transkriptionsfaktor PHA-4 (FOXA) ist für das Überleben notwendig, aber dessen Inhibierung in der postreproduktiven Lebensphase verlängert die Lebensdauer von *C.elegans* um bis zu 33%. PHA-4 reguliert die Expression zweier essentieller Gene der Autophagie, *bec-1* und *unc-51*.Überraschend ist, dass die Inhibierung dieser Gene in späten Lebensphasen des Wurms einen noch größeren Effekt auf die Verlängerung der Lebenserwartung von bis zu 63% hat. Der Mechanismus zur Verlängerung der Lebensspanne ist unabhängig von den kanonischen Hauptsignalwegen der Lebensverlängerung und liegt sehr wahrscheinlich in der Autophagiefunktion dieser Gene.

Die in meiner Doktorarbeit erhaltenen Ergebnisse demonstrieren eine neue Methode zur Erzeugung von gealterten *C. elegans* Populationen, welche eine Large-Scale Analyse ermöglicht. Die Effektivität des RNAi-Screenings verdeutlicht zudem, dass noch viele AP Gene unentdeckt sind. Dies könnte neue Wege in der Altersforschung und zur Behandlung von altersbedingten Krankheiten eröffnen. Zudem wird Autophagie generell als lebensbegünstigender und essenzieller Prozess angesehen. Die Identifikation von Autophagiegenen als schädliche Faktoren in der späten Lebensphase könnte unser Verständnis des Autophagieprozesses radikal verändern.

# Preface

The research and experiments described within this thesis were conducted in the group of Dr Holger Richly at the Institute of Molecular Biology in Mainz, Germany. This research was conducted as part of the international PhD programme on gene regulation, epigenetics, and genome stability offered by Johannes Gutenberg University Mainz, Germany. The work herein is presented for the awarding of a doctorate of natural sciences (Dr. rer. nat.) from the Faculty of Biology and Johannes Gutenberg University.

The contents of this thesis were written solely by myself and the experiments described therein are those designed and implemented by myself, unless noted otherwise. This project was part of a larger study on the ageing process that was performed in close co-operation with Thomas Wilhelm, Rebeca Median and Johannes Geisinger from the group of Dr Richly. Each member of this team was assigned independent sub-projects and collaborated on the interpretation and integration of each aspect into a final paper (Wilhelm *et al.* 2017). To help frame my thesis in the context of the larger project, I have referred to this final publication throughout my thesis and used some of the discoveries therein to elaborate on my own findings. I have also included 2 figures from work by Thomas Wilhelm and Johannes Geisinger with their permission and have annotated the figure legends accordingly. These are worm movement and pumping assays (Fig. 20, C & D) and day 9 & day 0 inhibition of *vps-34* and *epg-8* (Fig. 22, A & B). Some of the figures of my thesis have already been displayed by Thomas Wilhelm in his own thesis "*Post-reproductive inhibition of the autophagic vesicle nucleation complex extends C. elegans lifespan through the neurons*" as a means of providing context to his own project. All images of this nature were credited to myself.

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# 1 Introduction

Why do we get old? This eternal question has been in the psyche of man probably since we first learned to use stone tools. Humans have been shaped by evolution to be both observant and curious about the nature of things they see around them. Even in early human societies, with their high mortality rates and low median lifespans, the fact that the body grows old and has reduced functionality must have been evident. The question of why we as a species age could not have been long following, even given that these societies would likely have had little time for formal philosophy. In fact, it wasn't until the time of Plato that we see written recordings musing on the purpose of the ageing process. For Plato, getting older had benefits as "those who lived longer reached a philosophical understanding of mortal life, which lead to the desire in understanding everlasting ideas and truths, beyond the mortal world" (Baars 2012). Thus in Plato's view ageing was a desirable process, though not all have seen it so, "Old age is the supreme evil, because it deprives us of all pleasures, leaving us only the appetite for them, and it brings with it all sufferings" (Leopardi et al. 1905). Irrespective of philosophical discussions, the fundamental biological question of why we age remained poorly understood until the mid-1900s and is still heavily debated today. Indeed, a compilation of all known ageing hypotheses in 1990 resulted in a list of over 300 theories (Viña et al. 2007, Medvedev 1990). Despite the lack of a unified consensus on how or why we get old, it is inside this milieu that I am framing this thesis, which focuses on providing solid, observable, experimental evidence for genetic components of late-life longevity based upon the evolutionary origins of the ageing process. With the current changes in our social demographics, it has never been more imperative to start answering the questions of what causes ageing and what, if anything can be done about it. I hope this thesis will be a small stone in the foundation of that progression.

## 1.1 Ageing research, its context and implications within modern society

In engaging with the concepts of ageing including its origins, consequences and potential future treatment, we as scientists must first tackle some of the most difficult aspects of research in this area. To whit (i) what do we mean by the term the ageing, (ii) when does ageing start, (iii) why do we need research into ageing, (iv) what are the ethical questions and implications of ageing research, and is it even possible to engineer human ageing?

### 1.1.1 (i) What is meant by ageing?

Imagine asking a random person on the street, "what is ageing?" I think most people would have a ready answer, perhaps along the lines of "ageing is just getting old". Despite the perceived simplicity

in defining "what is ageing", it has proven to be an extremely difficult concept to pin down scientifically. Simply put, ageing means different things to different people and what it means will be determined by how they connect with the process. For the layperson on the street, it can mean anything from a loss of faculty, to retirement, to economic and social uncertainty. In contrast, biomedical scientists emphasise the absence of disease and the preservation of physical and mental function, whereas sociologists emphasise life satisfaction, social participation and social functioning (Bowling & Dieppe 2005). Unifying such disparate and wide-ranging ideas, combined with uncertainties about the ageing process itself has mired many definitions in imprecision and incompletion. What has emerged is a growing consensus that is common to all streams of enquiry, in that ageing is not a singular event that takes place and continues until the point of death. In fact, ageing is not, in and of itself, a physiological process. It is a multifaceted interaction of genetic and environmental factors combined with an element of stochastic variability leading to a progressive decline in the function, health, and reproductive capacity of an organism over time, culminating in death. The origins of this definition has its roots solidly in evolutionary theory (discussed in section 1.2.2). This definition is not new nor is it fully complete, as it fails to take into account observed plateaus in mortality and functional decline at extremely late-life (Flatt 2012). However, as a simple rephrasing of numerous other definitions (Flatt 2012, Rose *et al.* 2012, Kirkwood 1977, Hamilton 1966), which have been developed and iterated upon over time in the published literature, it serves as a solid basis for all future discussions in this thesis.

If we accept this definition then several aspects of its consequences need addressing. Firstly, ageing arises through the action of natural selection. It is a species-specific response to environmental conditions, whose variation accounts for the noticeable dissimilarities in the lifespan trajectories both within and across different species (Singer 2015). It likely arises as a non-adaptive response to external conditions, although some interesting arguments suggest the possibility of an active adaptive response (see section 1.2 for a detailed discussion). There is a clearly established genetic component to ageing (see section 1.3 for a detailed discussion). Genetic ageing effects typically derive from tradeoffs between other vital aspects, including energy metabolism, fecundity, and longevity (Partridge & Barton 1993), or from the stabilisation or removal of processes involved in the normal production of pro-ageing factors within the cell (de Grey *et al.* 2002). Despite extensive searching, no single control mechanism for the modulation of ageing has been discovered. This implies that ageing is a highly complex polygenic trait and will be difficult to ever fully control.

Secondly, there are clarifications to be made about mortality and senescence and their relationship to ageing. As stated in my definition, the end result of ageing is death and as such mortality is used as a primary proxy for evaluating the effects of any treatment on longevity. Nevertheless, mortality is a less than optimal gauge in this scenario. It cannot be used preemptively to determine the rate of ageing for an individual, it requires a large cohort of subjects to be interpreted with any accuracy (Petrascheck

& Miller 2017), it is extremely ineffective in determining ageing in early life, and is subject to external, non-ageing factors which are hard to control for (Milne 2006). Additionally, when examining conditions that disrupt vital processes, an organism will often die much faster than the control. This does not necessarily mean that an organism has specifically aged faster, increased death rates or the sudden onset of death is not the same as ageing. Attributing cause of death to old age, or a natural cessation of life is always difficult and often only makes sense when a treatment extends lifespan or acts epistatically in mitigating the effects of known longevity interventions. However, despite these caveats and given that there are few if any established biomarkers for ageing (Tuttle & Maier 2017), I will continue to use mortality in combination with measures of health as a proxy for ageing.

Thirdly, the general term senescence has a historical association with ageing, such as in the seminal paper discussing the origins of ageing "Pleiotropy, Natural Selection, and the Evolution of Senescence" by George Williams (Williams 1957). While senescence is defined in the Oxford dictionary as "the condition or process of deterioration with age", its synonymous use with the term ageing is no longer quite so clear-cut, having also become associated with cellular senescence. Cellular senescence refers to the decline in the reproductive capacity of individual cells to zero, combined with distinct phenotypic changes (van Deursen 2014). This senescent state is a programmed check to prevent the accumulation of mutations within a cell that could give rise to cancer if the cell could divide unimpeded. Cellular senescence is closely linked to ageing and indeed some excellent work showed that the removal of senescent cells in mice could delay the onset of age-related disorders (Baker *et al.* 2011). Moreover, their removal even from the mid-life period still extended lifespan (Vaiserman *et al.* 2017). Cellular senescence is not however ageing in the global, whole organism view that I use here and which also predominates the literature. In my definition of ageing, I included environmental factors as contributors to the process. These factors are generally stochastic, cumulative and often can be passed on in a transgenerational fashion. The presence or absence of these factors can modulate longevity at any stage in an organism's life and are often present from birth (Vaiserman *et al.* 2017). As the accumulation of the factors over time is integral to ageing they are commonly referred to as senescence factors. Thus, there is a clear distinction to be made between the decline in function of a single cell and the decline in function of a single organism. To avoid confusion I will here use the general term ageing to also mean whole organism senescence and clearly distinguish between senescence factors and cellular senescence.

### 1.1.2 (ii) When does ageing start?

When looking at mortality rates in humans it is well established that mortality rates are high following birth, dip between ages of 5-13 and subsequently then rise continuously for the remainder of life (GBD Study, 2015). This has sometimes been taken to mean that ageing must begin following this nadir in

mortality as "*ageing first becomes manifest as mortality [rates] turn upwards*" (Grimley Evans *et al.* 2003). While this is an attractive notion on its surface, it is clear that it does not necessarily hold up upon closer inspection. For example, the nadir of mortality has historically shifted over time and even within one country as seen in England and Wales between 1980 and 2001 where the low range in mortality shifted from 10-14 to 5-9 respectively (Milne 2006). Clearly, the onset of ageing is unlikely to shift so dramatically in a 20-year period. As ageing is separate from development, and as every developmental programme is optimised to produce individuals of maximum fitness capacity very shortly after the onset of complete reproductive maturity, it is then hard to consider that ageing could in any sense begin before this point. Subsequent to this, natural selection becomes weaker and allows for a de-tuning of adaptation and thus fitness with increasing age which essentially is ageing (Rose *et al.* 2012). As we have defined ageing as a decline in function, health and reproductive capacity over time, it is seemingly evident that ageing can only begin following the zenith of the developmental process. Nonetheless, the onset of ageing must also consider the influence of senescence factors that are independent of development and can influence ageing from birth. These factors, such as inherited epigenetic landscape or plentiful nutrition during development, can clearly influence how an organism ages (Dato *et al.* 2017) and more fascinatingly this influence can even be transmitted to subsequent generations (Vaiserman *et al.* 2017, Herskind *et al.* 1996). As such, ageing can be said to being immediately following reproductive maturity but its effects are modulated by senescence factors, which are stochastic and cumulative and can arise from birth onwards.

It is for these reasons that it is essential in any gerontological study to remove as many of these confounding factors as possible. This is only easily achieved in the controlled environment of the lab where the influence of extrinsic senescence factors can be minimised and populations established that are genetically heterogeneous and age synchronised. This standardisation allows for the use of mortality-derived data to examine the genuine life-course effects of an intervention. Nevertheless, as previously stated we must consider not just mortality but also measures of functional decline. To do this we must be clear about what aspects of functional decline we are examining and how these changes are different from a "normal" or median level of health at a defined time-point and judge their change relative to a fixed state. Thus, for example, an individual in their 50s is said to have aged if a measure of a marker for ageing has declined in comparison to the ideal of say at 25 year old. However, they may be considered to have aged well if, for example, they have an augmented capacity compared to a cohort of other 50 year olds. By examining both outcomes we can determine how any intervention not only impacts on the process of ageing but also on how that intervention will affect the remaining life history of the individual affected and gain a true measure for any effect on functional ageing. This is extremely important as any intervention, which extends life but does so at the cost of function or

increases the proportion of life spent with reduced capacity is perhaps of academic interest but likely of little social value.

### 1.1.3 (iii) Why do we need research into ageing?

The notion of social value brings me to the next question, why do we need research into ageing. One of the most difficult aspects of this thesis may be making a convincing argument that ageing is not in fact a normal, natural process. Ageing is in effect a disease state. Much like the HIV virus, ageing is not the direct agent causing death in an individual but rather the facilitator for other diseases and conditions, which normally absent in a young and healthy person now progress to the point of lethality. While this analogy could seem extreme, it is widely accepted that individuals infected with HIV are at significantly higher risk for age-related morbidity (Hunt 2014). Essentially both pathologies result in a weaker and more fragile state that is vulnerable to life-threatening illness.

Again, like for HIV, the number of diseases associated with the progression of ageing are manifold, and too many to explore in detail here. As a brief summary they include diseases involving: the immune system – increased rates of infection and autoimmune diseases; the endocrine system – including benign prostate hypertrophy and diabetes; the nervous system – including Alzheimer's (AD) and Parkinson's disease (PD); the locomotor system – including osteoporosis and sarcopenia; the cardiovascular system – including atherogenesis and atherosclerosis (Niccoli & Partridge 2012, Wick *et al.* 2000); and cancer – most cancer risks are increased in direct proportion to age (Hunt 2014). In addition to increasing the risk associated with certain diseases, the pathways known to modulate the ageing process have direct consequential roles in major diseases. If we look at the three most prevalent diseases with age (cardiovascular, neurodegenerative and cancer), a clear link between major ageing mechanisms: nutrient sensing; mitochondrial dysfunction; autophagy; and DNA damage and senescence (see section 1.2.1 for a detailed discussion of these pathways) and the aetiology of these diseases is observed.

#### *1.1.3.1 Cardiovascular disease and ageing*

The nutrient-sensing pathways of both the Insulin-like growth factor (IIS) and Target of Rapamycin (TOR) can encourage the onset of disease through the accumulation of fat and subsequent insulin resistance, both of which are significant risk factors in cardiac disease (Gray & Kim 2011, Curtis *et al.* 2005). Additionally, insulin resistance and hyperglycaemia have been shown to exacerbate the formation and progression of atherosclerotic lesions (Bornfeldt & Tabas 2011) and people who maintain high levels of insulin sensitivity, such as seen in centenarians, are at lower risk of disease (Curtis *et al.* 2005). The heart itself responds to insulin directly and can also become insulin resistant which gives rise to systolic dysfunction (Yu *et al.* 2011). Obesity can stimulate the TOR pathway, which,

through a downstream effector kinase S6K, reduces the strength of insulin signalling via down-regulation of Insulin receptor substrate 1, further exacerbating insulin resistance in the heart (Gray & Kim 2011). Older hearts have increased levels of mutated mitochondria (Shih *et al.* 2010), which can trigger apoptosis and lead to heart failure (Crow *et al.* 2004). Mouse models containing mitochondria in which the mutation rate is enhanced show traits of premature heart ageing (Trifunovic *et al.* 2004). Additionally, mutated mitochondria have increased levels of reactive oxygen species (ROS) leading to remodelling of the myocardium and subsequent heart failure (Tsutsui *et al.* 2011). The failure of autophagy later in life to degrade damaged organelles and proteins has been linked to heart failure. Patients with defects in the lysosomal-autophagosome fusion process, required for such degradation, have severe cardiomyopathy (Wang *et al.* 2012a). Generally, decreasing the levels of autophagy leads to hypertrophy and subsequent heart failure (Shirakabe *et al.* 2016). Interestingly, however, following injury, the heart induces autophagy as a protective mechanism (Lee *et al.* 2012a). This induction can be done to excess and inhibition of early autophagy through Beclin-1 in this context can have a protective function, wherein the heart is insulated from overloaded maladaptive remodelling (Wang *et al.* 2012b). The endothelial surface of the heart is constantly in contact with toxins present in our blood and suffers from high levels of DNA damage and increasingly shortened telomeres, leading to senescence (Kovacic *et al.* 2011, Shukla *et al.* 2010). This phenotype is suspected to induce the inflammatory cycle and lead to plaque deposition (Moore & Tabas 2011). In general, the accumulation of senescent cells is detrimental to whole body health and their removal in mice increased lifespan by 20% (Baker *et al.* 2011) while reducing plaque buildup by 60% (Childs *et al.* 2016). This agrees with data showing the internal thoracic artery is protected from atherosclerosis by higher levels of telomerase which inhibits senescence (Fuster & Andrés 2006).

#### *1.1.3.2 Neurodegenerative disease and ageing*

The process with the best intersection between ageing and the development of neurodegenerative disease is probably autophagy. The relationship between autophagy and neurodegeneration is discussed in detail in (section 1.3.6). Beyond autophagy, other ageing associated processes also play a role in neurodegeneration. The gradual accumulation of DNA damage over the lifetime of a neuron can cause a re-entry into the cell cycle, which for these cells triggers apoptosis and neuronal loss (Barzilai 2010). In the brains of both Alzheimer's disease (AD) and Parkinson's disease (PD) patients, there is evidence of increased DNA damage accumulation followed by cell cycle re-entry. The inhibition of this process leads to better synaptic retention and reduced neuronal death triggered by protein aggregates (Barzilai 2010, Weissman *et al.* 2007). Our neurons are highly metabolically active and it is therefore unsurprising that mitochondrial health is essential to neuronal survival. Indeed, mitochondrial genetic disorders are highly correlated with subsequent neurodegenerative disease

(Yang *et al.* 2008). Increased ROS production from damaged mitochondria may be exacerbated by reduced autophagic clearance (Green *et al.* 2011). In particular, a failure of the selective targeting of damaged mitochondria to the autophagic machinery through the disruption of PINK1 can lead to PD (Filosto *et al.* 2011). Reduced insulin signalling specifically in the neurons has a neuroprotective effect in animal models (Bishop *et al.* 2010) and also extends lifespan (Broughton & Partridge 2009). However, insulin is still critical for neuronal health, patients with AD have severely reduced insulin signalling and resistance to insulin promotes the deposition of amyloid beta plaques and is a known risk factor for AD (Cholerton *et al.* 2011). Thus, it seems the degree of insulin signalling and its regulation is a critical aspect of neuronal health.

#### *1.1.3.3 Cancer and ageing*

Clearly in the case of cancer one of the primary causes will be damage to the DNA where increasing genome instability gives rise to uncontrolled proliferation. DNA damage when it occurs is repaired by a complex orchestra of proteins in what is known as the DNA damage response. Genetic mutations affecting the function of key members of this pathway render individuals highly susceptible to cancer and often have decreased lifespans (Menck & Munford 2014). The processes involved in repairing DNA damage are themselves not perfect. Mistakes accumulate over the life of the cell contributing to genome instability and as a result, increased age correlates with increasing cancer incidence (Niccoli & Partridge 2012). A normal cell that has suffered significant DNA damage will be shuttled into senescence or destroyed via apoptosis (Childs *et al.* 2014). When these safeguards are bypassed through the mutation of tumour suppressor genes, such as p53 or checkpoint proteins like ATR or CHK2, cancers can progress from benign to aggressive becoming lethal (Muller & Vousden 2013, Craig & Hupp 2004). Interestingly, increasing p53 expression in mice reduced cancer incidence and extended lifespan (Matheu *et al.* 2007). Telomere length is a good predictor of mortality risk (Blackburn *et al.* 2015) and telomeres themselves play an important role in the inhibition of cancer by limiting their replicative potential through activation of replicative senescence (Childs *et al.* 2014). Cells that have become cancerous overcome the activation of this check by either re-activating telomerase or repairing their short telomeres through homology-directed repair (HDR) (Finkel *et al.* 2007). Proliferating cancerous cells are voracious in their requirements for energy and as such the IIS pathway functions as a key node in cancer progression. The presence of high insulin levels can in and of themselves induce cancer (Lawlor *et al.* 2004) and positive and negative regulators of insulin signalling have corresponding effects on cancer occurrence (Blume-Jensen & Hunter 2001). Rapamycin and metformin, which target the TOR pathway and liver glucose production respectively, show promise as chemotherapeutic agents (Andújar-Plata *et al.* 2012, Sharp & Richardson 2011). The role of autophagy in cancer is an ever developing one and seems to have a dual effect on cancer progression. Efficient

autophagy is necessary to prevent the formation of early cancers, possibly through the removal of pro-oncogenic substrates, and mice lacking key autophagy genes develop benign cancers (Kimmelman 2011). However, once a cancer is established within the host, the levels of autophagy in the tumour cells are increased enabling tumour survival and cell growth (White 2012). Thus, it is cancer and stage-specific as to whether enhancing or inhibiting autophagy is the correct approach. Numerous therapeutic cancer strategies have are now in trial where the levels of autophagy are manipulated in combination with other chemotherapeutics (Towers & Thorburn 2016). Once cancerous cells begin to proliferate they have to cope with reduced oxygen availability especially in the core of the tumour. This up-regulation of glycolysis impacts the function of the mitochondria directly, reducing ATP synthesis (Gogvadze *et al.* 2008), reducing apoptotic potential (Danial *et al.* 2003), and increasing ROS production (Lee 2015). These changes enable the cancer cell to survive longer and encourage further DNA mutations which eventually allows the cancer to become metastatic (Ralph *et al.* 2010).

#### *1.1.3.4 Demographics of ageing*

When we take all this into consideration it can be stated without hyperbole that ageing is, in fact, the single most prevalent disease state that exists on our planet today. This has not always been the case. Throughout evolutionary history ageing rarely progressed to the point at which humans died from age-associated disease. Death typically came from external factors such as violence, starvation, infection or other diseases. This is still the case for the majority of wild animals on our planet but no longer holds true for us humans. The highest causes of death in people in the 28 EU countries in 2014 were in order: circulatory disease, cancer, heart disease, respiratory diseases and diseases of the nervous system (Eurostat Mortality Statistics, 2017). This trend will only accelerate in the near future as in the next 12 years it is predicted that every single country on the planet will experience an upward shift in the median age of the population (Lutz *et al.* 2008). While there has been a general trend towards an older median population age for the last 60 years it has actually been slight, a shift of only 2%. The projections for the next forty years for this same group predict an unprecedented 22% increased share of the total population (Lutz *et al.* 2008). This can be considered as a triumph of modern society, for the first time globally, the majority of people can reasonably expect to live into their 60s (Beard *et al.* 2016) and their subsequent life expectancy is an additional 23 years in higher income countries (Mathers *et al.* 2015). The largest gains have arisen from improvements in access to healthcare and improved public policy and include reduced infant mortality, reduced tobacco use, reduced cardiovascular mortality, and for people over 60 reduced deaths from non-communicable diseases (Wilmoth 2000). Despite these improvements, ageing is a risk factor for all the highest mortality disease classes and these non-communicable diseases cause 70% of deaths globally. This trend is not quite the same in third world countries where the burden of infectious disease is still high and accounts

for 52% of deaths in this region, though, notably, the rate of non-communicable disease in these countries is rising (WHO, 2017). As such, when viewed in the context of the increasing disease burden associated with ageing populations it makes for a worrying prognosis as a so-called "silver tsunami" could roll over the world drowning the healthcare and economic systems of the world (Perry 2010). It is perhaps only due to the ubiquity of ageing across every individual and through every class and society, combined with an increase in the number of people reaching an advanced age in the last century, that allows us to consider ageing as a normal process rather than a health burden in desperate need of treatment.

#### *1.1.3.5 The need for ageing research in modern society*

If then ageing research will extend lifespan, does it not follow that in doing so we are simply accelerating the rate of disease occurrence in older age and driving our society towards a point of social implosion? Are we bringing our own "Struldbrugs" – immortal individuals in Jonathan Swift in Gulliver's Travels who live fantastically long lives but suffer all the ravages of ageing none the less – into the world? Modern science has yet to find a way to significantly increase longevity in humans without the accompanying rise in age-associated disease. This has often lead to the viewpoint that advances in medical technologies prolonging late-life have progressed far enough and need to be reined in to protect the economy. For example, this idea was expressed by Dr Peter Orszag who was the head of the U.S. congressional budget office under the Obama administration (Perry 2010). Such thinking is understandable but is predicated on the current models of disease treatment, which focus on a disease by disease, organ by organ strategy. Such a strategy is unlikely to work as most elderly people have multiple interacting morbidities that significantly complicate treatment. Even curing cancer tomorrow would not necessarily lead to longer and healthier lives for most people (Kennedy *et al.* 2014). Ageing research and ultimately controlling the ageing process has the capability to provide a new lateral approach to these issues. Ageing unsurprisingly connects together nearly all age-associated diseases and as illustrated above, the pathways involved in ageing have direct impacts on disease. Thus, if rather than focusing on improvements in gains of years lived we instead focused on improving healthspan by targeting the core processes of ageing, we could gain the ability to age without the accompanying diseases (Sierra 2016). This targeting of ageing pathways has already been carried out in animal models with positive reductions in late-life disease but is dependent on the strategy employed (Sierra *et al.* 2009). These ageing targets may prove to be the best route forward to attack the root causes of not one but many diseases and diffuse a medical time bomb before it can explode.

Even if one was to ignore the argument for ageing being something we need to tackle to reduce human suffering, the presence of an increasingly ageing population has numerous problematic and likely

unavoidable economic implications for countries with increasingly aged populations. For example, while more and more people will be living further into late-life, they will be dependent on social contributions over a timespan that could even exceed that of their working life. Moreover, they are at an increased risk of non-disease associated physical disability for every additional year they live (Kenny *et al.* 2008). As such, many individuals, even if they would like to work, are simply unable to partake in the modern workforce. This leads directly to reductions in economic output and national production; increased burdens on health and long-term care systems (Bloom *et al.* 2011); and increasing strain on government pension schemes, through combinations of retirement and disability (Turner 2006). The burdens imposed by these trends have historically fallen on family members but with declining fertility rates and shrinking family sizes, these burdens will be falling on an ever-shrinking portion of the population (Kohler & Ortega 2003). These factors combined are already placing increasing strain on global capital spending and economic stability (Bloom *et al.* 2009) and this trend will continue and exacerbate over time. The risk to the integrity of countries' economic situations and status are real, however, the opportunity to respond to these demographic changes and so offset some of the risks is still possible through long-term governmental policy changes (Rechel *et al.* 2013). Unfortunately, without further progress in alleviating the burdens of ageing from the population, all these policies do is buy time. As such, performing research on the processes of ageing, understanding its origins and outcomes, and using this knowledge to help alleviate its consequences is likely to become, in my opinion, one of the most pressing issues of biological research in the next 20 years.

Outside of pure monetary consequences, there are other benefits to be gained through the study of ageing. Subjective well-being and health are closely linked to age (Steptoe *et al.* 2015), as one gets older, in part due to the prevalence of chronic illnesses, measures of well-being tend to decline during the middle years (Blanchflower & Oswald 2008). Though it is worth noting that there is a subsequent upward turn in older age which is attributed to the accumulation of emotional wisdom leading to better decisions (Carstensen *et al.* 2003). Well-being itself may also have a protective role in maintaining good health during ageing. Data from the English longitudinal study of ageing showed the incidence of deaths over the next 8.5 years was 30% in people identified as having the lowest levels of well-being. This was in comparison to those with the highest levels of well-being were only 9% died in the same period (Steptoe *et al.* 2013). If we consider a potential future where the amelioration of age-related diseases is a valid reality, we could expect that well-being would be correspondingly dramatically increased and thus feeding back into the system to preserve that improved health.

### 1.1.4 (iv) What are the ethical questions and implications of ageing research?

If the previous section persuades for the need for gerontological research, then the subsequent question must be what are the ethical implications of the outcomes of such research and is there an ethical argument either for or against pursuing such research?

The notion that ageing could realistically become a controllable aspect of our lives is a relatively recent one, arriving as it does with the discovery in 1988 that mutation of the gene *age-1* extended lifespan in *C. elegans* (Friedman & Johnson 1988). Prior to this, ageing was viewed as an intractable problem with solutions found only in the realms of fantasy. With the identification of a genetic control to the ageing process, suddenly fantasy had an edge of reality to it and a path forward could be seen. 30 years later no individual therapy has broken through and given us eternal youth but the promise of such an event seems only to be a matter of time, albeit potentially centuries away. If or when therapies that can repair/halt the damage of ageing from within our own bodies ever became a reality, the effects would be epoch-defining (Bond *et al.* 2007). Not only would the notion of personal health and survival be radically altered but our very identity as humans will be called into question (Glannon 2002). Such potential upheavals, while unlikely in the near future are ultimately the end product of ageing research. As such, as scientists and members of humanity, it is critically important to judge the merits of such changes and to examine both the potential good and bad that such changes would bring about.

When discussing ageing research with people who are themselves gerontologists or even natural philosophers, there is often a mistaken assertion made about the end goal of ageing research. "You are trying to become immortal, that's a terrible idea!" The notion of genuine immortality – a complete inability to die – is extremely familiar to the human imagination. From Homer to Shakespeare and modern literature too (all men are mortal by Simone de Beauvoir is an excellent example) we see tragic examples of those who seek immortality only to find the curse it brings with it. We are primed as a society to view the quest for immortality to be about as clever as giving sentience to machines, it doesn't end well. There is, however, an important distinction to be made in the case of ageing research. Immortality as potentially granted through the control of the ageing process is not invulnerability. This is hugely important. Disease and accidents would still exert their cost and therefore, humanity would retain its link to death and impermanence, which are viewed as crucial drivers of human progress (Berger 2011). Instead, humans will progress through life freed from the burden of increasing disability and frailty and retain the youthful energy and vigour that allows us as a species to really push the boundaries of the possible. While this view is by necessity utopian, ageing research has to be framed through this lens, all the while keeping watch for the unintended and negative consequences along the way.

This futuristic, utopian view is a distant reality from now. So, at this moment we must ask what are the immediate benefits that justify even starting out on this road. When we preserve life we postpone that inevitable meeting with death for just a bit longer. What then is lifespan extension other than another form of preserving life. Generally, the preservation of life is a powerful, often overriding imperative as a failure to do so ultimately makes us directly responsible for a death (Harris 1987, 1980). In countries such as Germany, such imperative is enshrined into law for the common citizen, whereby, legally, aid must be rendered to someone in peril of losing their life as long as it can be done without peril to your own (section 323(c) of the German Criminal Code). Of course, modern liberal societies such as the Netherlands and Switzerland have acknowledged that this imperative does not apply to individuals suffering great pain and reduced life quality. Here it is viewed that the ending of such lives should, to a degree, be at the discretion of those suffering (Emanuel *et al.* 2016). Yet, despite such options, there is a great drive for extended life even in terminal cancer patients regardless of the associated costs in pain and life quality (Slevin *et al.* 1990).

There are, however, risks to advancements in longevity research. One of the major ones is that in our modern capitalistic world such technologies will be expensive and limited initially to the developed world, perhaps only to a small subset of people within that world (Hamzelou 2017). If we imagine that technologies such as CRISPR could be ethically applied to gametes, zygotes or developed adults – and the ethics of such modifications are a whole other issue themselves – then such facilities as would be needed would be specialised and rare and subsequently expensive. Nonetheless, such notions were similarly touted with the advent of artificial insemination (Ombelet & Van Robays 2015). While this technology is not universally available or cheap it has spread and is even provided for by some national health plans such as in the UK. Early subscribers to any ageing treatment, and one can imagine that there would be many, would create the classical demand that drives innovation, lowers cost and increases availability. However, until such advances become available to all, the issue of a two-tier population would arise. In this scenario, citizens of rich countries would gain further advantages over those in developing countries and there would be populations of mortals and immortals living alongside one another (Harris 2004). Such a stratification of society, even temporary, naturally turns many off the notion of any ageing therapy. It appears to them, on the face of it, to be both undesirable and unfair. However, such stratification between rich and poor, haves and have-nots is already endemic in the modern world and short of some new social revolution is unlikely to change. Ethically we are aware that not all public goods can be provided to all people simultaneously (Kaul 2003). This does not mean that such goods should not be provided for in the first place, rather it means that we have to be better in facilitating the availability of such services to those without access. I think it is fair to say, the idea of not researching a vaccine for HIV because it will not be immediately available to those most in need falls flat. Instead, governments need to pressure companies producing these

products to make their profits in the developed world where they can be afforded and provide them in poorer regions at close to cost (Kaul 2003). Also, we as a society have a duty to ensure that those who benefit from any treatment are chosen according to some just principle of distribution (Harris 2004). It is our obligation to structure our society in such a way that this is possible, rather than restrict the development of these treatments for fear of inequitable distribution. I also believe that the obvious disparity in manifest health between those who do and did not receive such treatments will drive us as a society to act more immediately and effectively than we have in the past to negate such inequalities. In this sense, the greatest good is achieved through the development and implementation of any such therapy.

It is important to add that control of the ageing process and subsequent immortality does not itself have to be the goal of gerontological research. Instead, if we frame the research in terms of eliminating diseases such as cancer, dementia, heart disease and others in a single blow, with a subsequent bonus of immortality, it becomes immediately more obvious where the ethical approach lies. I feel this is one point often missed in the discussion of controlling ageing. When presenting such possible interventions it is important to clarify that not only will there be longer life but that also the accompanying health and fitness would be that of a younger person. This is crucial as the prospect of facing into 50 or 60 years of extended life in an aged state is simply not appealing on any front. Thus, ethically and socially it is extremely important that the goals of ageing research are aligned with genuinely improved health. How much that health improves then becomes a determining factor in the viability of any proposed treatment.

Is a longer life in and of itself a good thing? Even if we take the view that the prospect of parallel populations is tolerable and that in tackling ageing we are curing disease, another point of contention arises i.e. whether there are downsides to living a longer life which outweigh other benefits? As stated by the ethicist Leon Kass "*is it really true that longer life for individuals is an unqualified good?*" (Kass 2001). Kass argues that the process of becoming immortal would disconnect us from what it is to be human. Additionally, he claims that psychologically, over time, the continuity of self would change, resulting inevitably in an immortal who is in effect not human anymore. While there is some truth to the idea that an immortal at 30 and the same immortal at 200 would not be the same in terms of self-identity, this is the case for everyone immortal or not. I am certainly not the same human I was in my 20s for example. Experience changes us and while centuries of experience may make it hard for any immortal to relate to a 30-year-old, they would still be human. Perhaps knowing that natural death is removed from the table could lead to a subsequent change in outlook or approach to life but surely this is no more drastic than the common sudden changes of fortune we all experience today, an unexpected child perhaps. After such an event, how one now views and interacts with the world will be different but still intimately human.

Another argument levelled against increasing longevity is that the drive for progress and innovation would drop. Without the looming shadow of death, humans will all become procrastinators with no drive to change things now. As the famous quote from Samuel Johnson says "*Depend upon it, sir, when a man knows he is to be hanged in a fortnight, it concentrates his mind wonderfully*" arguing that it is only by confronting our mortality are we driven to focus and apply our full attention. I can understand the implied criticism behind the argument for inevitably decreased productivity – that progress is itself a worthy goal. However, the inbuilt assumption causes, in my opinion, such an argument to fall at the first hurdle. There is no doubt that technological and social progress can be a great thing and I would much rather live now than 150 years ago. However, the idea that the pace of progress has to be maintained at current levels is similar to wanting economic growth of 3% or more year-on-year. It is an expectation built on current trends and should not be assumed to be a *de facto* good. If an ageless society did take a slower approach to life and allowed progress to arrive gently, I believe this would actively be a societal good. We are so bombarded with new technology that we have no opportunity to look at their unintended consequences, nor comprehend the ramifications for who we are as a society with this new technology (Volti 2017). I do not suggest that we should actively work to slow the entry of technology into our lives, merely that if it was slowed as a consequence of immortality that this would not necessarily be a bad thing. Take social media as an example, the democratisation of information and the ability to stay connected all around the world are fantastic developments. However, we are barely grappling with the consequences of having our lives and information out there for all to see as it is. Whether this is good or bad is irrelevant, it is more that we have no time to process and deal with these issues before a new technology building on the old arrives. In a world with immortals and a slower pace of progress, we might finally have the chance to get some solid footing under us and decide whether or not to embrace these changes or not. Of course, the reality of whether such a drop in productivity would actually happen or not is debatable. We are a curious species and we will probably always strive to see what's beyond the next bend in the road looking for something new. Increased longevity is only going to give us more time and opportunity to do that searching.

If we assume for a moment that the control of the ageing process was available tomorrow, in our world as it currently stands, then another of the biggest issues is how would our already overpopulated world cope (Glannon 2002). This argument implies that ethically we should not be pursuing the control of ageing because if we do, we doom our world through resource scarcity. To some degree, this may surprisingly be a non-issue from the start. As explained before it is suspected that the early adoption of this technology would be by a small percentage of the population in the developed world. The demographics of these countries show a general decrease in population size as mortality begins to overtake births (Rechel *et al.* 2013). Thus, any life-extending technology could, in

actuality, be a force for stabilising the productive healthy workforce in these countries. These demographic trends are expected to spread to developing countries as they achieve political and economic stability (Mathers *et al.* 2015). This would go hand-in-hand with the conditions necessary for the uptake of such a technology and it is unlikely that an immediate population boom would occur. In addition, those availing of such treatments would still be subject to mortality, to infectious disease, and to accidents and would therefore not remain in the population *ad Infinitum*.

When looking to the future we have the possibility of controlling diseases and paying the price with extended longevity. I cannot but see this development as anything but a public good. Nevertheless, this good will have associated societal costs and we have to develop sound ethical and social policies that allow us to manage such a transition without becoming lost along the way. It is only in our capacity to do this that the ethical equation for the control of longevity tilts from negative to positive.

## 1.2 The origins of ageing: Why we get old?

Given that we, as an intelligent, questioning species, have observed and wondered about the ageing process, combined with advances in modern technology, one would think that the question of why we age would be long resolved. However, despite numerous theories in the last 150 years, there are still continuing and heated debates as to how and why some form of ageing arose for practically every species on the planet. The current debates, predominantly between the three main branches of ageing theory described below, are perhaps a reflection of the current status of ageing research. We have and are accumulating more and more data that allows us to peer into the queer biology underlying ageing. However, classically, this wealth of data is forcing us to challenge what we think we know and to try and adapt our theories to fit the observed reality. If anything, the fundamental uncertainties that abound make this a fascinating time to be a gerontologist.

Questions about the origins and mechanics of ageing may at first seem to be a purely academic quarrel over subtleties of thought but are actually non-trivial, as their resolution could define how we proceed with tacking ageing into the future. Briefly, the three main branches of theories for the origin of ageing are divided into 1) deterioration of fundamental limitation theories, 2) non-programmed or non-adaptive ageing, and 3) programmed or adaptive ageing. Fundamental to the discrepancies between these theories is whether ageing is a deliberate evolving part of our biology or whether it arises through unintended consequences. These differences give rise to very different predictions in how age-related diseases are linked to ageing and thus propose very different approaches for identifying the underlying causes and the relevance of animal models for treatment. As an example, George Williams (on whose theory this thesis is largely based) stated "*Senescence should always be a*

*generalized deterioration, and never due largely to changes in a single system.…This conclusion banishes the 'fountain of youth' to the limbo of scientific impossibilities ...*" (Williams 1957). Such views held that looking for single genes that would have profound impacts on the ageing process was the equivalent of Ponce de León's search for the fountain of youth. Such critiques meant it was 30 years, following this statement, before scientist began to discover mutations in single genes that conferred impressive changes in longevity (Friedman & Johnson 1988, Kenyon *et al.* 1993).

The rationale for this thesis is grounded in and based upon assumptions from these theories of ageing. This has naturally informed my methodologies and conclusions. As such, in this chapter, I will give an overview of these three main branches and discuss some of the issues speaking for and against them to place my research in context.

### 1.2.1 Fundamental limitation theories

There are a multitude of theories and sub-theories that propose to explain the presence of ageing as a consequence of the biological processes needed to live (Jin 2010). The current versions of these theories trace their origins to August Weismann, who in extensions to his discussions on the evolutionary origins of ageing suggested that cells and tissues simply have vital components that wear out over time (Weismann 1882). The basic idea is that there is a slow, incremental accumulation of damage, even from the point of birth, that eventually culminates in the failure of a critical system such as the heart, liver or brain. This notion is simple and elegant and thus appeals to the popular science view of ageing where it is often explained with a version of the subsequent analogy. When a new animal is conceived, a large degree of energy is invested to create a functional system that can survive until the point of reproduction. As long as the organism reaches this point with a sufficient degree of fidelity to successfully mate then nothing more is required. This state of health at reproduction then slowly declines as accumulated damage mounts up, eventually leading to catastrophic failure and death. These notions of gradual decay are usually synonymous with a failure of repair processes that normally retard or reverse damage. I will discuss the theories that most prominently feature these aspects here.

#### *1.2.1.1 DNA damage accumulation*

Given the discovery of the DNA double helix in 1953 (Watson & Crick 1953) and the understanding of the critical nature of DNA to life, it is perhaps unsurprising that one of the earliest formalised theories for the gradual accumulation of damage leading to ageing is the DNA damage theory of ageing. First proposed by Gioacchino Failla in 1958 (Failla 1958) it broadly linked the process of DNA damage (mutations) with that of ageing and was succeeded a year later with an independent but more formal

mathematical analysis (Szilard 1959). These ideas essentially assumed that mutations would inevitably lead to the dysfunction or absence of a gene product(s), which would impact other systems or whole tissues, resulting in deterioration and eventual collapse. As our understanding of the types of DNA damage progressed over time so has the theory, which, while no longer claiming to be the ultimate source of ageing, still emphasises that "*DNA damage and its imperfect repair play a major role in human ageing*" (Freitas & De Magalhães 2011). It assumes primacy over other forms of damage as DNA, being the central storehouse of the cells operational instructions, has to last the lifetime of the cell, unlike other cellular components that can be replaced. There is considerable evidence demonstrating a rise in DNA mutations and chromosomal defects as both humans and mice age (Lu *et al.* 2004, Dollé & Vijg 2002). Additionally, the rate of mutation in somatic cells is about 20 times that in germ cells (Milholland *et al.* 2017). Despite the fidelity of DNA repair mechanisms they are not perfect, when challenged with approximately 50,000 events daily, mutations will occur (Fox *et al.* 2016). Defective cells may be killed or replaced but it is thought that the accumulation of genetic damage in stem cells may contribute to their replicative senescence, leading ultimately to an inability to replace essential cells (Rossi *et al.* 2007). An example of this was found in the blood of a 115 year old woman where the majority of her white blood cells originated from just two stem cells (Holstege *et al.* 2014). This suggests that her advanced age and health were in part due to the ability of these progenitor cells to survive and function into advanced age. A disruption in stem cell function forms one of the major modern hypotheses of this theory, that DNA damage activates signalling pathways that cause functional disruption or stem cell loss resulting in accelerated ageing (Freitas & De Magalhães 2011). DNA repair mechanisms are correlated with the rate of ageing, the most striking examples being in the accelerated ageing (progeroid) syndromes (Martin 2005). In these conditions, there is either a failure to deal with sources of DNA damage or a failure in DNA organisation. Additionally, mice undergoing caloric restriction (CR), a lifespan extending treatment, have reduced numbers of spontaneous DNA mutations (Garcia *et al.* 2008). However, from research in mouse models, the pure accumulation of mutations/damage alone does not seem to exclusively cause accelerated ageing. Introducing mutations to mice via X-ray bombardment failed to accelerate the appearance of ageing phenotypes (Cosgrove *et al.* 1993). Indeed, not all mutations in DNA repair genes lead to increased ageing rates (Narayanan *et al.* 1997), suggesting that only certain types of DNA changes are crucial to ageing. In an analysis of the published literature linking DNA repair to ageing, it was found that genes involved in the non-homologous repair pathway are more often linked to organismal ageing (Freitas & De Magalhães 2011), while those involved in transcription-coupled repair effect cellular ageing (Hoeijmakers 2009). If DNA damage and repair are crucial to ageing, then a logical prediction would be that improving the fidelity or rate of repair should lead to slower or postponed ageing. While there are some indications that by enhancing the DNA repair process we may be able to improve longevity (Baker

*et al.* 2013, Tomás-Loba *et al.* 2008, Zhou *et al.* 2001) the jury is still out as to the exact impact these enhancements have on qualitatively improving the ageing process. Recently, the identification of clock-like mutational processes that introduce mutations at different rates in different cancer tissues (Alexandrov *et al.* 2015) suggests a mechanism by which different tissues can age at different rates, as seen between skin and heart. This finding could help overcome one of the longtime obstacles to understanding how DNA damage could give rise to different rates of ageing across species and tissues when the mechanisms behind these processes are so highly conserved. It is clear that DNA damage and how it is dealt with are crucial components of the ageing process. However, given the caveats regarding the effect of different DNA repair pathways on ageing and the inability to robustly extend lifespan through enhancements of these pathways, it seems that DNA damage/repair is unlikely to be the central pillar from which all ageing derives.

#### *1.2.1.2 Protein damage*

With the proposed role for DNA and its alteration in ageing, it is unsurprising that the notion of damage to proteins being a central component of the ageing process followed not long after. The first attempt at combining both the genetic and protein production machineries as an explanation for ageing was proposed by Leslie Orgel in 1963 and became known as Orgel's hypothesis (Orgel 1963). Orgel reasoned that the consequences of transcription errors might be as vital to the ageing process as any mutation. Since cells inherit not only the DNA from their parent cell but also all the enzymes needed for faithful translation of genes into proteins, an inheritance of inadequate or defective enzymes could be more disastrous than a single mutation. Consequently, a cell could begin to deteriorate from a progressive decrease in the reliability of its transcriptional machinery. This reasoning suggests that once translational errors appear in proteins directly involved in translation, they propagate themselves and can then accelerate exponentially. This exponential increase correlated with the known exponential increase in mortality rates with age (Gompertz 1825) and suggested a cause and effect relationship to many at the time. While Orgel explicitly stated "*I am not proposing here that the accumulation of protein transcription errors is the mechanism of ageing*" (Orgel 1963) and retracted his initial postulations (Orgel 1970), the idea of protein's integrity being central to ageing has remained as a persistent hypothesis whose relevance has rapidly become apparent. It quickly became obvious that the fundamental assertions of Orgel's hypothesis were incorrect as there was no observed increase in errors in macromolecule synthesis over age (Rabinovitch & Martin 1982), nor were increased protein errors seen in ageing, cultured fibroblasts (Harley *et al.* 1980). Nevertheless, new ideas of how proteins could be relevant to ageing were not long in following. One notion was that over-age cells accumulate increasing numbers of cross-linked proteins that slow down the processes in the body (such as DNA repair) and lead to ageing (Bjorksten 1968). Here the hypothesis was that

crosslinking leads to tissue damage, a loss of elasticity and general "embrittlement". While this theory did not effectively explain ageing as a whole, it did highlight the importance of chemical reactions which could disturb protein function if not kept in check. One of the primary cellular proteins affected by crosslinking is collagen and this process is strongly linked to skin ageing (Yamauchi *et al.* 1988). Interestingly, perhaps the most common form of collagen crosslinking is a non-enzymatic reaction with glucose (glycation), which has been heavily implicated in both diabetes and ageing (Hause *et al.* 2017, Snedeker & Gautieri 2014, Ulrich 2001). The other mode of glucose protein reactions, the attachment of sugar molecules to proteins via enzymatic action (glycosylation), has also proven to be relevant to ageing. In contrast to non-enzymatic reactions, stabilisation of the unfolded protein response (UPR) through the addition of N-acetylglucosamine (a precursor for the glycans to be added to the protein) to the diet of mice, or an upregulation of the hexosamine pathway (the pathway responsible for N-glycosylation) via a *gfat-1* mutation significantly extended the lifespan (15-40%) (Denzel *et al.* 2014).

As research into the link between proteins and ageing has progressed, the area of focus has shifted to general protein quality control and homeostasis (proteostasis) mechanisms and the impact these have on ageing. It is now known that failures in these systems have severe negative consequences and underlie the pathologies of a wide range of diseases including neurodegenerative, amyloidosis, metabolic, myopathies and liver disease (Koga *et al.* 2011). Persistent imbalances in proteostasis have direct negative impacts on cellular and organismal lifespan (Taylor & Dillin 2011). The accumulation of misfolded, aggregated or damaged proteins are probably the best-known examples of protein damage with age. Normally, the cell has mechanisms such as the proteasome or autophagy that enable the removal and recycling of these problem proteins. However, when these mechanisms fail, the stress on the cell often leads to death and an impairment of the whole tissue with age. For example, in *C. elegans* by inhibiting the proteins that normally accumulate in human muscles cells, researchers were able to restore muscle health and mobility (Ayyadevara *et al.* 2016). The activity of the proteasome generally declines during ageing, which can be mediated through a decline in subunit expression, ability of the complex to assemble, or reduced catalytic activity (Vernace *et al.* 2007, Ferrington 2005, Petropoulos *et al.* 2000). An increase in aggregated proteins and a failure to clear them has been associated with ageing (Cohen & Dillin 2008) and has been similarly linked to neurodegenerative disorders such as AD, PD and Huntington's disease (HD) (Taylor & Dillin 2011), although the exact pathology of the aggregated proteins remains speculative. Interestingly, in older animals, the half-life of proteins appears to be extended, suggesting a cause and effect for ageing (Friguet *et al.* 2000). In longer-lived species the proteasome system appears to be under selection pressure, suggesting a potential role in determining species-specific lifespans (Li & De Magalhães 2013). Unsurprisingly therefore, there is intensive research on reactivating the proteasome in ageing as a means of reconstituting its function to improve both health and lifespan (Chondrogianni *et al.*

2015). Whereas the proteasome is concerned with the recycling of individual proteins, autophagy has the capacity to degrade large macromolecules, aggregated protein clusters, and damaged organelles within the cell. One of the most fascinating aspects of this bulk recycling system is that almost every significant longevity mechanism identified in *C. elegans* and other model organisms (insulin signalling, TOR signalling, germline signalling, mitochondrial respiration, and starvation) rely on autophagy to function (Lapierre *et al.* 2013, Gelino & Hansen 2012). Similar to the proteasome, autophagy has been shown to decrease with age (Cuervo 2008), its inhibition can shorten lifespan (Tóth *et al.* 2008), and it is linked to neurodegenerative diseases (Wong & Cuervo 2010). Additionally, controlling the levels of autophagy is emerging as a treatment for neurological disease, cancer and ageing (Towers & Thorburn 2016, Rubinsztein *et al.* 2015). Orgel's theory that translational fidelity would be the crucial factor in maintaining functional proteins was slightly off the mark. It is now clear instead that it is the systems governing protein turnover, folding and localisation that are the critical aspects in ageing.

#### *1.2.1.3 Oxidative damage*

The free radical theory of ageing encompasses the idea that through basic cellular processes reactive oxygen species (ROS) are produced. These have a range of toxic or damaging effects when inappropriately or insufficiently dealt with. ROS creation occurs primarily during the production of ATP in mitochondria during metabolism but also arises from the action of NADPH oxidases that are chiefly involved in pathogen defence (Nauseef 2008). Before the discovery of ROS as active mediators of metabolic damage, the idea that the metabolic rate could influence longevity was proposed based on the correlation between body size and longevity. Proposed in 1908 by Max Rubner, the basic cornerstone of this theory was that larger animals generally have longer lives than smaller ones and spend fewer calories for each gram of tissue they have. Thus, the rate of intrinsic metabolism would be what determines the lifespan of an organism (Rubner 1908). Early support was given through experiments in *Drosophila* reared at different temperatures, whereby lowering the metabolic rate in the cold increased lifespan (Loeb & Northrop 1917). Similar findings were reported in *Daphnia* where elevating the temperature from 8° to 18° reduced their lifespan by 80% (MacArthur & Baillie 1929). Using cantaloupe seeds and *Drosophila* as his experimental organisms, Raymond Pearl performed a series of experiments whose observations seem to confirm that a reduction in the rate of metabolism increased lifespan (Pearl 1928). Based on these and other findings he developed the hypothesis further stating that "*the duration of life correlates inversely with the rate of energy expenditure*" and this became formerly known as the rate of living theory (Pearl 1928). Additional mathematical weight was given to this notion with the discovery of Max Kleiber's law which showed that, in the majority of animals tested, their metabolic rate scales to ¾ of their mass (Kleiber 1932). This intuitive notion was based on the evidence available at the time, however, quality information for the real lifespan of

various organisms was in short supply. Since this time numerous exceptions to the lifespan-biomass-metabolism relationship and ageing have been discovered. To name but a few, both bats and birds live considerably longer than would be predicted based on their metabolic rates alone, naked mole rats despite their small size can live for 30 years, and despite being cold-blooded and having a reduced metabolic rate, marsupials live shorter lives than their eutherian cousins (Austad & Fischer 1991). Additionally, with the use of more advanced methods and statistical tools, results suggest the maximum lifespan is not associated with metabolic rates in mammals or birds (Magalhaes *et al.* 2007). Similarly, when rats are kept at lower temperatures they eat up to ~40% more food but do not age faster (Holloszy & Smith 1986) and surprisingly mice with increased metabolic rates even live slightly longer (Speakman *et al.* 2004).

Despite falling out of favour in the last decade or so, the link between metabolism and ageing proved to be a compelling hypothesis and considerable resources were dedicated to unravelling the underlying mechanisms. Due to the arrangement of its electrons, oxygen is one of the best oxidizing elements found abundantly in nature. Ever since biological cells discovered a way to harness this reactive power to produce energy, life has been fighting in a perpetual conflict. Oxygen acts as the terminal electron acceptor in the production of ATP and with the correct provision of four electrons and four hydrogens is directly reduced to water. However, the transfer of only one or two electrons produces the reactive oxygen intermediaries superoxide and peroxide. Both of these species and their reaction products, such as the hydroxyl radical, easily strip electrons from any proteins or DNA they encounter, causing severe damage (Mittal *et al.* 2014). Thus, biology is constantly striving to deal with and minimise the production of ROS especially in times of stress or metabolic need. Biology has developed a range of countermeasures to deal with this problem including improving the efficiency of Cytochrome-C in donating electrons to oxygen, such that only ~2% of oxygen used in metabolism is converted to free radicals (Viña *et al.* 2007). Additionally, cells produce their own antioxidants such as vitamins C and E or β-carotene and produce enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase, which together contain and degrade ROS into inert forms (Hensley & Floyd 2002). ROS can also originate from external sources such as ultraviolet and ionising radiation. This can be especially damaging as these are more easily produced in the vicinity of DNA, compared to metabolic ROS which has to escape the mitochondria and travel to the nucleus, and are, therefore, highly genotoxic (Cadet & Wagner 2013). The idea that ROS are toxic biological agents was first proposed by Rebecca Gerschman (Gerschman *et al.* 1954) and subsequently developed into the free radical theory of ageing by Denham Harman (Harman 1956). The theory proposed that "*aging and the degenerative diseases associated with it are attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connected tissues*" – organisms age due to the buildup of oxidative damage from exposure to ROS. Since its proposal there has been considerable support in the

literature to bolster the credentials of this theory. There is clear evidence that both ROS and oxidative damage increase with age (Stadtman 2006), in comparison to younger animals, old animals have a higher oxidation index and accumulate oxidised proteins, DNA and lipids (Agarwal & Sohal 1994, Sohal *et al.* 1993). Additionally, the activities of SOD, catalase and glutathione decrease sharply in old flies (Sohal *et al.* 1984). Increasing antioxidant defences, through direct administration of antioxidants or overexpression of antioxidant genes in *Drosophila,* increases lifespan (Viña *et al.* 1992). Indeed, it was observed that the level of several antioxidants in numerous organisms had an inverse relationship with both the metabolism and longevity of a species (Cutler 1991). Moreover, by measuring both the level of antioxidant and free radical damage at mid-life in members of the same fly population, long and short-lived individuals could be identified (Sohal *et al.* 1986). With the manipulation of the ageing process through genetic intervention, further evidence for the role of ROS in lifespan has been found. Through the selective breeding of only the oldest females in each cohort, long-lived fly strains have been created that show increased resistance to paraquat, a potent producer of free radicals (Mockett *et al.* 2001) and have increased expression of antioxidant genes (Bashirullah *et al.* 2003). Similarly, the production of transgenic flies with multiple copies of antioxidant genes showed decreases in protein oxidative damage and a 30% increase in lifespan (Orr & Sohal 1994). Mutations in the *C. elegans* genes *age-1* and *daf-2* double the worm's lifespan and show a strong increase in SOD and catalase activity combined with resistance to oxidative stress (Larsen *et al.* 1995). Given that the majority of ROS is produced in the mitochondria it is unsurprising that its effects on mitochondrial DNA are considered as critically important. These structures are particularly susceptible to ROS damage: they are contained inside lipid membranes that are highly vulnerable to peroxidation, they have at least 100 unique enzymes for energy production that show a significant decline of activity during ageing, and they have their own genetic material that is unprotected by histones (Coppotelli & Ross 2016). As a demonstration of this vulnerability, the amount of oxidative lesions found in mitochondrial DNA is found to be tenfold higher than in the nucleus and accumulates more rapidly with age, particularly in post-mitotic cells (Ames *et al.* 1993). Additionally, the efficiency of DNA repair is much lower in mitochondria when compared to nuclear DNA repair (Mandavilli *et al.* 2002). Mitochondrial gene expression decreases with age as does mitochondrial membrane potential and respiratory activity (Miquel *et al.* 1980). The rate at which ROS damage accumulates will inevitably affect the ability of the mitochondria to produce ATP and thus influence cell survival and ageing. Indeed, in short-lived species the rate of ROS production in the mitochondria is much higher than for comparatively longer-lived species (Sohal *et al.* 1990).

Despite these extensive findings that seem to imply a causal role of ROS in ageing there have been numerous exceptions that have called the theory into question, which I will highlight briefly. The levels of SOD antioxidant enzyme activity was found to be exactly the same in two strains of *Drosophila*

whose lifespan varied by 40% and similar trends have been found when comparing long and short-lived mice strains (Sohal 1993b). Similar discrepancies were found for glutathione peroxidase activity whereby an inverse correlation with lifespan was observed in six species and multiple tissues (Cutler 1985). Using mice as genetic models Alan Richardson and his lab demonstrated that not all antioxidant genes shorten lifespan when knocked out and that individual overexpression of these genes had no effect on lifespan (Pérez *et al.* 2009). SOD2 null mice did not show changes in median longevity or signs of accelerated ageing (Van Remmen *et al.* 2003). In *C. elegans* there are mutants that show higher levels of oxidative stress but still live longer than the controls but when this stress is removed they then live shorter, implying that their longevity is tied to increased ROS production (Yang & Hekimi 2010). Similarly, there are further reports in *Drosophila* that show the level of ROS production does not correlate with lifespan (Miwa *et al.* 2004). Direct feeding of antioxidants to mice did not either increase or delay ageing (Lipman *et al.* 1998). The long-lived naked mole rat does not have higher levels of antioxidants but does exhibit increased ROS generation and increased oxidative damage compared to control rats (Hekimi *et al.* 2011, Andziak *et al.* 2005). There exist several pathologies in both humans and mice that arise from mutations resulting in increased ROS leakage from the mitochondria (DiMauro *et al.* 2002) but these are not associated with an accelerated ageing phenotype (Wong *et al.* 1999). Though it is worth noting that mutations leading to increased ROS are associated with neurological disease (Patten *et al.* 2010) and the administration of SOD and catalase mimetics is associated with improved cognition in mice (Clausen *et al.* 2010). In summary, there is strong but conflicting evidence for the impact of metabolism and ROS on ageing and longevity, which implies that this mechanism is unlikely to be the ultimate cause of ageing as it was believed for so long. Nevertheless, the idea that ageing can be influenced rather than controlled by the reactive products of metabolism combined with a decline in the ability to repair their damage, particularly in post-mitotic cells such as the neurons, remains likely true.

### 1.2.2 Evolutionary theories of ageing

The fundamental limitation theories discussed above all imply that there exists a rate of inherent destruction that comes with being alive, and it is this, that ultimately causes us to age and die. In fact, these theories could and have been combined into comprehensive theories that take damage as a whole to be paramount. For example, the pure damage theory (Gladyshev 2014) or the reliability theory (Gavrilov & Gavrilova 2001). Yet, despite their success in explaining individual aspects of the ageing process, there is one critical issue they fail to tackle. This is that damage of all kinds is coupled to some form of repair. If this was not the case then life could not have evolved much beyond extremely short-lived uni-cellular species, and likely not even that far. The genetic machinery of

inheritance (DNA and the enzymes needed for its replication) must be passed onto subsequent generations with minimal flaws, otherwise the whole system would break down after a few generations. This, of course, is expressed in the idea of the immortal germline, which without adequate repair could not give rise to innumerable generations of a species. Additionally, in order to pass on this genetic machinery, a species must be capable of, and required to, reach sexual maturity without any of the accrued damage influencing their ability to reproduce. For these simple reasons, the ability to mitigate, control and remove the effects of all cellular damage becomes a highly selectable trait. That this trait can be selected for is clear from the marked difference in the capacity of repair systems between species (Kirkwood & Melov 2011). The question then becomes why has every species not evolved the capacity to repair at close to an optimal rate. Essentially, if damage causes ageing why is it still around and why have its countermeasures not been selected for, thereby removing it as a causal factor? This puzzle is larger than just how is cellular damage still such a prevalent force in biology but instead asks how can ageing arise in the context of natural selection. Is it there by chance (non-programmed/non-adaptive) or is it directly selected for in our evolutionary history (programmed/adaptive)?

Prior to the arrival of Darwin's theory of evolution by natural selection (Darwin 1859), there was no real reason to distinguish between the rate of ageing of a species and any other apparently inherent characteristic. Whatever it was that caused birds to have wings and a beak was presumably the cause of that species lifespan. The observed variation between individuals and species made apparent logical sense alongside their other characteristics and overall design (Goldsmith 2004). However, this revolutionary theory ran almost immediately into difficulty when faced with the paradox of ageing. Evolution by natural selection requires that any trait develops and changes to benefit the survival and fitness of an organism. Ageing is clearly detrimental to fitness while longevity is apparently indefinitely beneficial to the individual. This contradiction, therefore, provided almost immediate and compelling grounds for attacks on evolutionary theory, if ageing was a selectable trait, as it appeared to be from cross-species comparison, then how could natural selection explain it. Darwin responded to these criticisms in later editions of his book by suggesting that survivability could be viewed through the persistence of offspring, whose ability to persist would be derived from the actions of selection, and additionally quoted E. Ray Lankester, a prominent British zoologist, saying "*that longevity is generally related to the standard of each species in the scale of organisation, as well as to the amount of expenditure in reproduction and in general activity. And these conditions have, it is probable, been largely determined through natural selection*" (Darwin 1859, 3rd edition). This response suggested Darwin viewed ageing as a selected entity but that it could arise through the action of other evolutionary forces and that perhaps individual survival was not the only point of consideration.

However, Darwin could not explain how the presence of a limited lifespan could benefit a species nor why any such benefit could outweigh the clear reproductive disadvantage of a limited lifespan.

The first person to address these limitations in evolutionary theory was August Weismann who rejected the notion that the lifespan of an organism was in some way determined by its physiological construction but instead proposed that species-specific longevity was imposed by the needs of that species (Weismann 1891, 1882). One of his main arguments was the accumulation of injuries over time, which an organism could not heal perfectly, would result in older organisms having reduced fitness compared to the young. Thus, he reasoned there would be fewer fit organisms competing for resources that could be best utilised for the young, reproductively prolific individuals, creating a selective advantage for death at old age. This would be a programming for death, which would be an evolved (adapted) characteristic giving a benefit to the species as a whole despite a severe negative effect on the fitness of the individual (Weismann 1882). This idea can be viewed as the forerunner of group selection (discussed in section 1.2.3.1). He suggested that once this selective advantage had been created there would then be no barrier to any advantageous characteristic that traded off against immortality (Weismann 1882). In the battle of these tradeoffs, immortality would ultimately become a useless trait and he believed such traits escape the actions of natural selection and consequently gradually disappear. Although Weismann would later abandon his views on the adaptive nature of ageing (Kirkwood & Cremer 1982) his ideas regarding the trade-off of individual benefit for the benefit of the group/species as a whole quickly became the branching point for the fundamental differences between adaptive and non-adaptive ageing theories. The debate between these two forms has gone on for many decades now with each side claiming victory at different times. Here I will outline the history and arguments for each side.

### *1.2.2.1 Non-programmed/non-adaptive ageing*

Proponents of the non-adaptive theory of ageing were quick to dismiss Weismann's notions of programmed death. Based on their understanding of natural selection they viewed the benefit to the individual as paramount for evolutionary selection whereby the fittest individuals would always have an advantage in reproduction and thus pass on their genes. They pointed out that the cost of deterioration and death to the individual, arising from any programmed ageing, would likely exceed that of any benefit to the group as a whole. Longer-lived organisms would inevitably have more offspring than ones living a shorter life, which would balance the gene pool against the selection of a death mechanism. Despite their assertions that ageing could not be programmed by evolution, for over 50 years following Darwin and Weismann there was no good theory which explained how ageing could arise in a non-programmed sense. In this void ageing theorists developed the early fundamental

limitation hypotheses (discussed in section 1.2.1) that quickly gained favour in the absence of strong alternative evolutionary proposals for ageing.

The paradox of required individual benefit vs the obvious detriment of ageing remained unresolved until the insights of John Haldane lead to a new breakthrough that would inform the discussion of non-programmed ageing to this day. Haldane was puzzled by the prevalence of Huntington's disease. This neurological condition is now known to be caused by the expansion of CAG triplets in the *lamin A* gene (Ross & Poirier 2004). It is autosomal dominant, requiring only one gene to be mutated and is inherited in 90% of cases with 10% arising from *de novo* mutations. The disease begins with a gradual, subtle change in mood or mental abilities and progresses to full-blown movement impairment, the death of neurons in the brain, and is ultimately universally fatal. Haldane wondered how natural selection would not have removed such a disease from the gene pool given that unlike recessive diseases it couldn't hide from selection through allelic compensation and that it was so highly detrimental to fitness. His revelation came from the age at which sufferers of the disease first manifested symptoms, with onset usually in their 30s or later. He realised that a disease with these devastating effects could only occur because in our evolutionary pre-history such a disease would have no selective effect, as the overwhelming majority of our species would die before the disease could manifest. In essence, natural selection would have little if any force in later life under the natural conditions in which evolution did most of its work (Haldane 1941). This idea of a declining force of natural selection with age would become the cornerstone of modern ageing theory and it is not hard to see why. Much like in HD, ageing exerts a similar toll on the capacity and functionality of our bodies and is only readily manifest from our 30s onwards. Thus, from Haldane's view, it was conceivable that the evolutionary process that could allow for the persistence of Huntington's could also allow for the persistence of ageing.

Haldane's military research during the period of the second world war prevented him from pursuing his notions on the declining forces of natural selection and their relationship to ageing. His ideas were instead taken up by Peter Medawar and outlined in two essays "Old age and natural death" (Medawar 1946) and "An unsolved problem of biology" (Medawar 1952), which were originally presented as a lecture at University College London. As a starting point, Medawar described how a reduced force of natural selection with age would arise and generate ageing in a way that did not violate the individual benefit requirement of Darwinian evolution. He imagined an idealised non-ageing species, under some external selective force that randomly reduces population numbers e.g. predation, resources, disease etc., which are then replaced by a new generation. This replacement cycle means that the percentage of a population at a given age would exponentially decrease, resulting in few, if any, very old individuals. He then took the reproductive capacity of each individual and assumed that from the onset of reproduction it would remain stable over time, meaning that the

number of offspring produced would be linear. By multiplying these two factors he determined the reproductive effect for each cohort or age group. Since the number of older animals decreases exponentially over time but their reproductive capacity remains linear, ultimately their reproductive effect declines significantly over age. Therefore, the contribution of older individuals to the genetic biodiversity of the species would peak shortly after reproduction and then decline with age.

As natural selection acts to increase successful reproduction and exerts its strongest effects when reproductive capacity is highest, then in older age the selective pressure on detrimental or beneficial alleles will be diminished, validating Haldane's assertion. Medawar further went on to imagine that a mutation could be acquired that has no effect on fitness early in life but exerts a detrimental cost later in life when selection is weaker. As selection remains blind to these effects, he reasoned, mutations could accumulate over the evolutionary history of a species through genetic drift and give rise to the complex phenotype that is ageing. This hypothesis of the gradual accumulation of mutations giving rise to ageing became known as the "Mutation Accumulation" (MA) hypothesis and provided the first theory of ageing that was consistent with known evolutionary mechanisms (Medawar 1952). What is fascinating about this theory is its ability to apparently explain the variation in species lifetimes. In Medawar's theory, the lifespan curve of a species is determined by the sum of external mortality pressures offset by reproductive success. Each species will have its own unique environment and challenges to survival, if there is more death as adults then the average lifespan of the species will contract and reproductive rates will go up to compensate. This also allows more late-life ageing mutations to accumulate. If however, this external mortality pressure is applied preferentially towards juveniles, then selection will act to increase early survival mechanisms resulting in longer lives and an eventual decrease in reproductive rates. As these animals begin to live longer then the effects of the accumulated detrimental late-life mutations begin to manifest and then also come under selection until a new equilibrium is reached. In this way, ageing is a constant balance between the forces of the environment that push us to shorter and more highly reproductive lives against the advantage gained from the persistent selection of defensive survival traits that encourage longevity (Stearns 1992). Medawar's MA theory of ageing can be considered as a counterpoint to the programmed death idea of Weismann. It turns around the deficiencies of that theory, such as violations of Darwin's individual benefit requirement, and makes them into advantages. For ageing to become a programmed mechanism, Weismann required that wild animals live long enough to become a resource drain without a further addition of reproductive benefit and evidence for this assertion was lacking. Medawar on the other hand, by assuming that species are under constant external threat and thereby rarely achieving old age, removed this as an issue and allowed for the negligence of natural selection towards older individuals to create the recipe for ageing. The average lifespan would be evolved to fit the needs of each species, elongating and contracting in time with changes in the

environment and genetic fitness. It is worth noting that for Medawar's hypothesis to be true it requires that for any species there would be zero evolutionary advantage to having a longer lifespan, otherwise even a very minor benefit would eventually cause lifespan to increase towards immortality.

Five years after Medawar's lecture on the evolution of ageing, apparently initially unknowing of the research and findings of both Haldane and Medawar (Rose *et al.* 2008), George Williams published a paper with the title "*Pleiotropy, Natural Selection and the Evolution of Senescence*" (Williams 1957). In this paper, Williams proceeded to outline his issues with the in vogue fundamental limitation theories (wear and tear theories) and took particular aim at Weismann's evolutionary theory of senescence. He criticised Weismann on four fronts: " *1) The fallacy of identifying senescence with mechanical wear. 2) The extreme rarity, in natural populations of individuals that would be old enough to die of the postulated death-mechanism. 3) The failure of several decades of gerontological research to uncover any death mechanism. 4) The difficulties involved in visualizing how such a feature could be produced by natural selection*" (Williams 1957). In contrast to Medawar and another prominent gerontologist of the time, Alex Comfort (Comfort 1954), he viewed the relationship between age and selection pressures to be misapplied, in that pro-ageing effects during a valid reproduction window would still be relevant and selected against. He agreed that in a post-reproductive period, events such as mutation accumulation could occur and be of consequence but that this would lead to a necessarily sharp decline in health rather than the gradual one seen in most species. He, therefore, assumed that ageing is an unfavourable characteristic that would be directly opposed by selection at all reproductive ages. In order to account for the prevalence of ageing, he assumed the presence of another force acting in opposition, with the deviations in lifespan resulting from variations in the balance between the two forces. He believed that this force arose out of the indirect effects of selection and derived from the evolutionarily driven selection of genes that trade-off different effects of fitness at different ages. It is this last assumption that sets this theory apart from Medawar and others and the main reason that this theory of ageing is considered the gold standard today. Similar to Medawar, and with much the same reasoning, Williams agreed that there is a decreasing probability of reproduction with increasing age. He also agreed that the timing and the magnitude of a positive or negative fitness effect would determine the likelihood of that allele becoming fixed in a population or being lost. However, he reasoned that due to the relative weighting of natural selection towards effects with an impact in the early period of life, that if a gene had simultaneously a small positive effect on fitness early in life but a larger detrimental effect on fitness later in life, it would still be selected for due to this weighting imbalance. Evolution by its very nature would automatically and consistently select for such pleiotropic genes as long as the early vs. late-life tradeoff increased the total reproductive fitness of the species. In this fashion rather than a gradual accumulation of pro-ageing mutations by genetic drift as suggested by Medawar, there would instead be an evolutionary drive to select for pro-ageing alleles

regardless of their detrimental effects in late-life. Williams envisioned a constant selection of balancing and counterbalancing genes whereby if a "*later effect is eventually deleterious, a selective premium would be placed on any gene that might suppress [its effects]. As the suppression approached completion, however, the selection pressure for further suppression would diminish. Complete suppression would probably never be realized. Senescence might be regarded as a group of adaptively unfavorable morphogenetic changes that were brought in as side effects of otherwise favorable genes, and which have only been partly expurgated by further selection*" (Williams 1957). This theory of ageing was later coined the "Antagonistic Pleiotropy" (AP) theory of ageing by Michael Rose (Rose & Charlesworth 1981). Notably, this AP theory of ageing does not contradict the possibility of MA still contributing to the ageing process, rather it limits its contribution to the period of a species life when all possibility of reproduction has ceased, as without the early vs. late fitness tradeoff it argues that detrimental late-life mutations would be selected against if acting while reproduction is still possible. Much like the MA theory, William's pleiotropy notion provided an excellent fit for the observed inter-species variation in lifespan and simultaneously avoided the dilemma of individual benefit. However, it allowed for a more realistic scenario for the presence of pro-ageing genes as it did not require the negative effects of these genes to be negligible but rather it allowed them to be of varying strength based on the timing of when they act and the magnitude of their counterbalancing pro-fitness effects. One notable consequence of this difference is that the prospects for finding root causes of ageing became much less likely, as it was assumed ageing would arise from the action of a large number of AP genes and that their pro-fitness effects would preclude interfering with them in a meaningful way. For this reason, this non-adaptive evolution of ageing was seen at the time as banishing "*the fountain of youth to the limbo of scientific impossibilities*" (Williams 1957).

One of the fundamental corollaries of William's theory is that there will be, by necessity, a genetic trade-off between early fitness components such as development and reproduction with late fitness components such as genome maintenance and survival in old age. From this, it would be assumed that species with a high level of early fecundity would be shorter-lived than those with lower reproduction rates, which is for the most part borne out by species observations. In a similar vein to this tradeoff battle, Thomas Kirkwood proposed the "*disposable soma theory*" of ageing where, rather than a genetic tradeoff, there is a resource allocation tradeoff between the germline and the soma (Kirkwood 1977). Due to how unlikely it is that an organism will survive indefinitely, Kirkwood predicted that the optimal level of investment into maintaining the soma would be below that required for indefinite survival. Essentially an organism will only have a limited amount of energy and resources that have to be divided between reproductive efforts (germline) and the maintenance and repair of the non-reproductive aspects of the organism (soma). Repairing the inevitable accumulated damage obtained throughout life will require resources that could otherwise be spent on reproductive efforts

and the full benefit of such investments would never be realised due to extrinsic mortality. This type of resource allocation tradeoff can be viewed as a physiological extension of the AP theory.

The theories of Medawar and Williams were largely intuitive verbal constructs dealing with changing forces of selection and the impact of genes. While logical, they lacked a formal mathematical model that could be tested against real-world data. This insufficiency was addressed in 1966 by William Hamilton where he formally defined the force of natural selection, showing exactly how and under what conditions it could lead to the evolution of ageing (Hamilton 1966). Hamilton's mathematical model was in effect a restatement of the ideas of both Medawar and Williams in equations. He demonstrated how a mutation acting at a specific age would influence the fitness of the individual and additionally how likely it would be that such a mutated allele would spread within the population's gene pool. This work was subsequently expanded upon by Brain Charlesworth, who took the models of Hamilton and used them to clearly demonstrate how both MA and AP could lead to the evolution of ageing (Rose 1985, Charlesworth & Williamson 1975).

Unlike many other evolutionary theories, the models proposed by Medawar, Williams and Hamilton gave clear predictions as to how the lifespan of a species should be modified by changes in specific conditions. These predictions were 1) Increasing or decreasing the extrinsic mortality pressure on a species should lead to an inverse response in median lifespan. 2) Increasing or decreasing reproductive rates in an organism should correspondingly affect lifespan. 3) Influencing the time of first reproductive success should directly affect lifespan. Over the course of the next 50 years these clear, general predictions were tested under laboratory conditions and shown to be valid in most cases, making this non-programmed theory of ageing extremely robust. One of the first deliberate experiments that sought to address these predictions was performed by Robert Sokal, who examined what happens to life history traits when the reproductive window is artificially controlled. Using the flour beetle *Tribolium castaneum*, Sokal limited its reproductive window to a very short period early in life. Over the course of 40 generations, he demonstrated that this restriction sharply reduced the median lifespan compared to the freely reproducing controls (Sokal 1970). Michael Rose, a graduate student in Brian Charlesworth's laboratory also used changes in reproductive timing to examine the predictions of these theories. Rose reasoned that if he were to delay the first age of reproduction consistently through the generations, this could lead to the evolution of slowed or postponed ageing. Using *Drosophila* in a small-scale experiment and selecting for only the last offspring produced to go to the next generation, Rose observed an increase in both longevity and later life reproductive capacity, both of which came at the expense of early life reproductive capacity (Rose & Charlesworth 1981). The observations on the consequences of late-life reproduction have been validated in numerous other *Drosophila* studies since (Dekert-Cruz *et al.* 2004, Sgro *et al.* 2000, Rose 1984) and even in mice (Nagai *et al.* 1995). Interestingly the experiment initiated by Rose has been ongoing since 1975 and has

generated *Methuselah* flies which now live at least three times as long as their wild-type (WT) counterparts. In a similar experiment Laurence Mueller subjected flies to a limited early reproductive window for over 100 generations, which led not only to a shortening of the median lifespan but also to reduced fecundity later in life, as would be predicted by both MA and AP with a shift in selection pressure from late to early life (Mueller 1987). In an attempt to distinguish between whether this effect was being generated by MA or AP, Mueller crossed two separate strains that had been selected through curtailed early reproduction. In contrast to the results obtained by Rose (Rose 1985, Rose & Charlesworth 1981) whose experiments favoured an AP explanation for ageing, Muller saw significant hybrid vigour in late age reproductive capacity. This was only observed in crosses with the selected fly populations and not in normal fly populations, suggesting the accumulation of mutations rather than fitness tradeoffs as the origin of the changing lifespan trends. One of the most direct assertions from these theories is that only organisms where there is a separation of both germline and soma should age, since it is the distinction between the immortal germline and the ageing soma that allows ageing to arise in the first place. Organisms, such as prokaryotes, algae etc. should not age, otherwise how could the germline be permanently perpetuated. However, the observation of senescence in asexual metazoans such as *Paranais litoralis* and in the budding yeast *Saccharomyces cerevisiae* are at odds with this idea (Martínez & Levinton 1992, Mortimer & Johnston 1959). To remedy this discrepancy Linda Partridge put forward the idea that what mattered was not a distinction between a soma and a germline but rather a phenotypic difference, or asymmetry between parents and offspring (Partridge & Barton 1993). Without this asymmetry, she believed that ageing could not evolve as there would be no distinction between parent and offspring for natural selection to exert different pressures on. Ageing could therefore only exist where either cellular or subcellular asymmetry also exists. Recent evidence has now demonstrated ageing in asymmetrically dividing bacteria and shown that this form of ageing appears to conform to the predictions of the AP theory of ageing (Stewart *et al.* 2005, Ackermann *et al.* 2003). Additional experimental evidence for the evolution of ageing has been provided by controlling the pressures of natural selection through manipulating the extrinsic mortality rate. In fruit flies, nematodes and complex computer simulations, an increase in extrinsic mortality resulted in higher early-life fecundity and shorter lifespans, while a reduction in the same pressure extended lifespan, improved overall fitness and shifted reproductive capacity towards older ages (Shokhirev & Johnson 2014, Chen & Maklakov 2012, Stearns *et al.* 2000, 1998). Combined, this evidence strongly suggests that ageing is much like any other complex phenotype. It arises through the action of complex balancing forces that are moulded and shaped through evolution by natural selection and that both the accumulation of mutations and genetic tradeoffs underlie the life history traits of individual species.

### *1.2.2.2 Criticisms of the non-programmed theory of ageing*

Despite the success of the evolutionary non-programmed theory of ageing in explaining much of the conundrums associated with the origin and action of the ageing process, this theory is not without issues and fails to explain observations of ageing in some species. One point for critics is that non-programmed ageing requires rigid tradeoffs between fitness and lifespan. These theories claim that the evolutionary value of increased lifespan declines over age but never reaches zero. This implies that a longer life will always have some advantage, although small, in the perpetuation of a species. Ageing can then only evolve due to a rigid linkage of a late-life detrimental effect with an early life beneficial effect. The critics argue that natural selection would, over the incredibly long evolutionary history of a species, have found a way to decouple early vs. late tradeoffs such that the negative effects of ageing could be removed from the equation, since there is an assumed benefit in doing so. This could be accomplished through gene duplication events, allowing for a new gene variant that was beneficial for fitness in both early and late-life, or the evolution of new genes that would counteract the negative effects later in life. The fact that such a situation has not occurred despite the time frame of evolution and the incalculable number of species in which it could have, suggests to critics that such tradeoffs cannot be the fundamental reason for ageing. This is unless such tradeoffs are constantly being generated anew before previous versions have been decoupled, a situation they view as highly unlikely (Goldsmith 2013). Another criticism focuses on the fitness of individuals as they age. Here critics argue that the argument for the decline in the force of natural selection based on the concept of reproductive effect is not correct. In non-programmed ageing, the declining force of natural selection arises due to an imbalance in the contribution of older aged cohorts to following generations due to increased extrinsic mortality. Critics argue that the value of the reproductive effect in older individuals is incorrectly assumed to be equal to that of a younger cohort. They argue that say "*ten young individuals each producing one decedent do not have the same evolutionary importance as one older individual producing ten decedents*" (Goldsmith 2014). This is because older individuals, by virtue of having lived longer are more likely to exhibit adult survival characteristics that are beneficial to the species. Additionally, the longer an individual lives the greater the chance they contain superior genes compared to a younger member of the species. For this reason, the decline in the force of natural selection with age would not be as predicted by AP and MA. Instead, it would be buffered by successful old age events that would drive the selection of longevity genes and not the other way around. In essence for any given period of time, more of the less fit individuals in a species would die, meaning those that did survive would be on average more fit than a younger cohort. Thus, the average fitness of a non-ageing population would increase with age and result in fewer deaths of older individuals relative to their younger counterparts. This is in contradiction to the reproductive effect curve proposed by non-programmed ageing theory.

In addition to criticisms that non-programmed ageing makes unsupported assertions in generating its model of ageing, critics also point to examples of ageing that cannot be explained by this model. The senescence seen in most salmon species following breeding is considered as a particularly good example. Salmon are biologically required to return to the site of their hatching to initiate reproduction. These journeys are upstream and require incredible health and vitality and an adaptation from saltwater to freshwater conditions. Yet despite this vigour, within 2-3 weeks of laying and fertilising their eggs the majority of salmon will die of old age, in a programmed form of senescence that is essentially biological suicide. This is a very rapid process but it still represents a death from old age rather than from external causes or accumulated damage over life. Following reproduction, salmon undergo acute immune suppression, develop age-associated frailties to their skeletons such as spinal kyphosis and suffer from an acute decline in muscle strength (Meyer-Ortmanns 2001). This programmed death is seen as one of the strongest counter-arguments to non-programmed ageing as there appears to be no fitness trade-off strong enough to explain forgoing all future reproductive success. Proponents of non-programmed ageing suggest that the death of the salmon somehow creates a nutrient-rich environment that promotes the success of other species destined to become food for the offspring in 4 months time. This reasoning, however, has issues. If it is assumed that the young salmon benefiting from such a programme would be the direct descendants of the adults who died and that without such a programme they could never survive, then kin selection would allow for such a trade-off to exist. However, as thousands of salmon die in the same region at the same time, there is no clear benefit to an individual's genetic legacy. Instead, there is a group benefit that does not fit with classical Darwinian selection. Moreover, such a programme promotes biological cheating. This is the evolutionary paradox whereby there is an evolutionary advantage to be gained by circumventing a constraint at the expense of other members of the same species. In the case of salmon, the evolutionary pressure from the successful cheaters would remove programmed senescence as a character trait. As this has not happened proponents of programmed ageing argue that there must be an inherent benefit beyond that of the individual keeping such a death mechanism present in the genome. They argue that further evidence is provided by the variation in life histories of different salmon species. Some species become sexually mature at 2 years and others at 5, some will only live for 2-3 years other species will live for 10. In most species all the adults die after spawning, though in some up to 10% can return to spawn again and in others there can be several spawning seasons in a lifetime (Hendry *et al.* 2004). This suggests that the mechanism of programmed death is not fundamentally hardwired into the salmon's genome but can be adapted to fit the needs of the specific species. This necessarily requires a selective pressure that overcomes the individual benefit otherwise no such programmed death mechanism could still exist.

Other examples of ageing phenomena that do not fit within the non-programmed theory include: 1) Death through starvation in older elephants. Elephant's teeth have to be constantly renewed but elephants can only have 6 sets after which they die of starvation, occurring at around 60-70 years of age for African elephants in the wild (Lee *et al.* 2012b). This type of death, especially in a species with a very low extrinsic mortality pressure, pre-modern humans, appears to be programmed as the ability to have more teeth and thus live longer should surely have been selected for during the elephant's evolutionary history. 2) The lifespan of dog breeds. Through domestication and selective breeding humans have created varieties of dogs that share the same fundamental genetic components but vary primarily in single nucleotide polymorphisms (SNPs) at about one SNP per 900bp with ~73% of those being polymorphic (Lindblad-Toh *et al.* 2005). Despite this close genetic relationship, different dog breeds have quite different lifespans and atypical for most species larger dog breeds have shorter lifespans than their smaller counterparts. This is particularly relevant as larger breeds take longer to develop to full maturity suggesting that the strict ratio of developmental time and lifespan, as proposed in non-programmed ageing, can be subverted. 3) Programmed lifespans in the cicada. The cicada *Cicadidae homoptera* spends its 17 year lifespan living in the ground as a larva before emerging as a winged adult, laying eggs and dying within a one week span (Alexander & Moore 1962). The vast majority of insects emerge from the ground as adults within a 24 hr window, presumably to facilitate finding a mate and to reduce the impact of predation. While this fits with classical individual benefit, the precision of their lifespan means that each individual lives within 0.1% of its counterpart highlighting that a programmed lifespan is possible under strict Darwinian conditions. This programming also highlights that there is not always a benefit to living longer in the absence of further reproductive opportunities. 4) Bamboo's reproductive suicide. Bamboo propagates by extending lateral runners, which are clones of the original plant that eventually develop into mature plants. Around every 100 years a stand of clonal bamboo will produce flowers, then seeds and subsequently die. This occurs independently of the age of each part of the stand with younger shoots dying alongside their older counterparts (Keeley & Bond 1999). This example of a *semelparous* (a species that only reproduces once sexually) plant highlights that ageing is not necessarily coupled to growth and gives another example of programmed death despite the obvious benefit that would be gained through younger shoots living longer. 5) Programmed death in the octopus. Octopuses normally only reproduce once then stop feeding and die shortly after the birth of their young. Interestingly, this signal to die comes not from the action of the reproductive glands but from the eyes. Removal of the optical glands following reproduction allowed the animals to begin to feed and survive to another breeding season (Wodinsky 1977). In contrast to other *semelparous* events, here there is not just the gradual breakdown of internal organs but rather death through the deliberate control of a behaviour, namely feeding. This is a clear case of programmed suicide regulated by the control of a complex hormone

cycle, in this case hunger. 6) Negligible and negative senescence in a variety of species. In the formulation of the mathematical basis for the evolution of ageing, Hamilton asserted that "*senescence cannot be avoided by any conceivable organism…… and senescence is an inevitable outcome of evolution* " (Hamilton 1966). Despite this bold claim, some striking examples of negligible or negative senescence have been observed. The concept of negligible senescence was coined by Caleb Finch and describes a phenomenon whereby some species display very slow or negligible ageing post-reproductive onset (Finch 1990). Examples of negligible senescence include rockfish, sturgeon, turtles, bivalves and even the naked mole rat as a mammalian example (Finch 2009, 1998). Additionally, some species such as *C. elegans* can undergo a diapause in both a larval stage and in a starvation-induced adult reproductive diapause (Angelo & Van Gilst 2009). Such periods are clearly negligibly senescent as worms can survive for 3-5 times their normal lifespan in these states and return to full health and a subsequently normal lifespan with minimal reproductive consequences. Diapause is not limited to *C. elegans* but is present in butterflies, grasshoppers and fruit flies to name but a few (Tatar & Yin 2001). Similarly, many cases for negative senescence have been made with corals being considered as the best example. Coral mortality is inversely related to colony size and age and the fecundity of coral increases sharply with size and age (Babcock 1991). Other species that show some form of negative senescence include: the wild leek, brown algae, the forest tree *Garcinia lucida*, the neotropical tree *Cecropia obtusifolia*, the cushion plant *Limonium delicatulum*, and numerous mollusc species (Vaupel *et al.* 2004). These departures from the standard non-programmed ageing theories strongly imply that ageing is not an unavoidable consequence of life but only for specific approaches to life.

### 1.2.3 Programmed/adaptive ageing

The criticisms noted in the preceding paragraph along with other observations suggested to some that non-programmed theories of ageing were too simple to encapsulate all the variation and confounding aspects of ageing, as observed across different species. This lack of completeness is analogous to the current state of theoretical physics, where the standard model now encompasses and predicts nearly all known observations in the physical universe, however other observations such as dark matter, dark energy and quantum gravity are still absent from the model. This does not imply that the fundamentals of the model are incorrect but rather that there may be a more complete model still to be discovered, which would incorporate what is already known as a subset of a new larger theory. Similarly, proponents of programmed ageing do not dispute the likely contribution of the declining force of natural selection to the evolution of ageing but argue rather that it is only a subset of the whole and not the final word in and of itself.

### *1.2.3.1 Group therory and programmed ageing*

When looking at the issues surrounding non-programmed ageing, ageing theorists were drawn back to the notions of Weismann and programmed ageing leading to death (Weismann 1882). While Weismann's theory had been widely dismissed, the advent of a new biological theory, group selection (Wynne-Edwards 1968, 1963, 1962), created an original avenue of exploration for theorists looking for alternatives to non-programmed ageing. Group selection argued that animals engaged in social competition will evolutionarily develop mechanisms that "*override the advantage of the individual members in the interests of the group as a whole*" (Wynne-Edwards 1963). This theory suggested that animals are adapted to control their own population densities in relation to available resources and that the mechanisms depend on the substitution of conventional prizes, such as territory, social status or access to mates, for the desired resource, e.g. food. This type of selection substituted the individual as the prime conveyor of advantage with social groups "*as evolutionary units in their own right, favouring the more efficient variants among social systems*" (Wynne-Edwards 1963). The idea of evolved traits detrimental to the individual but beneficial to other members was not entirely new and had been explored and largely rejected at the level of the species but accepted for specific instances of kin selection that allowed for an explanation of altruism and the evolution of the social insects (Hamilton 1963). Interestingly, the proponents of kin selection and critics of group selection were the same individuals responsible for formulating the non-programmed theory of ageing, Fisher, Haldane and Hamilton. As group selection would form the basis for the counterpoint to their own ageing theory, it is perhaps unsurprising that their response to this new ageing model was highly critical (Smith 1964, Williams 1966). The idea of group selection appeared to address one of the primary criticisms directed at the notion of programmed ageing, that it would be too detrimental to the individual to be selected for by evolution. If, however, evolution could act on the needs of the species as a whole, placing the individual at a disadvantage, then ageing could then become an evolved characteristic tailored to improve the adaptation of any species to fit best within the local environment. One of the immediate benefits of this proposal is that it allowed for the presence of almost any lifespan programme imaginable, as evolved characteristics often seem bizarre with no obvious benefit to the trait in question. This neatly sidestepped many of the contradictions that have plagued non-programmed theories. It allowed ageing to adapt to the needs of the species rather than being a detrimental cost that needed to be balanced out. Despite heavy criticism (Kirkwood & Melov 2011) the idea of benefits to the group as a whole continues to be the fundamental basis around which almost all evolutionary programmed theories of ageing are based (Bourke 2007) and warrants further discussion.

A full review of the discussions, formulations, criticisms and counter-arguments in group theory is significantly beyond the scope of this thesis. However, as the idea of selection at the level of the group forms the core of perhaps the only strong argument remaining for programmed ageing

today, some clarification is required. 1) Group theory as proposed by Wynne-Edwards looks to the effects of evolution on groups within the same species that are competing against one another such as a selfish group and a co-operative group or mixtures of the two (Wynne-Edwards 1962). This is between-population (interdemic) group selection. 2) Modern group theory has been developed since the 90s and is focused on the evolution of behaviours and strategies within a reproductively intermingled population. Here, key interactions amongst small individual groups allow for the emergence of attributes beneficial to the whole but possibly costly to the individual (Yang 2013, Nowak *et al.* 2010, O'Gorman *et al.* 2008, Longo *et al.* 2005, Koeslag & Terblanche 2003, Koeslag 1997). This is within population (intrademic) group selection. 3) Intrademic group selection has been widely ciriticised, e.g. Nowak *et al.* received an unprecedented response from 137 evolutionary biologists criticizing the formulations in the paper (Abbot *et al.* 2011), and is viewed by the majority of evolutionary biologists as merely restating Hamilton's inclusive fitness theory (kin selection) (Hamilton 1964) with new words and without any substantive progress (Bourke 2011). 4) Interdemic group selection is considered by most evolutionary biologists as possible but only in specific circumstances, such as when there is extremely low genetic exchange between competing groups (Leigh 1983, Smith 1976, 1964, Williams 1966). 5) The proponents of both forms of group theory still argue the validity of specific group theories today and although none have achieved general acceptance their relevance to the issue of ageing, its origins and its consequences still warrants careful consideration for any ageing study.

#### *1.2.3.2 Evolved benefit of ageing theory*

Leading the case for programmed ageing arising from the benefit to the species as a whole are Josh Mitteldorf and Theodore Goldsmith. Both scientists have had a relatively unique path to becoming evolutionary biologists and neither are what might be considered to be traditional academics. This is perhaps why their theories have received little direct comment by established scientists in the field. Mitteldorf previously worked in astrophysics, optical design and energy conservation before becoming interested in the problems of ageing. Goldsmith was trained as an electrical engineer at MIT and worked for NASA specialising on issues of digital data communication mechanisms. This led to his interest in evolutionary biology and ageing as he contemplated the implications of digital genetics in these fields. It is likely that these non-traditional roots have helped their development of an ageing theory that challenges the orthodox view. The theory put forth by Mittledorf and Goldsmith synergises group theory with the theory of evolvability (Kirschner & Gerhart 1998) to suggest that ageing is a deliberate evolutionary strategy used to stabilise population dynamics and increase the long-term evolutionary fitness of a species (Goldsmith 2004, Mitteldorf 2006). The arguments of the theory

[lacking a name I have termed this the evolved benefit of ageing (EBA) theory] are relatively involved but I will do my best to summarise them and their implications here.

One of the central notions underlying the theory behind evolutionary mechanics is that of immediacy. The presumption of orthodox Darwinian evolution is that near-term or immediate benefits (e.g. those to the individual) will have a greater impact than a longer-term benefit (group benefit), which in turn would also be more impactful than a very-long benefit (species benefit). Such classical reasoning is one of the primary reasons that the idea of group selection was largely dismissed, as the benefit to the group would need to be exceptionally large to overcome a detriment to the individual (Leigh 1983, Williams 1966, Smith 1959). The fundamental plausibility of group selection depends on the time-frame of the evolutionary process. It argues that if evolution is an exceptionally long-term process there would be little selective difference between short-term and long-term benefits, assuming that the long-term is shorter than the time required for the benefit to be realised. If on the other hand, beneficial characteristics can be included in the genome rapidly, relative to the rate of speciation, there would be a large selective difference (Goldsmith 2014). [Clarification, the rate of speciation is important here as if a beneficial trait is to spread throughout a species it cannot occur in genetic isolation. If speciation occurs quickly, due to negligible gene transfer between co-evolving groups of the same species, then the sum benefit of a trait will be reduced as it will impact only a fraction of the total species population]. Thus, one of the first tasks of EBA theory is to establish that the rapid inclusion of beneficial characteristics (immediate benefit) is not the primary force behind evolution in complex organisms. It seems at first relatively clear that evolution can allow for the expression of beneficial characteristics quite rapidly, as seen in selective breeding or the artificial evolution of bacteria to metabolise citrate (Blount *et al.* 2008). However, selective breeding or artificial evolution produce a population that is adapted for a specific environment, which is not reflective of the normal environmental condition in the wild. Introducing accelerated phenotypic changes inevitably involves also introducing changes to other characteristics that are potentially adverse. In the lab, this is acceptable to the organism as the environment is controlled and free from the myriad pressures of the wild. If introduced back into the wild selectively bread organisms are often quickly out-competed despite the "beneficial" trait. An excellent example of this is mutations in the *age-1* gene of *C. elegans*. Mutations in this gene extend lifespan by approximately 70% with no obvious phenotypic detriment (Friedman & Johnson 1988). However, when the mutant was intermixed with a WT population and challenged with cyclical starvation the mutation was effectively lost (Walker *et al.* 2000). As cyclical starvation is a chronic problem for *C. elegans* in the wild this exemplifies issues with immediate beneficial selection. Of course when the only option is to adapt or die, such as with antibiotic resistance, then obviously any trade-off in fitness is worth it. However, examples of successful immediate adaptation to a novel pressure are extremely rare, especially in complex

organisms. For a benefit to be expressed at its maximum potency the benefit-cost trade-off has to undergo further selection allowing for a return to peak fitness. This rebalancing is a significantly longer-term process compared to the initial gain of benefit. The length of this process is augmented by the relatively limited proportion of inherited genetic data subject to selection between members of the same species. Humans have between 0.1% to 0.3% genetic variance when comparing two individuals of the same ethnic background (Frazer *et al.* 2009). Since selection can only act where there are differences in genetic sequence, this greatly reduced the number of combinations that can be tested for each generation. In all, EBA argues that these issues significantly shift the balance of selective force from short-term to long-term. It is only in the significantly longer term that the full value of a novel beneficial trait can be realized through adaptation in complex sexually reproducing organisms.

At the core of orthodox Darwinian evolution is the concept that single gene mutations can provide an immediate fitness benefit and that this is the only force behind natural selection. Each and every mutation is evaluated independently through the process of selection. This concept of immediate benefit is wholly incompatible with group selection and all theories requiring a longer-term benefit. As the complexity of an organism increased then so too do the number of interactions affected by any single mutation and, as such, the probability that a random mutation could be of a net benefit to fitness decreases. Additionally, the more complex an organism is the more it depends on the efficient functioning of its disparate parts. As such, when complexity increases the magnitude of any random change would have to decrease, otherwise the stability of the whole would be disrupted. The genetic transfer of information to a new generation is a digital rather than analogue process. This adds another layer of complexity to the evolutionary process. Evolution requires the ability to distinguish a fitness difference between two individuals for selection to act. However, because of granularity associated with digital information transfer, there is a minimum step size between one piece of information and another. This minimum size implies that there will be a limit to how minor a beneficial change can become before it is indistinguishable and invisible to selection. These ideas suggest that evolution should become progressively more difficult and slower as complexity increases. Consequently, a point would be reached where changes that cause a disadvantage should overwhelm those causing an advantage and maximum complexity would be reached preventing further evolution. This is evidently not the case, in reality, the pace of evolution appears to have increased as organisms become more complex (Rechtsteiner & Bedau 1999). This contradiction with the predictions of orthodox Darwinian selection implies, to the proponents of group-theory, that immediate benefit cannot be the sole mediator of the force of natural selection. This deficiency of Darwinian Theory has also been addressed by evolutionary biologists, through the fusion of genetics with evolution giving rise to modern inclusive fitness theory (Bourke 2011). This led to the acknowledgement that changing any one characteristic of an organism will interact with all of the other characteristics of the organism.

This means selection will operate on a mutation in its complete biological context, which includes long-term fitness characteristics (Hamilton 1966). This change in stance gives a foundation on which programmed ageing theorists have built. The GBA theory also highlights that while single mutations are the source of genetic novelty and the raw material of evolution, in complex organisms, natural selection acts on the properties of organisms that arise from the combination of different mutational differences. This, they suggest, this implies that the mechanisms of evolution in complex organisms are necessarily longer and more complicated than explained in classical selection theory. They claim that the evolutionary mechanisms are clearly evolved traits due to their complexity, organisation and absence in simpler organisms. For these reasons GBA makes a claim that is quite different from modern orthodox theory. " I*t is reasonable to believe that immediate single mutation benefit does not represent the primary mode of evolution in more complex organisms. Instead, mutations that are individually fitness - neutral or mildly adverse occur and are distributed rather widely in a population. Individual organisms, created by recombination, and possessing beneficial combinations of these mutations live longer and breed more. The nearly neutral mutational alleles underlying the beneficial combinations are therefore propagated more widely. Other, longer-term mechanisms cause genome organizational changes that affect subsequent evolution……. If immediate single mutation benefit is not the primary force behind evolution in more complex organisms, then the main conceptual barrier to longer-term evolution theories is removed. There is no individual fitness, just fitness. A tradeoff between a long-term benefit and an individual disadvantage would appear to be as feasible as any other tradeoff. "* (Goldsmith 2014). This bold statement that the value of a long-term benefit is equivalent to a short-term one is really the linchpin upon which the whole GBA theory is based, and is in direct conflict with modern evolutionary theory that still views short-term benefits as having a greater value in selection than longer-term benefits.

Further to this claim of long-term benefit equivalency, GBA goes on to suggest that ageing has a long-term benefit that outweighs the detrimental effect to the individual (who would gain an immediate benefit from its removal) by increasing the genetic diversity of the species and improving their ability to adapt and survive in new environment, i.e. their evolvability. Evolvability is the capacity of an organism to generate heritable phenotypic variation (Kirschner & Gerhart 1998). GBA argues that this capacity is, itself, under evolutionary selection as the features of inheritance differentially affect the inheritability of mutational changes (e.g. paired chromosomes, genetic linkage, pattern sensitivity, unequal crossover, transposons etc.). They imply that these mechanisms of unequal inheritance create a variance in the inheritance efficiency of survival traits from parents to offspring and that this encourages genetic diversity within a species. This diversity allows a species to adapt to new environmental challenges and increases mutational combinational frequencies encouraging the evolution of new beneficial traits. The evolution of such a system is not favoured by natural selection.

First because, ideally, clonal inheritance of genes would have the highest preservation rate of survival characteristics in inheritance and, secondly, because random variation is much more likely to be detrimental rather than beneficial. However, in complex organisms, a high degree of inheritance efficiency will increase the risk of population extinction due to the relatively slow response rate to environmental change. Environmental change is the medium through which natural selection is invoked and it is almost always in flux in natural settings, e.g. levels of available prey, temperature variation, climate change, new competitive species etc. If the ability to adapt was not maintained in the genome of a species and this population was unexpectedly exposed to a new environmental challenge, then the likelihood of their survival would be slim. For example, if an environment stayed highly stable for millions of years genetic variation amongst a population would be gradually lost. Populations that successfully survive a modular environment are those where variation is preserved. This creates a selection pressure that has left the survivors of billions of years of evolution as those with mechanisms for the encouragement of variation. This pressure is extremely long-term and acts on the species level, for without it species go extinct. It is combatted by shorter-term benefits that try to keep variation low and this balance has resulted in the observed genetic diversity of each species. It is possible that mechanisms of evolvability have become so fundamental to the biology of complex organisms that changes to their mode of action are almost universally lethal. For example, inhibition of the association of synaptonemal complex proteins in *C. elegans* through *htp-1, 2,* or *3* prevents chiasma formation and is embryonically lethal (Severson *et al.* 2009). If the inheritance of mechanisms designed to propagate genetic variation is a trait under positive selection, then, this suggests that species-level group selection would be a valid reality. If this is the case, then, group selection for ageing also becomes possible if there is a strong enough species level benefit.

In terms of evolvability, we see that the argument for species-level selection required the threat of extinction as a balancing force so that it could override individual benefit. Mitteldorf argues that the same case can be made for ageing. He argues that "*ageing has been selected for its ability to help stabilize population dynamics*" (Mitteldorf 2006). In short, group populations of species are subject to violent swings and chaotic fluctuations in number, which risks extinction (Lotka 1920). The development of demographic homeostasis can be an evolved trait of populations to counter this risk. He proposes that natural selection on the individual will push reproductive success higher and higher as this is an immediate benefit. When the growth rate of the populations exceeds the ecosystem's recovery rate, in his model by threefold, a tipping point is reached and chaotic population dynamics then ensue leading to a swift sharp decline in population numbers (Brannstrom & Sumpter 2005). The extinctions following such events will be frequent and often, as selection for individual reproductive benefit is a short-term event. Thus, the species where group selection overpowers this individual benefit will be the ones to survive and enforce growth restraint. This will be achieved by either

returning populations from high to moderate numbers when resources or low, or inhibiting the ability of unrestrained reproduction in the first instance. One of the most effective ways a species could evolve such control mechanisms would be through the development of programmed ageing patterns. Ageing would help buffer the volatility of population growth to below that of the chaotic threshold. The environment in which the species evolved would provide the framework for how rapidly such a programme would act and give rise to environmentally responsive lifespans. Additionally, once such a programme was implemented then its presence would allow for the action of other pro-ageing pressures such as MA and AP to arise as secondary consequences. Both MA and AP could be viewed as an attempt to maximise fitness that inadvertently feeds into a programmed ageing system. Goldsmith also argues that the majority of characteristics that affect evolutionary capacity involve negative tradeoffs with fitness. He states that programmed ageing is another route to maximizing evolutionary capacity (Goldsmith 2004). Natural selection ensures that on average each successive generation is fractionally more adapted to its environment (fitter). Thus, amongst hypothetical identical organisms, there would be an advantage to a group with a shorter lifespan as they could accumulate this increased fitness more rapidly. In sexually reproducing organisms variation is introduced in each successive generation. Meaning, a pressure on adult lifespan, and the effects this has on increasing early reproductive rates, acts to increase the total variation of the species group. Thus, ageing can be a trait that serves to minimise extinction events through variable population control and increase adaptive variation for the ultimate long-term benefit of the group/species. While at odds with traditional views of evolutionary biology (and this theory is not alone in that fact e.g (Martins *et al.* 2011, Travis 2004)) it offers a new perspective on ageing that asks us as gerontologists to examine our findings in terms of whether they fit a model of programmed or non-programmed aging more completely.

#### *1.2.3.3 Implications for future research*

The final verdict on which theory is ultimately correct is quite consequential as it will influence the direction of future anti-ageing research and determine the likelihood of success. Both theories imply that there may be common causes to ageing that have been preserved across the evolutionary history of different species. However, programmed ageing suggests that such commonalities are much more likely, as they would have been grandfathered into the genome from the early advent of multi-cellular complex organisms. Non-programmed ageing allows for the retention of fitness tradeoffs as species diverged but implies that it is significantly less likely for any one tradeoff to have been maintained over time. This suggests that many of the processes influencing the ageing process will be relatively unique to the species examined or its closer relatives. These differences advise different approaches to studying the ageing phenomena. The value of model organisms, especially non-mammalian species

such as *C. elegans* or *Drosophila*, is lower in the non-programmed ageing point of view. This could suggest that experiments utilising such species are unlikely to produce results applicable to humans. Additionally, non-programmed ageing, such as AP and MA, strongly implies that the individual contribution of any gene to ageing should be small and it is only with the combination of many such events that ageing arises. As such, in designing experiments to detect ageing it would seem fruitless to look at individual genes in a model organism such as *C. elegans*. Arguments like these were in fact often put forward by leading evolutionary biologists in the second half of the 20th century (Charlesworth & Williamson 1975, Hamilton 1963, Williams 1966). Had such orthodox views held total sway, then the revolution in identifying conserved genetic components of ageing, including those which identified the IIS pathway, may never have been conducted. Non-programmed ageing has provided a convincing *post hoc* explanation for the existence of these control systems but would not and did not predict their presence. This implies that experiments using these theories as their foundation would likely never have discovered single gene ageing effects, demonstrating how important the continued discussion on the evolution of ageing remains.

## 1.3 Mechanisms of longevity modulation

The story behind the discovery that individual genes of the *daf-2* insulin-like growth factor (IGF) signalling pathway could significantly extend the longevity of *C. elegans* is a fascinating tale of hard work, luck and being in the right place and time for such a discovery. It is also an excellent example of why it is important to question scientific dogma. Cynthia Kenyon, who was largely responsible for the identification of the *daf-2/daf-16* axis in longevity, sums this up in an exceptional article that chronicles her road to this monumental discovery. "*Not only was ageing thought to be merely a passive, entropic process, evolutionary biologists had argued forcefully that ageing could not be regulated. For example, they felt that mechanisms for regulating ageing would have no way to evolve, as ageing takes place after reproduction. These theories were thought provoking, but to my mind, they had the effect of discouraging searches for regulatory genes. It seemed to me, a molecular geneticist from the outside, that one should keep an open mind and just have a look*" (Kenyon 2011). This is one of the first articles I read when starting my PhD in 2011. It inspired me to question, even in the face of apparent truths and to look into the cracks where theory and observed reality did not quite meet. It also served as a great starting point in understanding the complex world of genetic longevity regulation, to which I will now give the highlights relevant to this thesis.

### 1.3.1 The insulin-like signalling pathway

Ageing in *C. elegans* is primarily controlled through the Insulin/Insulin Growth Factor 1 signalling pathway (IIS). This pathway is not limited to worms and is conserved in both flies and mammals where it has been shown to also extend longevity (Blüher *et al.* 2003, Tatar *et al.* 2001). The first gene identified as a modulator of longevity, *age-1* (Friedman & Johnson 1988), is a phosphatidyl inositol 3-kinase (PI3-kinase) and is a member of this pathway, although this wasn't confirmed until later (Morris *et al.* 1996). The pathway itself was initially identified not for its role in longevity but for its role in dauer larva formation processes (Swanson & Riddle 1981). Dauer is an alternative developmental stage that nematodes use to survive harsh conditions such as high temperatures, low food abundance or overcrowding. In their normal life-cycle progression, *C. elegans* develop into an adult form following four larval stages L1-L4. In conditions of adversity, worms can halt development after the second larval stage and enter an alternative L3 stage (dauer stage). In this state, worms do not feed but are highly resistant to stress and can live up to three times the normal lifespan of a worm. In particular, this stage is considered to be an ageless stage as lifespan following dauer entry is not affected by the duration of the stage itself (Klass & Hirsh 1976). Despite their apparent quiescence during the dauer stage, several thousand genes continue to be expressed, which are explicitly important for survival (Wang 2003). There have been over 30 genes identified in controlling the entry into the dauer state (Albert & Riddle 1988) and of these only two were found to extend lifespan: *daf-2* (Kenyon et al. 1993) and *daf-23, daf-23* was later identified as *age-1* (Malone *et al.* 1996). *daf-2* encodes the *C. elegans* homologue of the human insulin and IGF-1 receptors (Kimura *et al.* 1997) and modulates lifespan through negative regulation of the FOXO transcription factor DAF-16 (Ogg *et al.* 1997), the heat-shock transcription factor HSF-1 (Hsu *et al.* 2003), and the stress response factor SKN-1 (Tullet *et al.* 2008). DAF-2 responds to environmental cues such as nutritional availability or growth conditions (Li *et al.* 2003) and may be regulated by as many as 38 insulin-like ligands (Pierce *et al.* 2001). These peptides act as either agonists or antagonists to modulate DAF-2 signalling and thus control metabolism, growth and lifespan. DAF-2 is a receptor kinase that upon activation phosphorylates and activates AGE-1 (Morris *et al.* 1996). AGE-1 then generates secondary messengers (3-phosphoinositides) that are required for the activity of downstream effector kinases. These include AKT-1, AKT-2, PDK-1 and SGK-1 (Riera *et al.* 2016). These kinases, in turn, regulate DAF-16 through its phosphorylation level. Under normal conditions, DAF-16 remains phosphorylated and sequestered in the cytoplasm by 14-3-3 proteins (Brunet *et al.* 1999). Decreased or suppressed DAF-2 activity prevents the activation of the downstream effector kinases and subsequently a loss of DAF-16 phosphorylation resulting in its translocation to the nucleus (Lin *et al.* 2001). Here DAF-16 predominantly upregulates gene expression but also has a repressive effect on some genes including: neuronal signalling, apolipoprotein binding, RNA-binding, DNA replication and protein degradation (Murphy 2006). Many of the genes upregulated by DAF-16 are involved in

protection from stresses including: antioxidant defence genes, heat-shock proteins, pathogen resistance genes, and heavy metal detoxification (Murphy 2006). Interestingly, *daf-2* mutants increase fat storage and shift their metabolic activities to make use of this resource (Rea & Johnson 2003). In fact, metabolic genes represent some of the most highly regulated genes in the *daf-2/daf-16* pathway. Outside of *daf-16*, DAF-2 also regulates the expression of HSF-1 in a response that appears to primarily be a boost to the innate immune system (Singh & Aballay 2006). Its regulation of SKN-1 though is of particular interest, as this transcription factor controls a partially overlapping set of antioxidant and detoxifying genes along with lipid and metabolic genes (Blackwell et al. 2015). Increased expression of SKN-1 has been shown to be lifespan extending in both worms and flies (Sykiotis & Bohmann 2008, An & Blackwell 2003) and is expressed both in chemosensory (ASI) neurons and in the intestine. Its constitutive activation in the nuclei of intestinal cells results in longevity independent of DAF-16 (Tullet *et al.* 2008). SKN-1 inhibition also partially suppresses the longevity of *daf-2* mutants (Wang et al. 2008). Three of the downstream DAF-2 target kinases, AKT-1, AKT-2 and SGk-1 act on SKN-1 in a similar fashion to DAF-16, where they actively phosphorylate SKN-1 resulting in its cytoplasmic sequestration (Blackwell et al. 2015). The intestine is a major site of action for DAF-2 where it activates both SKN-1 and DAF-16 independently. Indeed, similar to SKN-1, the specific expression of DAF-16 in the nucleus of several tissues in *daf-2:daf-16* mutant worms is enough to restore a longevity phenotype (Libina *et al.* 2003).

### 1.3.2 The target of rapamycin pathway and dietary restriction

The target of rapamycin (TOR) pathway is a nutrient signalling pathway evolutionarily conserved from yeast to humans with relatively small changes in overall structure and function (Evans *et al.* 2011). TOR is encoded by two genes in yeast (*TOR1* and *TOR2*) and by a single gene in multicellular organisms (*let-363* in *C. elegans*). TOR forms a functional part of two complexes TORC1 and TORC2, which have different downstream targets. Individual components of both complexes have been linked to longevity along with TOR itself (Jia *et al.* 2004). TOR is vital for the total viability of developing organisms and is embryonically lethal when deleted in most instances (McCormick *et al.* 2011). The TOR complexes are primarily activated by nutrients, through amino acids (Nobukuni *et al.* 2005) and independently through glucose and the IIS pathway (Jia *et al.* 2004). TOR signalling is also activated by other pathways including the AMP kinase and MAP kinase pathways and in response to stress through the tumour suppressor TSC-1—TSC-2 complex (McCormick *et al.* 2011). TOR displays some interesting synergy with the IIS pathway which activates TOR through phosphorylation of AKT-1 and AKT-2 (Hahn-Windgassen *et al.* 2005). Much like reduced DAF-2 signalling, reduced TOR activation extends longevity, but does so in both a DAF-16 dependent and independent manner (Vellai *et al.* 2003), likely through the de-

repression of SKN-1 (Robida-Stubbs *et al.* 2012). TOR is itself a serine/threonine effector kinase that, outside of the induction of stress responses through DAF-16 and SKN-1, actively controls several key cellular processes including: rates of protein translation, autophagy, mitochondrial activity, and hypoxic responses (McCormick *et al.* 2011). Two of the best-known substrates of TOR are the S6 kinase (S6K) and eIF4E in *C. elegans* that control translation initiation and elongation (Sanchez *et al.* 2015). The inhibition of protein synthesis is a direct modulator of longevity in worms and mammals (Selman *et al.* 2009, Pan *et al.* 2007). It is through its regulation of this and the stress response pathways that TOR exerts its primary effects on longevity.

Dietary restriction (DR), also known as caloric restriction (CR) is perhaps the most successful intervention that delays the progression of ageing and the development of age-associated chronic diseases. CR is defined as a significant reduction in calorie intake without essential nutrient deprivation, usually around a 40% reduction in normal calorie intake (Speakman & Mitchell 2011). It has now been demonstrated to extend both health and lifespan in worm, flies, mice and even monkeys (Mattison *et al.* 2017, Testa *et al.* 2014). CR is considered to be an active and highly conserved stress response that works to increase the survival chance of an organism during adversity (Sinclair 2005). While the exact mechanisms by which a reduction in the intake of calories is translated into longevity remains elusive, in mice at least it has been shown to be independent of either fat content or metabolic rates (Masoro 2000). Indeed, the specific macronutrients provided as part of a CR diet can have quite differing effects on the success of dietary interventions (López-Lluch & Navas 2016). The longevity mechanisms known to be modulated by CR include: the IIS pathway, the TOR pathway, NAD-dependent sirtuins, reduced mitochondrial ROS production, activation of SKN-1, and activation of autophagy. As discussed, when nutrients are plentiful both IIS and TOR signalling are active and repressing their downstream effector transcription factors. When nutrients are limited CR can activate one or both pathways (dependent on the type of nutrient limited) to upregulate stress response genes and to inhibit other energetic processes such as mitochondrial activity and protein synthesis. The role of sirtuins in CR comes from several sources. CR was found to upregulate Sir2 in yeast (Lin *et al.* 2002) and its orthologue SIRT1 in humans (Cohen *et al.* 2004). SIR-2.1 (*C. elegans*) and SIRT1 are both involved in ageing and age-related disorders (Wątroba & Szukiewicz 2016, Tissenbaum & Guarente 2001). Finally, a sirtuin activator, resveratrol, extended lifespan suggesting an underlying mechanism to CR through sirtuin upregulation (Wood *et al.* 2004). A low-calorie diet is correlated with reduced oxidative damage either through increased ROS scavenging or reduced mitochondrial activity. Both activities have been implicated in CR mediated lifespan extension (Bordone & Guarente 2005). Both transcription factors PHA-4 and SKN-1 have been shown to be required for an extension of life following a CR regime (Bishop & Guarente 2007, Panowski *et al.* 2007). PHA-4 regulates autophagy induction following inhibition of the TOR pathway. It is suspected that the upregulation of autophagy

is required during CR to maximise resource turnover in the limited nutrient conditions (Hansen *et al.* 2013). In comparison to DAF-2 signalling where SKN-1 expression in the intestine was critical, in CR mediated longevity this role is dispensable and instead requires SNK-1 activity in the neurons (Bishop & Guarente 2007). This marks a point of interesting contrast between the two nutrient-depravation signalling systems as both depend on SKN-1 activity but in quite different tissues.

### 1.3.3 Germline mediated longevity

The trade-off between reproduction and survival is a cornerstone of the evolutionary theory of ageing and is best represented in the disposable soma theory of ageing (Kirkwood 1977). In order to maintain the germline in a functional state, a vast complex machinery operates that is highly energy intensive. As resources are limited, organisms, therefore, divert resources from the soma into germline maintenance. When the germline is removed reproduction will free up resources that can then be allocated to the survival of the whole organism (Maklakov & Immler 2016). Evidence for a trade-off between reproduction and longevity was readily found in model organisms (Stearns *et al.* 2000, Rose & Charlesworth 1981). However, other work showed that complete germline removal was independent of longevity effects (Kenyon *et al.* 1993) and that the longevity benefits gained through mutations in the IIS pathway could be uncoupled from reduced reproductive rates (Gems *et al.* 1998, Larsen *et al.* 1995, Johnson *et al.* 1993). Indeed the idea of a pure energy trade-off between reproduction and longevity has now largely fallen by the wayside. A more nuanced integration of the two pathways is now favoured "*reproduction and lifespan are not constrained by direct competition for limited resources but are rather connected by common molecular signaling networks optimizing metabolism differently for growth and fecundity versus somatic maintenance and longevity*" (Maklakov & Immler 2016). The gonad in *C. elegans* is comprised of both the germline and the somatic gonad. Removal of both tissues, which includes the support cells surrounding the germline stem cells (GSC), has no effect on longevity. However, the specific removal of the germline precursor cells, which leaves support cells intact, by laser ablation, results in an impressive 60% increase in longevity (Hsin & Kenyon 1999). These results suggested that it is the somatic gonad that generates pro-longevity signals and the germline which antagonises this action (Antebi 2013). There is little information regarding the nature of these signals though some have been identified in the last few years. In *C. elegans* the nuclear hormone receptor DAF-12 is a critical component of germline longevity (Hsin & Kenyon 1999). These receptors are transcription factors that directly regulate gene expression after binding to fat-soluble hormones including steroids and fatty acids (Antebi *et al.* 2000). In particular, DAF-12 binds to bile acid-like steroids called dafachronic acids (DA). DA biosynthesis contributes to the germline longevity pathway and its dietary supplementation can restore longevity in germline mutants with incapacitated

DA biosynthesis or absent somatic gonad (Yamawaki *et al.* 2010, Gerisch *et al.* 2007). DA is produced as a final end product in the somatic gonad but its biosynthesis begins in other tissues including the intestine. The somatic gonad represses the DA/DAF-12 signalling through repression of DAF-12's transcriptional activity, but in GSC(-) worms this repression is released, allowing for DA/DAF-12 signalling to promote longevity (Antebi 2013). Under starvation conditions, the production of DA is markedly increased and signals to reduce the number of germline nuclei. If this signalling is blocked and the number of GSC remains high, then the lifespan extension normally imparted through DR is lost (Thondamal *et al.* 2014).

Beyond DAF-12 transcriptional control, removal of the germline activates other key factors required for its lifespan extension including DAF-16, autophagy, SKN-1 and lipid metabolism. GSC(-) longevity (as in *glp-1* mutants) has a strict requirement for DAF-16 as its absence completely abrogates the lifespan extension (Hsin & Kenyon 1999) even when knocked down in adulthood (Arantes-Oliveira *et al.* 2002). Unlike for *daf-2* mutants, intestinal expression of *daf-16* is the critical factor in longevity determination (Libina *et al.* 2003). Also, it appears that DAF-16 responds to removal of germ cells in a manner distinct from that of IIS signalling, as *glp-1:daf-2* double mutants have an additive longevity effect living 4 to 5 times longer than WT (Hsin & Kenyon 1999). Indeed, some of the genes required for GSC(-) longevity are superfluous for IIS longevity and only a subset of DAF-16 genes are induced following germline removal (Ghazi *et al.* 2009, Berman & Kenyon 2006). Strikingly, there is an apparent crosstalk between DA/DAF-12 signalling and the nuclear localisation and activity of DAF-16 that is absent from other modes of DAF-16 activation (Berman & Kenyon 2006). This leads to a two-tiered activation of specific gene programmes with 230 *daf-16* and 130 *daf-12* dependent genes differentially regulated in *glp-1* mutants (McCormick *et al.* 2012).

The role of lipid metabolism in GSC(-) longevity is complex and still under development. Removal of the germline induces fat accumulation, particularly through the actions of SKN-1, NHR-80 and NHR-49. In addition, the activation of specific metabolic genes such as triglyceride lipase and acyl-CoA reductase through DAF-12 and DAF-16 is important, as they are proposed to generate putative lipid signals that modulate-lifespan extension (McCormick *et al.* 2012). The nuclear hormone receptor NHR-80 is required for GSC(-) lifespan extension but has little to no effects on WT, IIS, DR, or mitochondrial longevity (Antebi 2013). NHR-80 regulates the fatty acid CoA-desaturase genes *fat-5, fat-6,* and *fat-7* that convert saturated fats to monounsaturated fats, which have been shown to extend lifespan (Han *et al.* 2017). Indeed, removal of this desaturation process abolishes longevity extension in GSC(-) worms (Goudeau *et al.* 2011). NHR-49 regulates β-oxidation in the mitochondria through the mitochondrial acyl-CoA synthase *acs-2* and works in concert with NHR-80 (Pathare *et al.* 2012, Goudeau *et al.* 2011). The primary role of this complex appears to be in balancing the ratio of saturated and unsaturated fats. Deletion of NHR-49 increases fat content and shortens lifespan and this can be

buffered by overexpression of *acs-2* (Pathare *et al.* 2012). Curiously, *acs-2* deletion does not affect lifespan, suggesting that reduced β-oxidation levels are neutral to longevity. GSC(-) worms activate the transcription factor SNK-1 which in turn leads to a broad transcriptional reprogramming in somatic tissues (Steinbaugh *et al.* 2015). In particular, SKN-1 increases proteasome activity upon GSC removal, which is in stark comparison to WT where the activation of proteasome subunit genes is only activated by SKN-1 following proteasome inhibition (Li *et al.* 2011). GSC(-) worms accumulate fat but do so through an early accumulation of yolk lipoproteins (vitellogenins) by the first day of adulthood. These proteins are normally supportive of reproduction and in its absence are converted to stored lipids. The overexpression of vitellogenin reduces the lifespan of both *glp-1* and *daf-2* mutants but not WT worms, likely by impairing autophagy (Seah *et al.* 2016). Fascinatingly, SKN-1 acts to reduce this fat accumulation and its absence leads to rapid fat deposition and an absence of subsequent longevity. Thus, SKN-1 acts both in concert with and independently from NHR-49 to maintain a healthy fat balance. A main element of the distinction between these two pathways is the control of different lipase genes. Longevity through GSC(-) requires LIPL-4 (Wang *et al.* 2008), whose fatty acid products act through the NHR-80:NHR-49 complex (Folick *et al.* 2015, Lapierre *et al.* 2011), and LIPL-1/3 which act through SKN-1 (Steinbaugh *et al.* 2015). As such, GSC(-) mediated longevity involves metabolic signals generated by an alteration in the nutritional balance of the whole organism.

Autophagy connects with lipid metabolism in the longevity of GSC(-) worms as it functions not only to remove and recycle proteins but is also important for lipid breakdown and the alleviation of toxic lipid accumulation (Ward *et al.* 2016). As with other longevity pathways autophagy is also required for lifespan extension following germ cell removal (Lapierre *et al.* 2011, 2013b). Germline removal activates autophagy through TOR signalling and *pha-4*. Autophagy additionally modulates lipid breakdown through activation of LIPL-4 and in turn requires LIPL-4 for full induction (Lapierre *et al.* 2011). Interestingly, the inhibition of autophagy can abolish *glp-1* longevity even when initiated in early adulthood, suggesting that an absent germline provides pro-longevity signals beyond just the developmental period (Lapierre *et al.* 2011). The process of lipophagy, which is the breakdown of lipids via autophagy and lysosomal degradation (Singh *et al.* 2009), is an emerging mechanism for the prolongation of lifespan. Lipolysis in the lysosome is tightly coordinated with nutrient abundance through two nutrient-responsive transcription factors MXL-3 and the autophagy regulator HLH-30. In food abundant conditions MXL-3 suppresses lipase activity but following fasting HLH-30 is activated and subsequently activates both autophagy and lysosomal lipolysis (O'Rourke & Ruvkun 2013). Indeed, this transcriptional circuit is required for normal lifespan and the lipids generated through this process signal further via NHR-80:NHR-49 (Seah *et al.* 2016). The complex interaction of these processes demonstrate a deep interaction of lipids and autophagy in modulating lifespan in worms.

### 1.3.4 Mitochondria and longevity

Mitochondria as the energy centres of the cell are involved in several ageing processes including: cellular metabolism, ATP synthesis and the production and subsequent detoxification of ROS. Beyond even its role in energy production, mitochondria are factors in the regulation of apoptosis, buffering calcium release, signalling to the immune system, activation of nuclear gene transcription, assisting in steroid synthesis, and controlling both the cell cycle and cell growth (Sun *et al.* 2016). It is therefore unsurprising that dysfunctional mitochondria are implicated in ageing and age-related diseases, which include: cancer, diabetes, neuropathy, and neurodegeneration (Coppotelli & Ross 2016). The speculation that a decline in mitochondrial function might add to the age-dependent decline in organ function is an old one (Rockstein & Brandt 1963) and indeed an age-related decline in mitochondrial activity has been observed in worms and humans (Regmi *et al.* 2014, Shen *et al.* 2014, Petersen *et al.* 2003). Counterintuitively then, disruption of mitochondrial function often leads to an overall increase in lifespan. The first mutation in mitochondrial function identified to extend lifespan was a nuclear gene *clk-1* involved in ubiquinone biosynthesis (Wong *et al.* 1995). Subsequently, mutations in *isp-1* (a complex III subunit in the electron transport chain), *gro-1* (a tRNA transferase), and *lrs-2* (a leucine tRNA synthase) were identified as lifespan extending (Li *et al.* 2003, Feng *et al.* 2001, Lemieux *et al.* 2001). Numerous other genes involved in various aspects of mitochondrial function have been discovered through RNAi screens and forward genetics (Hwang *et al.* 2012). What is common to the evolutionarily conserved modes of lifespan extension through mitochondria is that the degree of inhibition must be mild and not produce a functional collapse in the organelle (Hwang *et al.* 2012). The mechanisms by which reduced mitochondrial activity can extend lifespan have become increasingly disputed. Initially, there seemed to be a relatively straightforward correlation with reduced activity and decreased ROS production, which, based on fundamental limitation theories of ageing, made sense. However, it was demonstrated that lifespan extending mitochondrial mutants exhibited different responses to free-radicals and other stressors (Kim & Sun 2007, Lee *et al.* 2003). Indeed it appears that increased ROS can directly promote longevity through hormesis, a process whereby mild stress exerts beneficial effects through the activation of stress response genes (Calabrese *et al.* 2015). Also, in long-lived mitochondrial mutants, increasing oxidative damage through reduced detoxification does not affect their longevity (Yang *et al.* 2007). As with other longevity pathways, a main source of the longevity derived from reduced mitochondrial activity may come through cellular signalling and the activation of alternate metabolic routes to compensate for reduced activity. Long-lived mutants in both yeast and *C. elegans* activate a retrograde signalling pathway that causes a shift in metabolism away from the Krebs cycle and towards the glyoxylate cycle (Ruzanov *et al.* 2007). This shift may directly contribute to the longevity of mitochondrial mutants through reducing the AMP/ATP ratio and activating AMPK, known to be required for longevity in both IIS and mitochondrial mutants (Apfeld *et*

*al.* 2004). Different mitochondrial mutations differ in their requirement for DAF-16 with many extending lifespans additively with reduced IIS signalling but other exhibiting dependence on DAF-16 activation (Hwang *et al.* 2012). Both *clk-1* and *isp-1* mutations upregulate hypoxia-inducible factor 1 (HIF-1), which is a master regulator of the cellular adaptation to reduced oxygen conditions. Curiously, hypoxic conditions extend lifespan in *C. elegans* and do so dependent on HIF-1 and DAF-16 but independent of SKN-1 whose overexpression shortens life in hypoxia (Leiser *et al.* 2013). CR enhances mitochondrial respiration through increased mitochondrial biogenesis and requires functional, active mitochondria to extend life. The role of TOR is slightly more confusing with reports that TOR both activates and reduces mitochondrial activity in a manner that may well suggest a tissue-specific effect (Hwang *et al.* 2012).

The connection between autophagy and mitochondria and its link to longevity is a burgeoning field of study. As part of their respiratory activities, mitochondria and their organelle specific DNA are subject to numerous insults and accumulate damage over time. In order to keep mitochondria at optimal efficiency, the cell has developed several methods that control the turnover of these organelles. One of the first is the ability of mitochondria to undergo either fusion or fission to control their numbers, shape, size and distribution, allowing them to shuttle between a networked and an individual state (Okamoto & Kondo-Okamoto 2012). Fission (through DRP-1) can result in the division of healthy resources preferentially into one mitochondrion, leaving the unhealthy one to be recycled and the healthy mitochondrion to re-enter the mitochondrial network via fusion (Lackner & Nunnari 2009, Griffin *et al.* 2006). Mitochondria cannot be synthesised from scratch and must be generated by increasing the mass of already existing mitochondria which then undergo either fusion or fission to enter the life cycle. The fusion process actively selects for a high inner membrane electrical potential, which excludes damaged, depolarised mitochondria. These are subsequently surrounded by an isolation membrane that ultimately forms an autophagosome, leading to degradation (Okamoto & Kondo-Okamoto 2012). This selective form of autophagic mitochondria degradation is known as mitophagy. Unsurprisingly, inhibition of autophagy disrupts the mitophagic process leading to impaired mitochondrial degradation, swelling and decreased respiration (Inami *et al.* 2011, Komatsu *et al.* 2005). In *C. elegans, Drosophila* and mammals, the identification of damaged mitochondria for autophagic destruction involves two genes, *pink-1* and *pdr-1* (*PINK1* and *PARK2* in humans) that are both mutated in early-onset Parkinson's disease (Lücking *et al.* 2000). Mitochondrial dysfunction is intimately linked to Parkinson's disease and the lack of recycling of dysfunctional mitochondria through autophagy is a critical factor for subsequent neuronal loss (Kalia & Lang 2015). Outside of Parkinson's mitophagy is generally critical for neuronal health and has been shown to decline over ageing and notably upon human huntingtin overexpression (Costa *et al.* 2010). Fascinatingly, increasing the rate of mitochondrial uncoupling (uncoupling the electron transport chain from ATP synthesis), through

overexpression of *ucp-4* in *C. elegans*, reduces neuron degeneration in a manner dependent on mitophagy. Loss of mitophagic activity decreases lifespan in flies and its enhanced activation extends longevity without a compromise in fertility or food consumption (Rana *et al.* 2013, Greene *et al.* 2003). Inhibition of key mitophagy genes *dct-1* and *pink-1* reduces the enhanced autophagy of *daf-2* mutants and reduces the longevity of *daf-2, eat-2, clk-1* and *isp-1* mutant strains (Palikaras *et al.* 2015). This implies that these mutants upregulate mitophagy as part of their longevity programmes. Mitophagy impaired worms are highly sensitive to stress but lifespan normal in unstressed WT conditions, likely through upregulation of SKN-1 and DAF-16. SKN-1 itself regulates the expression of *dct-1* and its inhibition impairs mitochondrial networking and increases membrane depolarisation (Palikaras *et al.* 2015). Thus, there is a control pathway using SKN-1 and DAF-16 to enhance mitophagy under stressed conditions, which includes when mitophagy itself is impaired, preserving mitochondrial balance through selective autophagy and subsequent mitochondrial biogenesis. The absence of any components of this pathway prevents lifespan extension and renders the organism highly sensitive to stress.

### 1.3.5 Epigenetics and longevity

The faithful control of gene regulation through the modulation of transcription and translation rates is evidently of critical importance to the viability of every cell and subsequently the whole organism. Many of the questions surrounding ageing ultimately ask what changes in genetic regulatory events give rise to an ageing phenotype. For example, which age-associated changes in RNA and protein levels are causal, how are hallmarks of ageing actually related to each other, what are the control hubs that control ageing, when does ageing begin and can it be therapeutically controlled (Booth & Brunet 2016). Because of the role of transcription factors and chromatin states in defining gene regulation, they represent one of the most powerful ways an organism can affect the ageing process, either passively or actively, and are probably a central hub linking many ageing processes together. These epigenome changes (alterations in transcription factor binding and activity, histone marks, DNA methylation, and non-coding RNAs (Booth & Brunet 2016)) have already been shown to have a variety of functions in ageing and age-related disease and more influences are constantly being discovered.

Due to their role in controlling gene expression, either up or down-regulating, transcription factors both create and respond to changes in the epigenetic landscape. The resulting changes in gene expression can be drastic over age, perhaps representing a loss in transcriptional control, and are a major focus of longevity investigations (Booth & Brunet 2016). A newly emerging trend in ageing research is focusing on global declines in histone levels over age (O'Sullivan *et al.* 2010), which may be directly influencing changes in transcriptional control in later life. So far, this trend has only been

identified in yeast, where the loss of histones is associated with cell-cycle exit and histone degradation via autophagy (Feser *et al.* 2010, Kvam & Goldfarb 2007). Indeed, increasing the amount of histones available dramatically extended the replicative lifespan (Feser *et al.* 2010). The loss of histones with age leads to a global transcriptional amplification. This is due to less precise nucleosome positioning and a lack of a nucleosome-depleted region specifically at the promoter, resulting in aberrant transcription factor binding (Hu *et al.* 2014). The histone mark H3K36me3/me2 functions in lifespan determination by the suppression of spurious cryptic transcription from within gene bodies (Sen *et al.* 2015). The deletion of a H3K36 methyltransferase shortened replicative lifespan, while deletion of its cognate demethylase extended lifespan by ~30% (Sen *et al.* 2015). Similarly, in worms, the loss of MET-1 (a H3K36 methyltransferase) shortens lifespan and inhibition of the respective demethylase, JMJD-2, had no lifespan effect (Hamilton *et al.* 2005). Inhibition of JMJD-2 was later identified as lifespan extending in a separate RNAi screen (Ni *et al.* 2012). Indeed, the level of H3K36me3 has a direct negative correlation with variations in gene expression over ageing (Pu *et al.* 2015).

Stem cells and somatic tissue senescent-cells from aged mice show a distinct upregulation of transcripts from repeat elements, which are a common feature of amyloid-forming proteins and normally transcriptionally silenced, indicating a loss of silencing control in heterochromatin regions. Indeed constitutive heterochromatin loss from telomeres and centromeres and increased unintentional transcription from these regions is a hallmark of ageing (Tsurumi & Li 2012, Villeponteau 1997). Heterochromatin is marked by di- and tri-methylated H3K9 and high levels of DNA methylation and these are reduced and disorganised in cellular senescence (Haithcock *et al.* 2005). The actions of the longevity-associated transcription factors DAF-16 and SKN-1 are affected by ageing, with changes in transcriptional targets and dysregulated activity seen over age (Webb *et al.* 2016, Rahman *et al.* 2013). As both DAF-16 and SKN-1 bind to open chromatin, especially enhancer regions in the case of DAF-16 (Eijkelenboom *et al.* 2013, Webb *et al.* 2013), disruptions in chromatin states in late-life will affect their activities and subsequent stress responses. DAF-16 and SKN-1 can themselves remodel chromatin through recruitment of SWI/SNF complexes, without which transcriptional control is disrupted and in the case of DAF-16 prevents longevity extension (Riedel *et al.* 2013, Zhang *et al.* 2006).

Histone modifications (histone marks) are covalent post-translational modifications that affect chromatin structure and DNA-protein binding. The differential recruitment of histone-modifying enzymes and chromatin remodelling complexes by transcription factors form the patterns of modifications seen at each region of the genome (Booth & Brunet 2016). There are eight types of confirmed histone modifications, which cover at least 37 individual site-specific modifications. These include: acetylation of lysine (Kac), methylation of lysine or arginine (Kme1, 2, 3 and Rme1, 2a and 2s), phosphorylation of serine or threonine (Sph and Tph), ubiquitination of lysine (Kub), sumoylation of lysine (Ksu), ADP ribosylation of glutamic acid (Ear), Deamination of arginine to citrulline, and proline

isomerisation (Kouzarides 2007). In the context of ageing only acetylation, methylation and ubiquitination have been shown to have functional relevance so far. These marks predominantly regulate transcription and the control of gene expression, with ubiquitination being also heavily involved in DNA repair. The exact mechanisms underlying this control of gene expression are not yet completely understood but most likely involve the promotion or inhibition of transcription factor recruitment and DNA binding and the subsequent movement of the polymerase through the gene (Kouzarides 2007). Indeed, there is a widespread remodelling of histone modification patterns during ageing, across both species and cell types (Benayoun *et al.* 2015). Two marks, H3K4me3 and H3K27me3, and their cognate enzymatic modifiers have particularly pronounced roles in ageing. These marks have opposing effects on gene transcription, activating and repressing it respectively. In *C. elegans* inhibition of the SET-2 H3K4 methyltransferase complex increases longevity and decreases global H3K4me3 levels. Conversely, inhibition of the demethylase *rbr-2* shortens lifespan while its overexpression extends longevity (Greer *et al.* 2010). Fascinatingly, these effects require an intact germline and can be inherited by offspring, giving remarkable evidence of transgenerational epigenetic inheritance (Greer *et al.* 2011). The pattern of H3K4me3 changes with age in *Drosophila*, aged murine hematopoietic stem cells, and senescent human fibroblasts (Booth & Brunet 2016). Additionally, increasing the abundance of this mark shortens longevity in male flies (Li *et al.* 2010). Two additional SET domain proteins have demonstrated pro-longevity effects when inhibited in *C. elegans*, *set-9* and *set-26*. The proteins of both genes are almost identical and exhibit some tissue-specific expression pattern differences. Neither seem to exhibit any direct action on histone methylation levels but instead appear to affect the general status of chromatin and modulate longevity through the regulation of DAF-16 transcriptional targets (Ni *et al.* 2012). Manipulation of the opposing, transcriptionally repressing mark H3K27me3 also affects longevity but does so in a context-specific manner and varies in effect across different species. In *C. elegans* H3K27me3 is generated by the PCR2 complex and removed by UTX-1. Over ageing in the worm, there is a marked global decrease in H3K27me3 levels and a corresponding increase in *utx-1* expression. Inhibition of *utx-1* extends lifespan and increases global H3K27me3 presence. Curiously, in comparison to *set-2* inhibition, *utx-1* mediated longevity is germline independent but *daf-2* dependent (Maures *et al.* 2011). Another H3K27me3 demethylase, JMJD-3.1, when over-expressed in worms can extend lifespan and showed increased stress resistance (Labbadia & Morimoto 2015). Similar findings found that overexpression of *jmjd-3.1* and *jmjd-1.2* act to induce the mitochondrial stress response and their inhibition abolished the lifespan extension of ETC mutants (Merkwirth *et al.* 2016). JMJD-1.2 is also a demethylase but specific for the removal of methyl groups from H3K27me2 and H3K27me1 (Kleine-Kohlbrecher *et al.* 2010). Mice with higher JMJD-1.2 levels are longer lived (Merkwirth *et al.* 2016). The changes exerted by both these demethylases appear to be related to the specific de-repression of stress response genes, as other

similarly related demethylases showed no effect on stress response when inhibited (Merkwirth *et al.* 2016). The participation of H3K27me3 in longevity is further complicated by results from other species. The loss of UTX-1 in female mice leads to severe developmental defects and a shorter life (Welstead *et al.* 2012). However, Loss of the H3K27me3 methyltransferase *mes-2* in flies and less H3K27me3 extends the lifespan of males but not females (Siebold *et al.* 2010). H3K27me3 increases in the brains of the African killifish over age and quiescent satellite cells in mice but decreases in both *C. elegans* and in human models of progeria. Comparing young vs old in both hematopoietic stem cells and senescent fibroblasts shows a locus-specific increase or decrease in H3K27me3 levels (Booth & Brunet 2016). Thus, a simple model of global gene transcriptional repression being beneficial to ageing does not fit in all contexts.

Both histone acetylation, and its deacetylation through sirtuins, and histone ubiquitination are relevant to the ageing process as both function to control transcriptional rates and DNA repair process. Sirtuins and their effect on longevity have been discussed in the context of caloric restriction mediated longevity already (see section 1.3.2). As such, that element of their function will not be repeated here. Acetylation of histones generates a more open chromatin structure facilitating transcriptional activation. Conversely, its deacetylation through NAD+ dependent sirtuins leads to more compact chromatin and transcriptional repression (Feser & Tyler 2011). In agreement with improved control of gene activation in late-life, high levels of both SIRT3 and SIRT6 are associated with increased longevity in mammals (Kanfi *et al.* 2012, Bellizzi *et al.* 2005). Mice lacking sirtuin genes have a reduced lifespan due to accelerated ageing or increased cancer incidence (Wątroba *et al.* 2017). Also, decreased sirtuin activity in some organisms has been shown to contribute to numerous ageing phenotypes (Chandra *et al.* 2012). The polyamine spermidine inhibits the activity of histone acetyltransferases and its levels decline with age across species (Sen *et al.* 2015). External supplementation of spermidine increases longevity through increased transcription of stress response and autophagy genes (Eisenberg *et al.* 2009). Sirtuins and deacetylation are vitally important for genome stability and the DNA damage response. Deacetylation of telomeres is required to keep these regions transcriptionally silent. In yeast, this silencing is maintained through Sir2 acting on H4K16. Older yeast have less active Sir2 and consequently more transcription from telomere-proximal genes, which are normally maintained in a silent, heterochromatic state (Gottschling *et al.* 1990). In humans, this effect is modulated through SIRT6, rather than the Sir2 homologue SIRT1 (Tennen *et al.* 2011). Damage to telomeres themselves can give rise to a reduction in core histone expression and thereby modulate the chromatin landscape in ageing cells (O'Sullivan *et al.* 2010). Beyond telomeres, both SIRT1 and SIRT6 are important in the DNA damage response. SIRT6 is actively recruited to DNA damage sites and facilitates a remodelling of the local chromatin landscape through the deacetylation of H3K56, which enables access of the DNA repair machinery to the site of damage (Toiber *et al.* 2013). Deletion of SIRT6 in mice results in a more

rapid ageing phenotype and increased genomic instability (Mostoslavsky *et al.* 2006). In mice, SIRT1 acts to repress a functionally diverse group of genes and also the expression of repetitive DNA sequences. Upon DNA damage, SIRT1 shifts its positioning from the promoters of its specific genes and repetitive loci to the site of DNA damage where it also assists in remodelling the chromatin environment. This shift can lead to the de-repression and transcription of individual genes and additionally transcription of repetitive DNA segments from normally heterochromatic regions. As organisms age, they suffer constant DNA damage. Accordingly, the continuous shift of SIRT1 to damage sites could contribute to ageing through the action of stochastic gene expression (Oberdoerffer *et al.* 2008). Histone ubiquitination on H2A is also intimately connected with DNA repair and potentially therefore to ageing. However, no direct links between H2Aub and ageing have yet been observed. A H2A variant (macroH2A) is prominently associated with senescence-associated heterochromatin foci (specialized domains of facultative heterochromatin that contribute to silencing of proliferation-promoting genes (Narita *et al.* 2003)), which lead to unintentional gene silencing (Adams 2007). MacroH2A's function is presumed to be important for the formation of these foci and transcriptional repression (Zhang *et al.* 2005). Additionally, the abundance of macroH2A increases during the replicative senescence of cultured human fibroblasts in an age-dependent manner (Kreiling *et al.* 2011). The role of H2Bub in DNA repair is less well defined, though its link to ageing is clearer. H2Bub is linked to the action of RNA polymersase II, which upon transcriptional activation monoubiquitylates histone H2B at Lys 123 (K123ub) in yeast and K120 in mammals (Hartzog & Quan 2008). H2Bub directs SET-1 and DOT-1 complexes to di- and tri- methylate H3K4 at and H3K79 respectively (Nguyen & Zhang 2011, Dover *et al.* 2002), both of which are activating marks. H2Bub then acts to promote transcriptional elongation into the gene body, increasing levels of histone acetylation in those regions. The de-ubiquitination of H2B leads to deacetylation and polymerase stalling at gene promotors (Batta *et al.* 2011). H2Bub is also associated with the repression of gene expression from cryptic start sites (Du & Briggs 2010). H2Bub has been demonstrated to be important for yeast replicative lifespan. Yeast strains lacking components of the SAGA/SLIK complex de-ubiquitinase module are exceptionally long-lived (McCormick *et al.* 2014). This same complex controls development of the neuronal components of the *Drosophila* visual system (Weake *et al.* 2008). The lifespan extension through SAGA/SLIK also requires Sir-2 activity suggesting the promotion of multiple longevity events through the control of gene transcription (McCormick *et al.* 2014).

### 1.3.6 Autophagy and its role in lifespan

Autophagy is the primary means by which cells deliver cytoplasmic substrates to lysosomes for degradation and recycling. It is divided into macroautophagy, chaperone-mediated autophagy, and

microautophagy. For the purposes of this discussion, the focus will be on macroautophagy (referred to as autophagy throughout this thesis). This is both a selective and non-selective mechanism of self-cannibalism, through which portions of the cytoplasm, damaged organelles and misfolded proteins are enclosed in double-membraned vesicles that fuse with endosomes and lysosomes, enabling the destruction of the internal cargo (Rubinsztein *et al.* 2011). The stages of autophagy can be divided into, activation of autophagy genes through upstream signalling, formation of the phagophore (isolation membrane), engulfment of cytoplasmic contents by the phagophore, elongation and closure of the phagophore membrane, and fusion with endosomes and lysosomes to form the autolysosome. The autolysosome is the stage in which the contents are degraded and the breakdown products released through permeases and recycled in the cytosol (Ravikumar *et al.* 2010). The regulation of these specific steps is derived from the sequential action of autophagy-related genes (ATG). More than 30 ATGs have been identified in yeast that are required for autophagy and most show conservation between yeast and mammals. This makes autophagy a highly conserved evolutionary process, implying that aspects of its regulation and function identified in the ageing of model organisms are likely to be relevant to the human condition (Nakamura & Yoshimori 2018).

The initiation of autophagy begins with upstream signalling processes, often stress related, which activate the key autophagic transcription factors PHA-4 and HLH-30. Both transcription factors are regulated independently and often by different upstream signalling events. HLH-30(TFEB) was identified as a master regulator of lysosomal biogenesis and was shown to link autophagy directly to this process (Settembre *et al.* 2011). HLH-30 is negatively regulated by TOR, IIS signalling, mitochondrial respiration, protein translation, and germline removal (Nakamura & Yoshimori 2018) and is required for extended longevity in all these conditions (Lapierre *et al.* 2013a). Its activation rapidly induces expression of autophagy-related proteins including BEC-1 and is considered as a master regulator of autophagy induction. PHA-4(FOXA) is regulated by TOR signalling to activate autophagy through BEC-1(BECN1) and UNC-51(ULK1) (Lapierre *et al.* 2011, Zhong *et al.* 2010) and is required for the longevity of the TOR, Caloric restriction, and germline removal pathways (Lapierre *et al.* 2011, Sheaffer *et al.* 2008, Panowski *et al.* 2007). Although both genes regulate autophagy, they do so in a different manner with PHA-4 required for the regulation of phagophore forming genes and HLH-30 for later steps in the autophagic process including lysosome biogenesis (Füllgrabe *et al.* 2016). In combination with transcription factor activation, TOR inhibition also directly results in the dephosphorylation of the UNC-51 ATG-13 complex (Ganley *et al.* 2009), causing its activation. Activation of this complex is critical for the recruitment of the phagophore forming complex that consists of BEC-1, VPS-34, EPG-8 and VPS-15 (Itakura & Mizushima 2010, Matsunaga *et al.* 2010). UNC-51 also directly phosphorylates and activates BEC-1 in a process that enhances autophagy induction (Russell *et al.* 2013). Together this group exerts PI3 kinase activity that recruits additional ATG proteins

for autophagic membrane formation and extension. This process involves two ubiquitin-like conjugation cascades (involving ATG-5, ATG-7, ATG-12, and ATG-16) that results in the eventual deposition of LGG-1 on the forming isolation membrane (Hanada *et al.* 2007). LGG-1 guides cargo loading into the forming autophagosome and assists in trafficking closed autophagosomes to lysosomes (Itakura *et al.* 2012, Pankiv *et al.* 2007). As such LGG-1 is typically used as a marker for identifying autophagosomes (Klionsky *et al.* 2012).

Considering its essential function in maintaining cellular homeostasis it is unsurprising that autophagy is intimately linked to the process of ageing. It has been demonstrated that ageing decreases autophagy, through activation of the TOR pathway, while also increasing resistance to apoptosis (Romero *et al.* 2016). SIRT1, which is an inducer of autophagy, exhibits decreased expression in aged tissues (De Kreutzenberg *et al.* 2010). Specific autophagy genes are downregulated in aged brains and in cartilaginous tissue (Caramés *et al.* 2010, Lipinski *et al.* 2010). Additionally, the calcium signalling receptors $IP_3$ (inositol 1,4,5-triphosphate) are upregulated with age and specifically in neurodegenerative disease. Calcium signalling is an important determinant of cellular health and eventual cell fate through modulation of apoptosis induction and autophagy inhibition (Decuypere *et al.* 2011). In yeast, multiple short-lived mutants have been identified that harbour defects in autophagy (Matecic *et al.* 2010) and similarly, in both *C. elegans* and *Drosophila,* the inhibition of core autophagy genes shortens lifespan (Rubinsztein *et al.* 2011). There is some evidence that increasing autophagy may be beneficial to longevity. Modulation of longevity through IIS, TOR and the germline enhances autophagy induction and inhibiting this reduction removes this longevity gain (Lapierre *et al.* 2013a, 2013b). The TOR inhibitor rapamycin also induces both autophagy and longevity in yeast, worms, flies and mice (Ehninger *et al.* 2014), although the longevity extension through rapamycin is unlikely to be solely through autophagy. Overexpression of ATG5 in mice enhances lifespan and improved ageing hallmarks (Pyo *et al.* 2013) as does HLH-30 overexpression in *C. elegans* (Lapierre *et al.* 2013a).

Almost every neurodegenerative disease shares a common pathogenesis, the age-associated accumulation of undegraded proteins. Classically, autophagy is considered as a protective mechanism against neurodegenerative disease, as it has the ability to degrade the associated toxic protein aggregates (Nixon 2013). Post-mitotic cells such as neurons are particularly reliant on autophagy due to an absence of cell division mediated dilution of intracellular aggregates and debris (Lindner *et al.* 2008). Moreover, as neurons are at best poorly replaced by stem cells, there is no mechanism for their replacement when damage becomes excessive. Surprisingly then, rather than maintaining a generally high level of autophagy in older age, the level of autophagy instead decreases over time (Rubinsztein *et al.* 2011). Autophagy is clearly still necessary, at least early in life, as the genetic deletion of key autophagy genes in animal models leads the development of age-associated neuronal pathologies very similar to those seen in humans (Jia *et al.* 2007, Ravikumar *et al.* 2010). Neuron-specific deletion of

*Atg5* and *Atg7* in mice sharply increases the accumulation of intra-neuronal aggregates (Hara *et al.* 2006, Komatsu *et al.* 2006). Double deletion of both ULK genes, also in mice, triggered neuronal death through association with the unfolded protein response pathway (Joo *et al.* 2016). Despite gene-specific effects on different pathologies, the general result of autophagy inhibition in neurons in all model organisms is reduced survival and an early onset progressive neurodegeneration (Menzies *et al.* 2017). In AD autophagy functions to dispose of amyloid-β and tau aggregates but eventually becomes overwhelmed by the aggregates leading to impairment. Impaired autophagic vesicles can then accumulate and aggravate amyloid-β toxicity by functioning as a concentration point for these proteins to further entangle. At this stage, inhibition rather than induction of autophagy is beneficial in controlling amyloid-β levels and toxicity (Haung Yu *et al.* 2005). The role of autophagy in PD has previously been discussed in the context of mitophagy and autophagy associated mitochondrial dysfunction (see section 1.3.4) and will not be recapitulated here. In HD the normal huntingtin protein (HTT) is mutated and becomes aggregate-prone. As with other aggregation diseases, autophagy is required for clearance of the mutated HTT but, interestingly, is itself suppressed by mHTT, which inhibits autophagosome mobility and capacity for fusion with lysosomes (Wong & Holzbaur 2014). This defective degradation results in increasingly induced autophagy, which, itself, can be toxic at high enough levels (Scott *et al.* 2007). As with other neurodegenerative diseases, including motor neuron disease (Zhang *et al.* 2011), autophagy inhibition rather than induction may be more beneficial in progressive stages of the disease (Menzies *et al.* 2017). In the brains of patients with AD, PD, and HD, there is an accumulation of autophagic and lysosomal vesicles, suggesting that the process of autophagy may be completely blocked in these diseases (Banerjee *et al.* 2010). Reactivation or enhancement of autophagy via generic chemical manipulation has shown only limited effectiveness in ameliorating neurodegenerative phenotypes in animals through preservation of neuronal function (Nah *et al.* 2015, Tan *et al.* 2014, Pan *et al.* 2008). That autophagy is beneficial when functioning is beyond doubt but the evidence of its disruptive and toxic nature when defective suggests that its role as a benefactor needs to be considered in the context of its environment.

## 1.4 Experimental rationale and design

For our purposes, we decided to use the genetically tractable model organism *Caenorhabditis elegans*. *C. elegans* is one of the most widely used species to conduct ageing research due to its extremely quick life-cycle (~3 days) and attenuated lifespan (~20 day median). *C. elegans* are hermaphrodites capable of self-fertilisation. This is their primary mode of reproduction as males are very rare < 1/1000. This feature is extremely advantageous for genetic studies as it is relatively facile to maintain a homozygous mutant strain. Additionally, self-reproduction allows for the easy generation of large numbers of

worms without the need for mating but comes at the cost of unwanted progeny in experiments using older worms. Fertilisation of eggs occurs *in utero* after which the eggs are usually expelled from the body to hatch. Under stressful conditions such as starvation, eggs can be retained inside the parent where they hatch, devouring the mother from the inside. This matricidal hatching (bagging) is a regular issue in the generation of age-synchronous cultures of *C. elegans*. Following hatching, *C. elegans* undergoes a defined transition through four larval stages L1, L2, L3 and L4, after which they are young adults until the production of their first eggs. In the absence of food, *C. elegans* can enter three paused developmental stages called diapause: L1 diapause; dauer state, a specialised L3 stage; and adult reproductive diapause (Fielenbach & Antebi 2008). The dauer stage is of particular interest to ageing researchers as worms can survive for months in this state and re-enter into a normal life cycle following the re-introduction of food. *C. elegans* serves as an excellent model organism for identifying pleiotropic ageing effects in the post-reproductive period, as it has a well-defined and limited egg laying duration (~5 days) followed by several more days of activity before a gradual decline to death. In addition, the genome of *C. elegans* has been comprehensively studied and annotated and worms can be grown in large quantities with relative ease (The C. elegans Sequencing Consortium 1998, Fabian & Johnson 1994). Genetic interference in *C. elegans* is greatly facilitated by the ability to feed the worms with bacteria expressing double-stranded RNA (dsRNA) corresponding to the gene of interest. This feeding results in the uptake of the dsRNA through the gut and its eventual processing into short interfering RNAs (RNAis), which can affect a rapid and robust downregulation of the mRNA species of the targeted gene (Hammell & Hannon 2012). This feature is supplemented by the availability of RNAi libraries that target almost every coding gene in the worm (Kamath *et al.* 2003, Rual *et al.* 2004).

### 1.4.1 Experimental approach 1

The discovery of individual genes capable of greatly modifying the lifespan of simple organisms has invigorated the field of ageing research. Through the use of forward genetic screens, mutagenesis, and targeted RNAi gene knockdown, the entire genome of *C. elegans* has been probed for genetic contributions to longevity (Klass 1983, Ni & Lee 2010). These screens and other studies have identified that genetic contributors to ageing appear to largely work through a small set of common pathways, such as metabolism, hormone control and reproduction (Kenyon 2010). That ageing is influenced by crucial nodes controlling multiple, essential downstream processes, suggests that however ageing has evolved its greatest effects arises from pathways or systems that influence the biology of the entire organism. The disruption of these master systems, not-unexpectedly, usually results in a fundamental change in the quantities and types of genes expressed (Heinonen *et al.* 2008, Halaschek-Wiener *et al.* 2005, Parisi *et al.* 2004). Ageing is a complex phenotype that represents the interplay of an organism's biology with the environment in which they live. How an organism responds to changing conditions

such as nutrient abundance, temperature and exogenous stress within their life often has profound impacts on their longevity (Zhou *et al.* 2011). As discussed in section 1.3.5, one of the most powerful ways an organism has at its disposal to respond to environmental signals and simultaneously control the actions of multiple genes is through the action of epigenetic modifiers. Changes in the epigenetic marks present on histones bound to DNA or directly on the DNA itself can have a profound effect on gene expression levels and initiate a coordinated response to changing stimuli (Bird 2007). Thus, as a nexus of control capable of modifying the genetic response to multivariate signals, epigenetic mechanisms seem like a probable focus for the action of pro-ageing signals. This notion has been bolstered by the identification of specific epigenetic modifications and modifying enzymes with pro- and anti-ageing effects (Maures *et al.* 2011, Greer *et al.* 2010, Dang *et al.* 2009, Viswanathan *et al.* 2005, Rogina & Helfand 2004, Tissenbaum & Guarente 2001). The top-down approaches of these experiments, while generating new discoveries, have been necessarily limited in their scope for finding new epigenetic regulators of longevity. We wished to tackle this limitation by switching to an unbiased bottom-up approach hopefully maximising the potential for new discoveries. For this reason, we developed the following experimental approach (Figure 1). We would grow large-scale cultures of age-synchronous *C. elegans* and take samples across the lifespan of the worm. From these samples, we would identify: changes in epigenetic modifications using western blot analysis and mass spectrometry (MS); changes in gene expression using RNA sequencing and quantitative PCR (qPCR); and changes in gene regulation using chromatin precipitation (CHIP) and CHIP-sequencing. From this data, we would identify the best candidate genes and epigenetic modifications, and assess their contribution to ageing using targeted gene inhibition with RNAi. This purely unbiased examination of the differences and changes between young and old worms should ideally give the broadest possible scope for identifying crucial, unknown components to the ageing process.

*Figure 1*

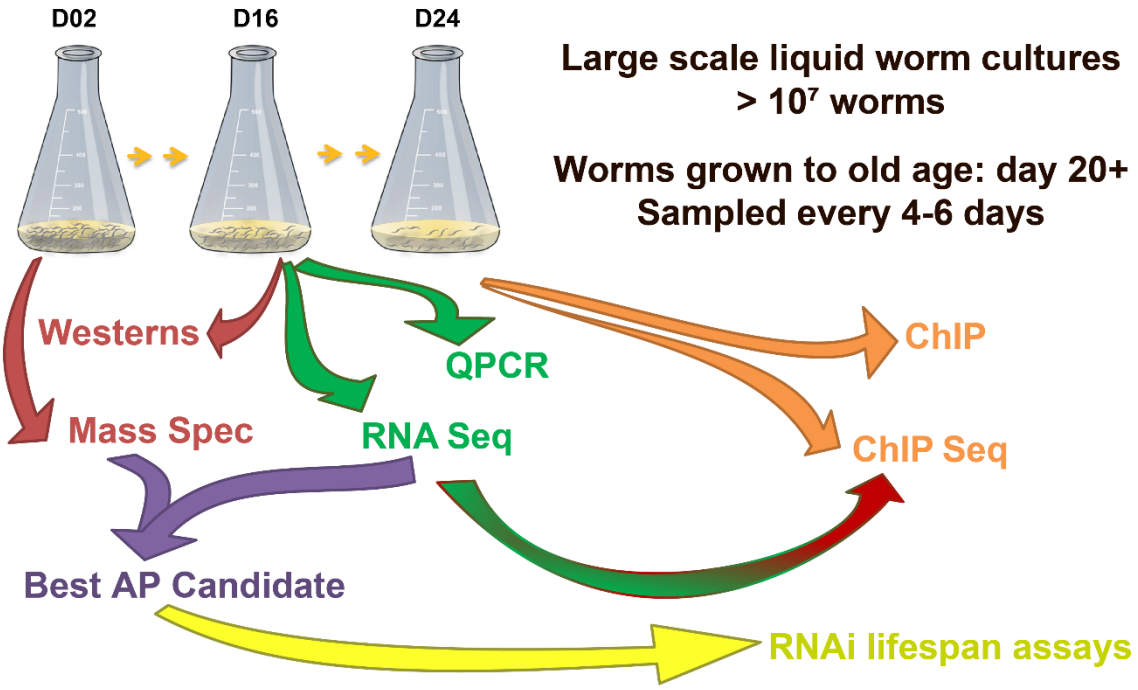


**Figure 1:** ***Schematic diagram of the first experimental approach****. C. elegans* are cultured in large quantities in Erlenmeyer flasks for time course analysis. Samples are taken at different time-points and interrogated through: Western blots, mass spectrometry, qPCR, RNA sequencing, ChIP and ChIP sequencing. Strong AP candidates are identified and then tested for lifespan effects with RNAi treatment in late-life.

### 1.4.2 Experimental approach 2

The identification of epigenetic modifiers of lifespan has occurred through the same process used to identify almost every other component of the ageing process: genetic mutation or inhibition of the gene in question from the embryonic or juvenile stages of life (Ni & Lee 2010, Sen *et al.* 2016). However, if we view this strategy through the lens of the evolutionary biology of ageing it appears to be a sub-optimal strategy. AP theory predicts that due to fitness tradeoffs the strongest effects of a pro-ageing gene will be exhibited later in life when the consequences to fitness are no longer relevant (Williams 1957). Additionally, it suggests that interfering with genes early in life could result in missing a later pro-ageing effect if the detriment to early fitness was too strong. As such, genes that are essential for early life fitness but detrimental and pro-ageing later in life have largely remained undetected. One of only two experiments to address this gap in conventional longevity screens was performed by Garry Ruvkun. His lab used RNAi treatments of known lethal *C. elegans* genes commencing in the L4 stage of their development, thereby bypassing developmental defects prohibiting previous lifespan characterisation (Curran & Ruvkun 2007). This strategy yielded 64 more unknown longevity genes, out of 2,700 essential development genes tested. It compared very favourably with previous whole-genome screens, where for example 129 novel longevity genes, out of 16,000 screened, were identified (Hamilton *et al.* 2005, Hansen *et al.* 2005). Parallel to this screen,

another group examined 57 genes known to cause developmental arrest and showed 24 of them extended longevity when inhibited in the late larval stages (Chen *et al.* 2007). The success rate of these AP screens suggests that even more genes impacting on longevity could be identified if the genetic intervention was delayed beyond developmental stages. According to this logic, we developed the following experimental strategy, which was designed to identify novel AP genes specifically acting detrimentally late in life. Using an unbiased approach, we would screen for AP longevity candidates from a library of gene-regulatory factors involved in chromatin or transcriptional regulation. To reduce the fitness cost of gene inhibition we would initiate gene knockdown only in the post-reproductive period, where it is predicted the majority of AP longevity genes will exert their effects. We expected that if a gene has detrimental ageing effects, its inhibition specifically at this stage will avoid detrimental fitness costs and subsequently extend lifespan. Using this strategy, we would specifically inhibit gene-regulatory factors involved in chromatin or transcriptional regulation in the post-reproductive period of life aiming to uncover new AP epigenetic regulators of longevity (Figure 2).

*Figure 2*

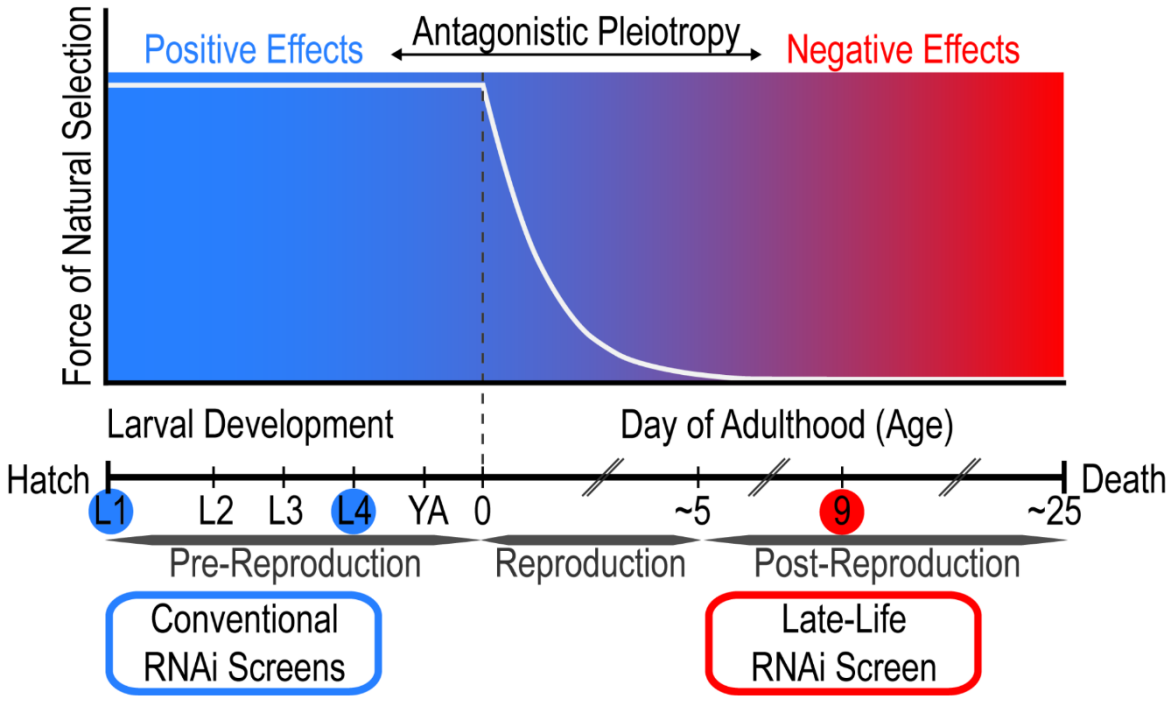


**Figure 2:** ***Screening approach for AP genes.*** The force of natural selection (white line) is high during development and declines sharply post reproductive onset. Genes with an AP effect will have a positive lifespan effect in early life (blue region) but a negative effect later in life (red region). Previous screens have only disrupted gene function in early life (blue circles), consequently failing to identify AP longevity genes. Our screen begins at day 9 (red circle) to maximise the possibility of discovering novel longevity genes with specific pro-ageing effects in late-life.

# 2 Results

## 2.1 Experimental approach 1

### 2.1.1 Large-scale culture of age-synchronous *C. elegans*

The preponderance of *C.elegans* ageing research is performed between the L1 and L4 stage of the worm's life. This strategy has several advantages, the greatest of these is the ease with which large-scale cultures of age-synchronous worms can be produced, maintained, and extracted for subsequent biochemical analysis. Several strategies have been employed to generate such large-scale young worm cultures including: the use of sterile strains (Fabian & Johnson 1994), using the drug FUDR to inhibit reproductive development (Mitchell *et al.* 1979), and liquid culture systems to generate large worm numbers (Win *et al.* 2013). Due to the increasing death rate in the population of worms in culture and the amount of sample needed for each assay type planed (5K-50K), it was clear that the only viable system for generating such large worm cultures would be a liquid culture system. This system would allow for a starting population of around 1-million worms, at a density of 1 worm/µl, theoretically allowing for the extraction of viable samples until day 26 of the worms life, assuming a 90% mortality at this age. Agar plate growth would run too many risks for localised worm starvation and sample loss due to worms burrowing into the agar, a common phenotype in overcrowded conditions (Beron *et al.* 2015). This liquid culture system was based on that devised by Fabian and Johnson who, in short, successfully grew synchronous cultures of *C. elegans* in Erlenmeyer flasks with rapid shaking (Fabian & Johnson 1994). Concerns with this system were that the use of temperature sterile mutants would overly influence our ability to uncover data relevant to ageing in the WT condition. Also, the rate of leakage, meaning unexpected reproductive events, while low in sterile mutatants, would quickly overwhelm the culture with 2$^{nd}$ and 3$^{rd}$ generations of offspring. For this reason, the drug FuDR was used to prevent the production of viable offspring, though this resulted in the package of unfertilised eggs into the worm, which they often found impossible to expel. This added a cost to their vitality and made it more difficult to cultivate them to their theoretical maximum age.

DR or starvation can have profound impacts on the transcription profile, health and longevity of worms (Garcia-Segura *et al.* 2015) and different pathways are activated dependent on the methods used to induce starvation (Greer & Brunet 2009). To avoid any complications potentially introduced by food shortage the worms were maintained in a high density of *E. coli* bacteria that were added fresh to new media on a daily basis. Optimal food concentrations to avoid DR are considered to be greater than 1^10$^9$ CFU/ml (Greer & Brunet 2009). Food was therefore added at a concentration of 3^10$^9$

CFU/ml (OD 2.5) to fresh cultures and refreshed before concentrations of less than 1.2^$10^9$ CFU/ml (OD 1) were reached.

One of the most significant challenges in performing a long-term culture of *C. elegans* was maintaining a culture free from external contamination. In general, cultures would become contaminated with bacteria able to grow in the nutrient-limited S-basal conditions despite the presence of concentrated antibiotics or the use of sterile conditions, including aseptic culture in a laminar flow hood. This problem was noted by Fabian and Johnson: "*attempts to prevent bacterial and fungal contamination of liquid mass cultures by performing all manipulations in a tissue culture hood using stringent sterile procedures were unsuccessful*" (Fabian & Johnson 1994). For this culture system, to limit contamination issues food was changed daily in combination with a sucrose preparation, for the removal of bacteria and debris. However, such frequent handling of the cultures depleted the number of worms available for biochemical analysis faster than expected. As such, it was generally impossible to extract samples at time-points later than day 24. In spite of these difficulties, and numerous failed cultures, three separate experiments were conducted, including biological replicates, wherein samples of worms were extracted at continuous time points across the lifespan of the worm. In addition, despite the harsh conditions including constant high-speed shaking and regular sucrose preparations, we were able to obtain healthy, age-synchronous worms with no offspring contamination at ages not achieved by any previous experiment involving large-scale culture (Bazopoulou *et al.* 2017).

### 2.1.2 Analysis of changes in histone marks over life

Having established a method that allowed for the production of age-synchronous cultures of *C. elegans*, changes in the epigenetic state of the worms over life were analysed. Two synchronous cultures of WT *C. elegans* were established, from which samples of worms were extracted at days: 0, 6, 12, 16, 20, and 24 (days are the number of days post first egg lay). For an unbiased approach, antibodies for all histone modifications (marks), where available, were used. For 16 of these, reliable signals were obtained. The presence of extremely high numbers of unfertilised eggs in day 0 worms created an artificially high baseline and as such these time-points were discarded and samples were instead profiled from day 6 when eggs are depleted. We compared the level of each histone mark to the level of Histone 3 (H3) expression, which was used as a normalising factor. Examining the trends for all marks, we observed a general decrease in abundance for the majority of the modifications (11 out of 16), an increase in 4 marks and stable expression for one mark (Table 1 & Figure 3, A-D). Specifically, the identification of declining levels in H3K27me2 is a finding that has not been previously associated with lifespan or longevity and may prove of interest in an AP context (Figure 3C).

*Table 1*

| Modification | Trend | Identified by western blot | Identified by MS | Publications associating histone mark with ageing |
|---|---|---|---|---|
| H3K4me | Decreasing ~55% | Yes | | (Avrahami *et al.* 2015) |
| H3K4me2 | Decreasing ~70% | Yes | | (Han *et al.* 2012) |
| H3K4me3 | Increasing ~250% | Yes | | (Larson *et al.* 2012) (Greer *et al.* 2010) |
| H3K9me | Decreasing ~85% | | Yes | (O'Sullivan *et al.* 2010) |
| H3K9me2 | Decreasing ~85% | | Yes | (Larson *et al.* 2012) (O'Sullivan *et al.* 2010) |
| H3K9me3 | Decreasing ~90% | | Yes | (Wood *et al.* 2010) (Liu *et al.* 2013) (O'Sullivan *et al.* 2010) |
| H3K9ac | Decreasing ~95% | Yes | Yes | (Peleg *et al.* 2016) (Kawakami *et al.* 2009) |
| H3K18ac | Decreasing ~95% | Yes | Yes | (Peleg *et al.* 2016) (Gong *et al.* 2015) |
| H3K23ac | Increasing ~90% | | Yes | (Peleg *et al.* 2016) |
| H3K27me | Stable | | Yes | |
| H3K27me2 | Decreasing ~70% | Yes | Yes | |
| H3K27me3 | Decreasing ~70% | Yes | Yes | (Wang *et al.* 2010a) (Maures *et al.* 2011) (Bracken *et al.* 2007) |
| H3K27ac | Decreasing ~85% | Yes | | (Avrahami *et al.* 2015) (Gong *et al.* 2015) |
| H3S28p | Decreasing ~99% | Yes | | |
| H3K36me | Stable | | Yes | |
| H3K36me2 | Increasing ~50% | Yes | Yes | (Su *et al.* 2018) |
| H3K36me3 | Stable | Yes | Yes | (Wang *et al.* 2010a) (Sen *et al.* 2015) |
| H3K79me2 | Decreasing ~75% | Yes | | (Wang *et al.* 2010a) |
| H4R3me2 | Increasing ~110% | Yes | | (Gong *et al.* 2015) |
| H2Bub | Decreasing ~80% | Yes | | (McCormick *et al.* 2014) |
| Pan H3ac | Decreasing ~60% | Yes | | (Ryan & Cristofalo 1972) |
| Pan H4ac | Decreasing ~95% | Yes | | (Ryan & Cristofalo 1972) (PIÑA *et al.* 1988) |

**Table 1:** ***Histone modifications examined over life.*** Individual histone modifications tested for changes in abundance over the life of WT worms are listed. Changes in modifications were identified through either western blots, with the respective antibody, or through histone enriched mass spectrometry as indicated. The general trend of each modification is listed, along with the change in abundance in comparison to the maximum value (decreasing trends) or minimum value (increasing trends) obtained over life. Previous publication where each histone modification has been identified in the context of ageing or lifespan modification are indicated.

*Figure 3*

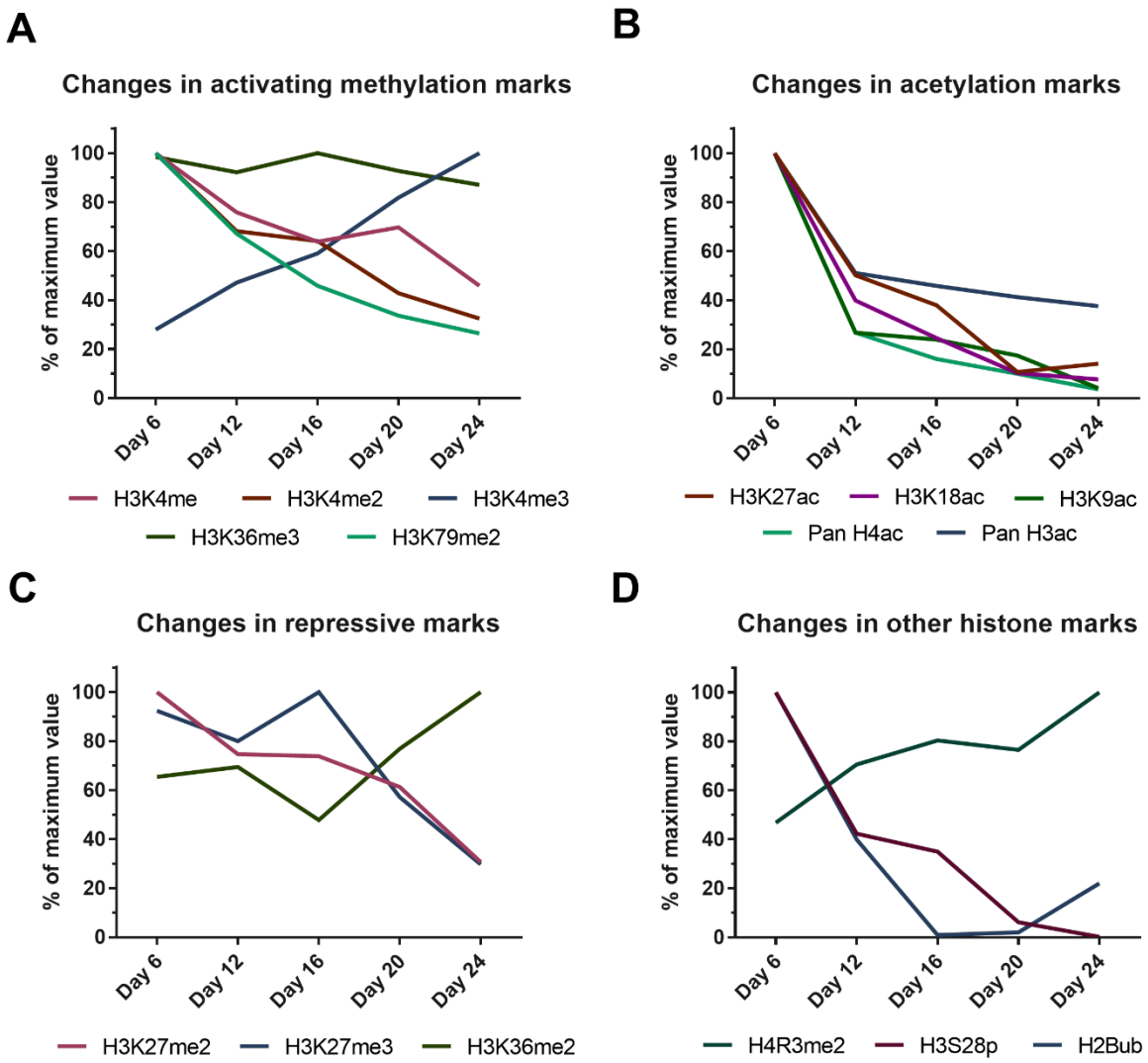


**Figure 3*: Changes in histone modifications over life measured by western blot*.** Changes in histone modifications are depicted as a percentage of the highest signal value detected across life, normalised to total H3. Graphs depict a single time-course experiment. (A) Changes in H3 methylation marks that correspond with active transcription. (B) Changes in acetylation marks that are usually associated with active gene transcription. (C) Changes in H3 methylation marks normally associated with repressed gene transcription. (D) Changes in histone modifications associated with functions beyond transcription.

The level of replicability between the different biological replicates in these experiments was good for the general trends, quantities of a modification moved in the same direction between both replicates. However, it was poor for the relative abundance of each mark, and the degree of change from time-point to time-point. This uncertainty made it difficult to determine the relevance or validity of each histone modification to the ageing process. To address this uncertainty we collaborated with Prof. Axel Imhof (LMU, Munich), who is an expert in the analysis of quantitative changes in histone modifications through mass spectrometry. Using a direct quantitative approach could overcome the

limitations of western blots, including a lack of functional antibodies, balancing issues, and only semi-quantitative data, and allow for profiling of many histone modifications rather than a limited subset. To enhance the ability to detect changes in low abundance histone marks, samples were enriched for histone proteins using an acidic concentration gradient (Govaert *et al.* 2016) combined with the addition of artificially labelled histone fragments corresponding to marks of reference as an internal control.

*Figure 4*

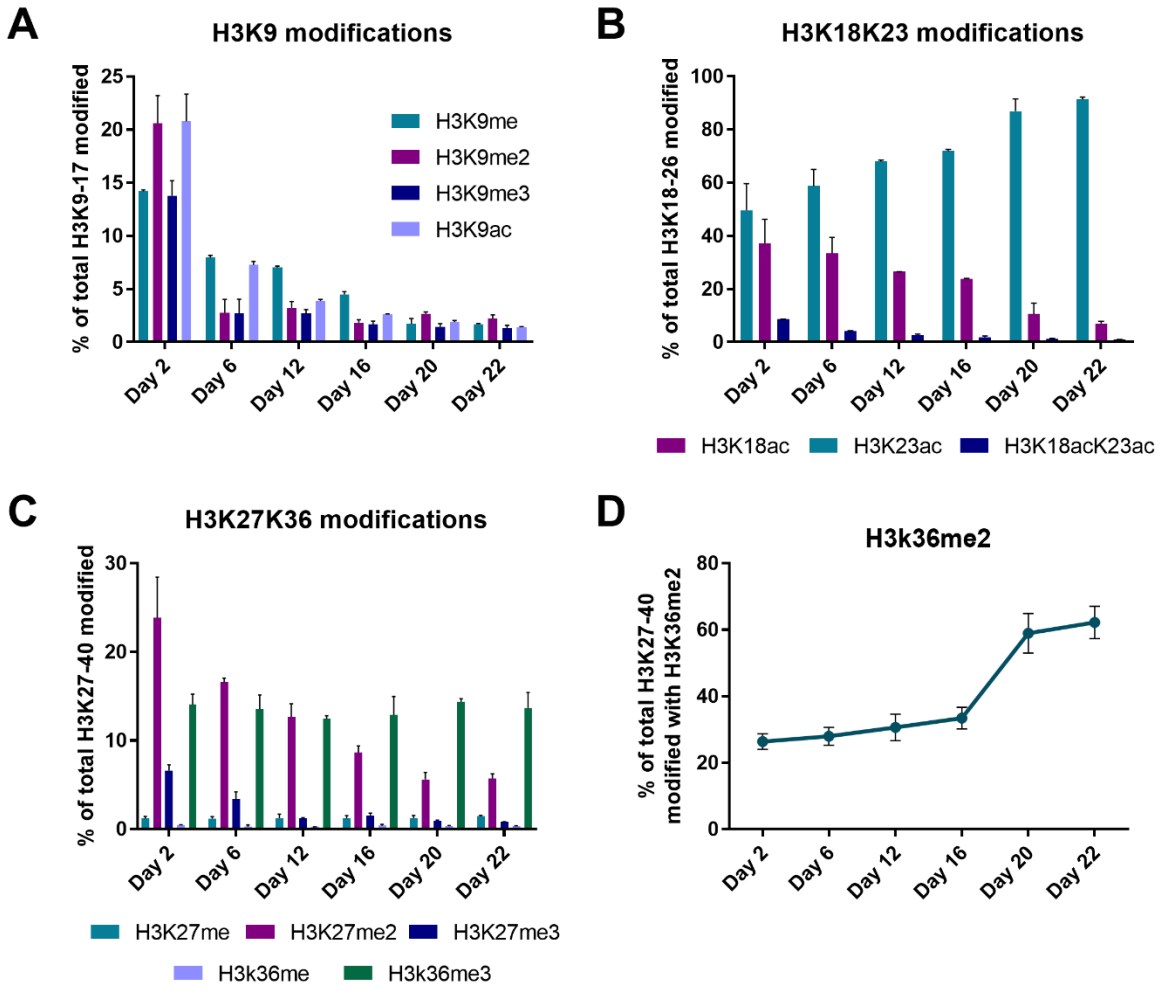


**Figure 4:** ***Mass spectrometry measurement of changes in histone modifications over life.*** Changes in histone modifications over life are shown as the percentage of the total modified histone fragment detected at each time-point. Error bars show the standard deviation of three biological replicates. (A) Changes in H3K9 marks over life. (B) Changes in the acetylation marks of H3K18 and H3K23 and change where both marks were identified in the same digested fragment. (C) Changes in methylation marks on the histone residues H3K27 and H3K36. (D) Changes in the histone modification H3K36me2, graph was drawn separately from (C) to allow for better resolution of small values in (C).

Despite a seemingly high success rate of this method, as published in other contexts, only limited information from these experiments was extracted, namely changes to modifications of H3 and none of the other histone proteins. Additionally, due to the short 5-residue peptide sequence obtained for residues H3-3-8, modifications to H3K4 were not detectable. Similarly, the detection of phosphorylated histones was beyond the capacities of this experiment. This meant that of the 16 identified changes by western blot only 6 marks could be reconfirmed through MS: reduction in H3K9ac, H3K27me2, H3K36me2 and H3K18ac and H3K36me2; and stable levels of H3K36me3 (Table 1 & Figure 4, A-D). The re-identification of H3K27me2 in particular (Figure 4C), suggests that changes in this mark over the lifespan of *C. elegans* could be consequential. In addition to the reconfirmed modifications, we further identified changes in other histone modifications. The methylation marks H3K9me, H3K9me2, and H3K9me3 decreased over life as did the acetylation mark H3K23ac, while H3K26me remained stable. The behaviour of these five marks were generally consistent across both experiment types despite deriving from different experimental samples. This suggested that there was a reasonable chance that other changes seen but not replicated across each experiment could also be used to guide further experimental investigation.

One of the results of greatest interest was the unexpected increase in the levels of ubiquitylated H2B (H2Bub), as seen by western blot, from day 20 to day 24 (Figure 4D). H2Bub is a mark generally associated with transcriptional elongation (gene activation), DNA repair, and is required for the deposition of the transcriptional activators H3K4me2 and H3K4me3 (Johnsen 2012, Shahbazian *et al.* 2005). Its relationship to DNA repair suggested this mark as an interesting candidate, since DNA repair is closely linked to ageing (Lombard *et al.* 2005). That the mark climbed from day 20 to day 24, a change not seen with any other histone mark checked, suggested that H2Bub may be exhibiting changes in abundance consistent with those predicted by AP theory. AP theory suggests there may be a complicated relationship between the time-dependent expression of a gene and its effect on fitness. In a simple model, a gene's expression remains consistent over life but the direct effect on fitness changes as the organism ages. Another potential AP expression pattern is a period of expression early in life with a direct positive effect on fitness followed by a detrimental re-activation later in life, which then has subsequent negative impacts on fitness (Leroi *et al.* 2005). Such a model could also describe the presence of a histone mark capable of modifying global gene expression. The trend of H2Bub reappearing in late-life was re-examined in the long-lived *daf-2* mutant in order to observe its behaviour over a longer period. This was combined with a re-confirmation of the trend in an independent WT experiment where the culture was sampled until day 28, when less than 5% of the total population remains alive. The spike in H2Bub levels was recapitulated in the WT samples and displayed a further increase in abundance in very late-life, between day 24 and day 28 (Figure 5A). Levels of H2Bub rose from 3% to 20% at day 20 and to 26% by day 28. In addition, very similar trends

were observed in the *daf-2* mutant worm strain. The lowest levels of H2Bub were seen at day 20 (11%) and rose precipitously as the worms aged (82% by day 32) (Figure 5A). There was a slight drop again at day 37 but overall the levels of H2Bub remained high in late-life *daf-2* worms.

*Figure 5*

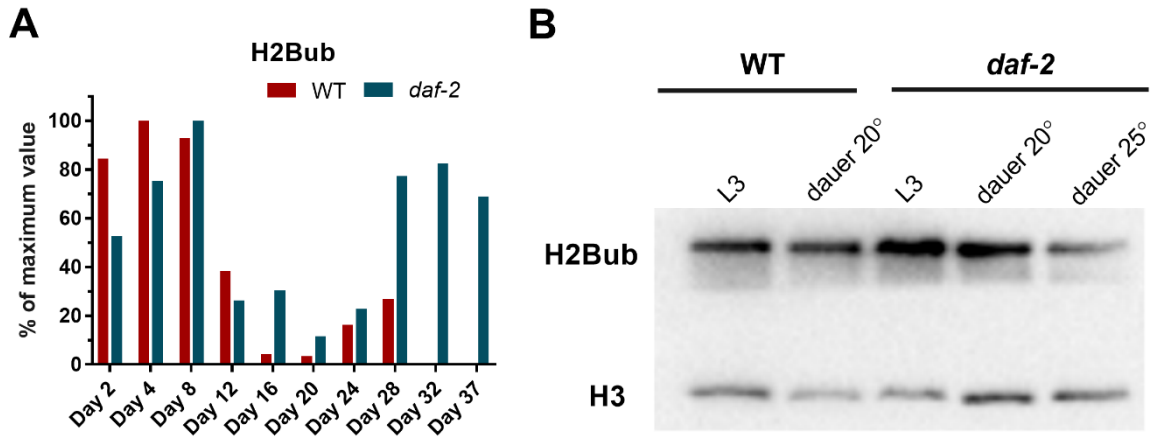


**Figure 5:** ***H2B ubiquitin and its relationship to ageing***. (A) Change in the levels of H2Bub over life, as measured by western blot, in both WT and *daf-2* mutant worms. Values are normalised to total H3 and depicted as relative changes compared to the highest signal detected. Absolute values between the two strains will differ. WT worms were sampled until day 28, after which insufficient live worms remained. Graph depict a single time-course experiment. (B) Representative western blot of H2Bub levels in L3 and dauer worms from both WT and *daf-2* mutants. Dauer state was induced by starvation for both strains (dauer 20°) and additionally via temperature (dauer 25°) in the *daf-2* strain. Histone H3 used as a balancing control.

In these experiments, the dauer stage of the worm's life, a developmental arrest stage where ageing is suspended that occurs in response to harsh environmental conditions including the absence of food, could serve as an indicator to the relevance of H2Bub in ageing. Dauer worms up-regulate pro-survival genes including oxidative damage response genes *sod-3* and *sod-4*, heat shock proteins e.g. *hsp-12.6*, and undergo changes in histone-like genes suggesting a different regulation of chromatin in this pro-survival state (Jones *et al.* 2001). If H2Bub levels were altered in dauer worms this could indicate a pro-survival signal. When compared to their respective larval stages (L3) in both WT and *daf-2* dauer worms the total level of H2Bub is reduced in starvation-induced dauer worms (dauer 20°) (Figure 5B). Interestingly, this reduction was more severe following temperature induced dauer formation in the *daf-2* worms (WT worms cannot be induced into dauer formation in the same manner so there is no comparative sample). A reduction in H2Bub in the dauer stage suggests that lower levels of H2Bub correlate with pro-survival signals and reduced gene transcription. This implies that the accumulation of this mark in old worms could be a detrimental reactivation of H2B ubiquitination, a phenotype predicted for an AP process.

### 2.1.3 Age-associated changes in gene and protein levels

In addition to changes in the epigenetic marks themselves, changes in the abundance of gene transcripts and proteins of epigenetic regulators were investigated. The first approach used label-free quantification of protein abundance with mass spectrometry. Using this system, we submitted a time course of samples (day 6, 12, 16, 20, 24) with two biological replicates for analysis. Initial analysis identified over 1300 different proteins present in the sample for at least one time-point. The analysis was refined to only include proteins identified by a minimum of three peptide fragments and proteins where peptide fragments were identified at all time-points in both replicates. With this filter, the total number of proteins was reduced to 505. To identify proteins relevant to the ageing process the data was subject to further filtering. Proteins were only included where there was a significant change in abundance between at least two contiguous time points (FDR 1%). Further, the identified proteins were divided into four categories of behaviour and proteins were included in these categories only when their behaviour profile was consistent across the two biological replicates. These four behaviour profiles were: 1) Rising, proteins whose abundance increased consistently over time. 2) Falling, proteins whose abundance decreased consistently over time. 3) Peaking, proteins where abundance rises to a peak and then decreases. 4) Trough, proteins where abundance declines to a nadir before rising again. 247 proteins fell into these 4 categories with 37 in rising, 62 in falling, 71 in peak, and 77 in trough (Supplemental Table 1). Proteins that behave in a cyclical fashion over age were excluded by this automatic filtering and proteins of interest with this pattern had to be identified through manual analysis.

To identify gene classes and functional terms enriched in each of the four abundance patterns we performed GO-Term analysis for each pattern using the freely available DAVID software (Huang *et al.* 2009a). Rising genes showed the least number of enriched terms, probably reflecting the limited number of genes in this category. The analysis identified the highest enrichment for the key term “signalling” and, in addition, identified “secreted” and “extracellular region” as an enriched component and key-term respectively (Supplemental Table 2). This could suggest that extra-cellular signalling is an activated process with ageing. Additionally, we identified “regulators of adult lifespan” as a final enriched term. The falling protein category, unsurprisingly, showed an enrichment of terms involving development (embryo, larval and nematode) along with reproduction. Interestingly, there was also an enrichment for nucleotide binding, possibly suggesting decreased metabolic signalling through AMP/ATP in late-life. The term “determination of adult lifespan” was again significant in this group (Supplemental Table 2). The proteins in the rising and falling categories were analysed for effects on lifespan or ageing through an examination of listed phenotypes in Wormbase (WS 258). Surprisingly both lists showed little correlation between whether they extend or shorten life and if they are falling or rising, suggesting no direct correlation with longevity and the patterns of these groups. From an AP

perspective, it is interesting to note that the majority of the falling proteins are lethal when mutated or inhibited (88%) whereas none of the rising proteins are. Peaking proteins were enriched for the terms: signalling, adult lifespan and transthyretin-like domain (Supplemental Table 2). Trough proteins showed an interesting enrichment for metabolic activities with the terms Carbon metabolism, mitochondrion, thiolase, fatty-acid oxidation, metabolic pathways, and Acetyl-CoA acetyltransferase (Supplemental Table 2). This enrichment in metabolic terms appears to suggest that older worms begin to struggle to find food and probably upregulate the breakdown of fat storage for their energy requirements. When combining all proteins from the four classes into one analysis there are some notable occurrences. Firstly, "determination of adult lifespan" becomes a highly enriched term, strongly suggesting that changes in abundance of known lifespan determining proteins are directly influencing the ageing process. This is highlighted by changes to proteins affecting lifespan, which represented 21% of the proteins identified (Table 2). Secondly, new terms were identified, including translation and hydrogen ion transmembrane transporter activity. Changes in the level of protein translation has recently been proposed as a central role in the ageing process (Gonskikh & Polacek 2017) as have alterations in transmembrane transporter activity (Colacurcio & Nixon 2016). While GO-Terms are not definitive, they do point towards several processes that could be highly relevant to ageing.

*Table 2*

| Rising | | | Falling | | | Peaking | | | Trough | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *hsp-16.2* | *sip-1* | *sod-1* | *asb-2* | *lem-2* | *ant-1.1* | *hsp-16.1* | *rpn-1* | *rpl-34* | *eef-2* | *cox-6A* | *dld-1* |
| *rpn-12* | *tbh-1* | *atp-5* | *asg-2* | *pab-1* | *pyk-1* | *hsp-16.48* | *rpn-2* | *eif-2β* | *npa-1* | *tba-7* | *pdha-1* |
| | | | *icl-1* | *pas-5* | *idh-1* | *lmn-1* | *rps-22* | *atp-4* | *pbs-3* | *tpi-1* | *cox-4* |
| | | | *gpd-3* | *pat-6* | *sodh-1* | *rpl-30* | *ttr-5* | *nol-2* | *pyc-1* | *vit-2* | *hsp-110* |
| | | | *hel-1* | *phb-1* | *lbp-9* | | | | *sca-1* | *tkt-1* | *nuo-5* |
| | | | *inf-1* | *rps-5* | *erm-1* | | | | *prdx-2* | | |

**Table 2:** ***Gene names of proteins associated with lifespan function changing over life.*** Genes belonging to proteins identified through MS that fall into the "determination of adult lifespan" GO term category are listed. Gene names are given for each protein identified and grouped based on the pattern of protein accumulation observed.

To delve deeper into these processes the individual proteins in each group were examined with the assumption that the different profiles of protein behaviour could suggest different relevancies to the ageing process. Falling abundance could suggest proteins vital to health as their absence leads to decline, or they could be proteins relevant to development and reproduction that are no longer needed. Rising abundance suggests proteins either aggravating or attempting to counter the ageing process, e.g. cellular stress proteins. Peaking and trough abundance could both indicate proteins with AP behaviour, though trough proteins are more likely to represent uncontrolled re-activation through spurious transcription/translation.

*Figure 6*

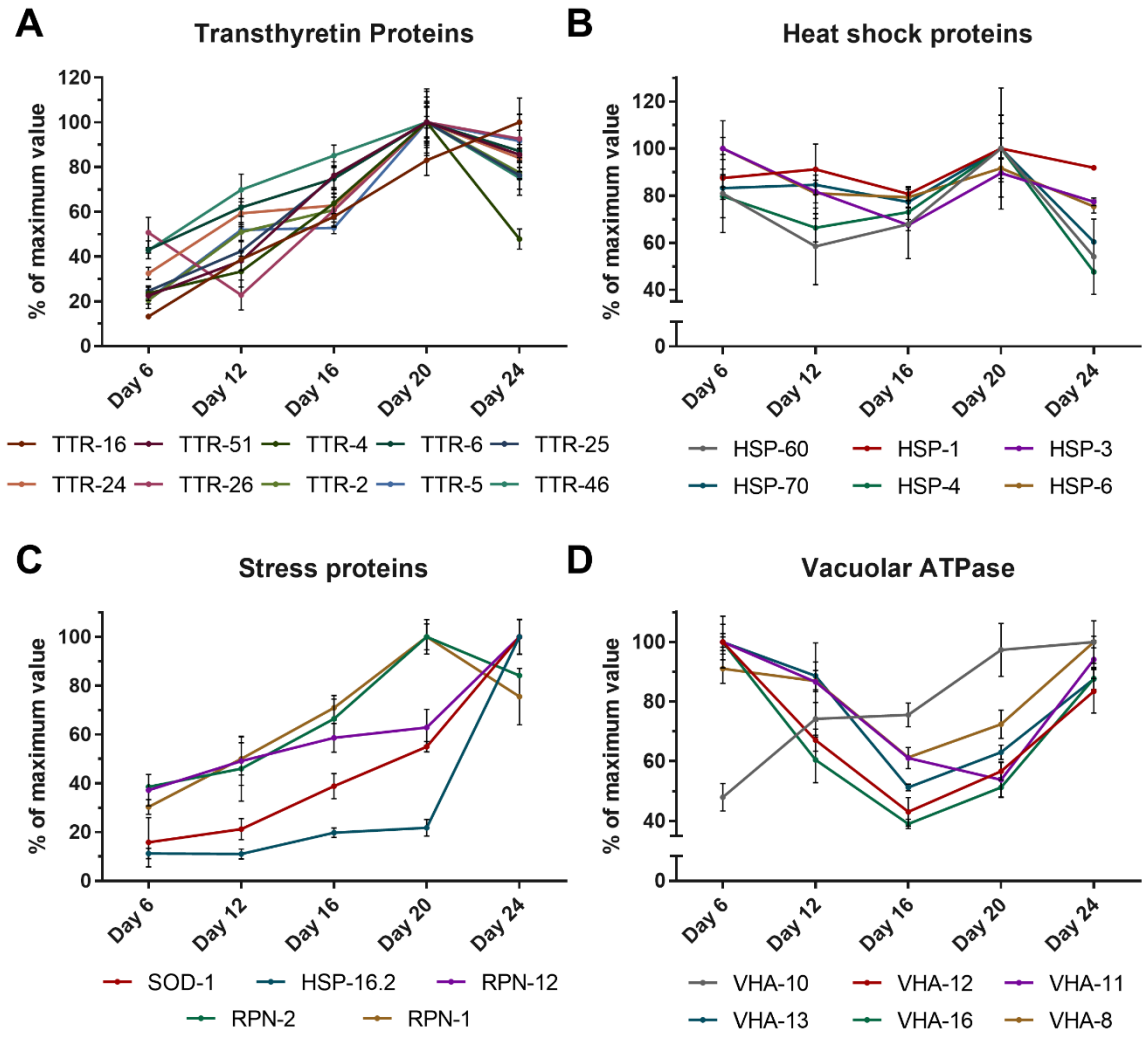


**Figure 6:** ***Patterns of protein classes over life.*** The abundance of protein signatures detected by label free MS are depicted as a percentage of the highest value obtained for each across life. Values are presented as the average of two biological replicates +/- SD. (A) Change in abundance of transthyretin proteins over life. TTR proteins display a peaking profile with the exception of TTR-16, which has a rising pattern. (B) Heat shock proteins display an oscillating pattern of abundance over life. (C) A selection of stress proteins rise in abundance over life, including SOD-1, HSP-16.2 and three UPS proteins RPN-1, -2, and -12. (D) Vacuolar $H^+$ ATPase proteins generally decline in abundance over life but are subsequently reactivated in late-life.

From the examination of protein abundance trends, transthyretin (TTR) proteins were identified in the peaking category and appeared to exhibit an AP like behaviour. Of the 15 TTR proteins identified, 9 exhibited a maximum abundance peak at day 20 followed by a drop in abundance at day 24 (Figure 6A). Only TTR-16 showed a continually rising pattern (Figure 6A). TTR proteins, when functioning correctly, are neuroprotective in mice and humans (Fleming *et al.* 2009). However, amyloidosis associated with TTR proteins is common and directly linked to cardiomyopathy and neuropathy (Galant *et al.* 2017). The pattern observed for the TTR proteins could, therefore, suggest

increased neuronal challenges over ageing, against which the TTR proteins are activated in compensation, possibly with subsequent neurotoxic effects as protein folding efficiency decreases. If this were the case one would expect a commensurate increase in stress-related proteins such as heat-shock proteins (HSPs). However, of the 12 identified HSPs the majority exhibited a modular oscillating abundance pattern (Figure 6B) rather than a consistent increase, as would be expected due to increased folding errors. The only stress proteins behaving in such a fashion were HSP-16.2 and SOD-1 (Figure 6C). Both of these proteins rose consistently in abundance over life with the steepest rise between day 20 and day 24 (80% for HSP-16.2 and 45% for SOD-1). These results, while not general to other stress-related factors, could imply a crucial role for both proteins in ageing. Additionally, the identification of 5 out of 6 detected vacuolar H(+)-ATPase (VHA) proteins grouping in the trough behavioural category is further evidence for proteotoxic stress in late-life (Figure 6D). Though the change in their abundance is not large, it is significant from the lowest to highest levels (FDR <1%). These proteins are necessary to acidify lysosomes required for protein degradation by systems such as autophagy (Mijaljica *et al.* 2011). Their failure is linked to accelerated ageing and neurodegeneration (Colacurcio & Nixon 2016). In addition, 3 of 4 identified proteins involved in the 28s proteasome complex (RPN-1,-2,-12) exhibit increasing abundance with time, suggesting an upregulation of the proteasome in order to deal with increased protein folding errors (Table 2 & Supplemental Table 1). These patterns indicate increased stress in protein management with ageing possibly resulting in a final collapse of the organism when the systems become overwhelmed or fail.

In all, while some insights were gained from the protein MS analysis, we failed to identify any potential epigenetic regulators of longevity, which was the primary goal. To address this issue we continued with an RNA sequencing (RNAseq) analysis of a similar time-course over life with 6 time-points and 3 biological replicates. The sequencing analysis mapped reads to more than 35,000 individual transcripts (~15,000 piRNAs) with the rest assumedly including the 19,000 genes and their alternative splice forms comprising the *C. elegans* genome. Examination of the change in gene variance showed that the number of differentially expressed genes (DEG) (DEseq2, FDR <5%) ranged from less than 1000 genes to nearly 6000 depending on the time-points compared (Figure 7A). Principle component analysis showed that each replicate for the different time-points clustered together, implying clear changes in the transcriptional output between different ages (Figure 7B). Notably, the number of DEGs increased between day 2 and each successive time-point until day 16, subsequently the number of DEGs decreased slightly at day 20 and further at day 22 (Figure 7A). This suggests that similar to the protein data the majority of DEGs change the trajectory of expression (Peaks or troughs) from day 16 or 20 onwards.

*Figure 7*

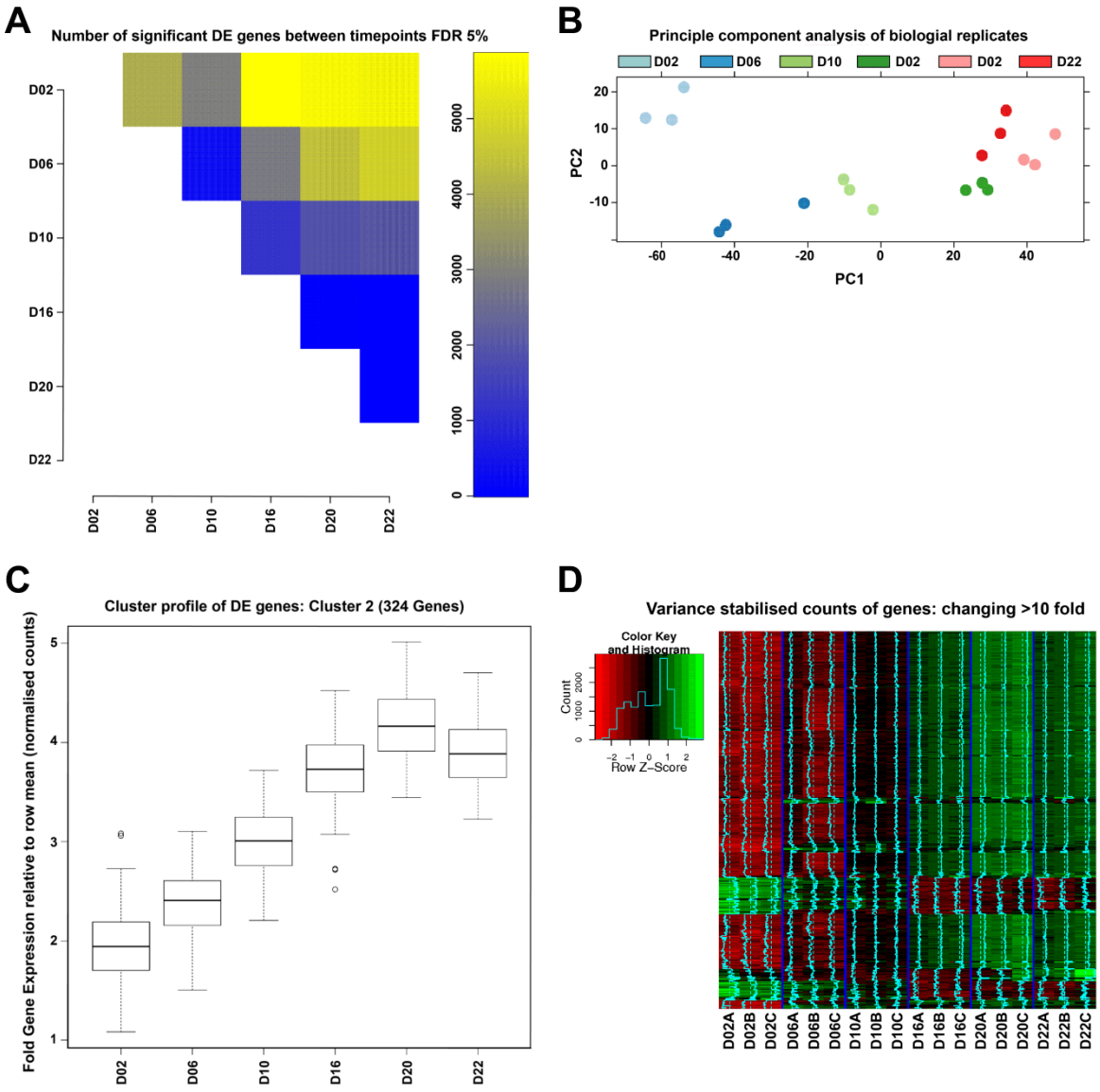


**Figure 7:** ***Analysis of gene expression variability over life in RNAseq data.*** (A) Heatmap of DEGs between each time-point sampled over life. Each time-point sampled is compared to each other time-point and DEGs identified with FDR <1% are counted. Number of DEGs ranges from <500 to ~6000 depending on time-points examined. (B) Principle component analysis of the three biological replicates sampled for RNAseq. Replicates cluster together, with the exception of a single day 6 replicate, demonstrating consistent gene expression across replicates. Samples from day 22 are the most variable. (C) An example of a cluster profile for the 727 highly variable genes. Cluster 2 of 16 is depicted as it contained the greatest number of genes (324). The genes in this cluster represent a peaking expression pattern with a maximum peak of expression at day 20. (D) Heatmap of the expression of all highly variable genes. Heatmap indicates that the majority of genes identified have lower expression in early life, which rise until day 20 before declining slightly.

Clustering analysis of all DEGs supported the pattern trends, identifying the major pattern of expression for DEGs as peaking at day 20 followed by a drop to day 22. Clustering analysis generated 16 expression patterns of which 5 were peaking, representing 47% of all DEGs (Figure 7C). Rising, falling and trough genes represented 11%, 16%, 21% of clusters respectively. Surprisingly the majority of DEGs showed a relatively small degree of total expression variance across lifespan. Only 727 genes displaying a greater than 10 fold change in expression across the whole lifespan, the majority of which were increasing in expression to day 20 (Figure 7D). Using the DAVID platform, a Gene Cluster Analysis looking for common genetic pathways was performed, which identified several highly enriched clusters including: growth and metabolism, protein phosphatase, protein kinase, innate immune response, and major sperm proteins (MSP) as being some of the most represented gene groups (Table 3). Similar to the global expression trends, the patterns of these gene classes were peaking in late-life, with the exception of the MSPs which showed a trough pattern, rising again in late-life. The overrepresentation of both phosphatase and kinase terms, with both having the same upward trend in gene expression, suggests a highly dynamic signalling process occurring during ageing. GO-term analysis of all 727 highly variable genes supported these findings (Supplemental Table 3 & Supplemental Figure 1) and highlighted that the activation of MPS in late age (covered in the pseudopodium GO term) appears to be a standout in the dataset. Examination of the decreasing and trough genes identified further processes of interest with lipid glycosylation, ion transport, transmembrane transport, and g-protein coupled receptors being the most enriched terms (Supplemental Figure 2).

*Table 3*

| Gene Clusters | No. of genes | Enrichment score |
|---|---|---|
| Major Sperm Proteins | 45 | 25.02 |
| Protein Phosphatases | 40 | 15.09 |
| Innate immune response | 9 | 8.9 |
| Protein Kinase | 41 | 7.01 |
| SCP-like extracellular proteins | 6 | 5.84 |
| C-type Lectin | 19 | 4.26 |
| Growth and Metabolism | 173 | 3.21 |
| Not Grouped | 366 | |

**Table 3:** ***Gene cluster analysis of highly variable genes over life.*** Identification of gene classes over-represented in the RNAseq data for genes that display a greater than 10 fold variance in expression. Analysis by DAVID gene cluster algorithm. Each gene class identified is listed along with the number of variable genes belonging to that class in the RNAseq data. Enrichment scores are ranks of the biological significance of gene groups. Cutoff for enrichment was set at >3. Genes not grouped (366) are genes that could not be assigned to any significantly enriched gene class.

*Figure 8*

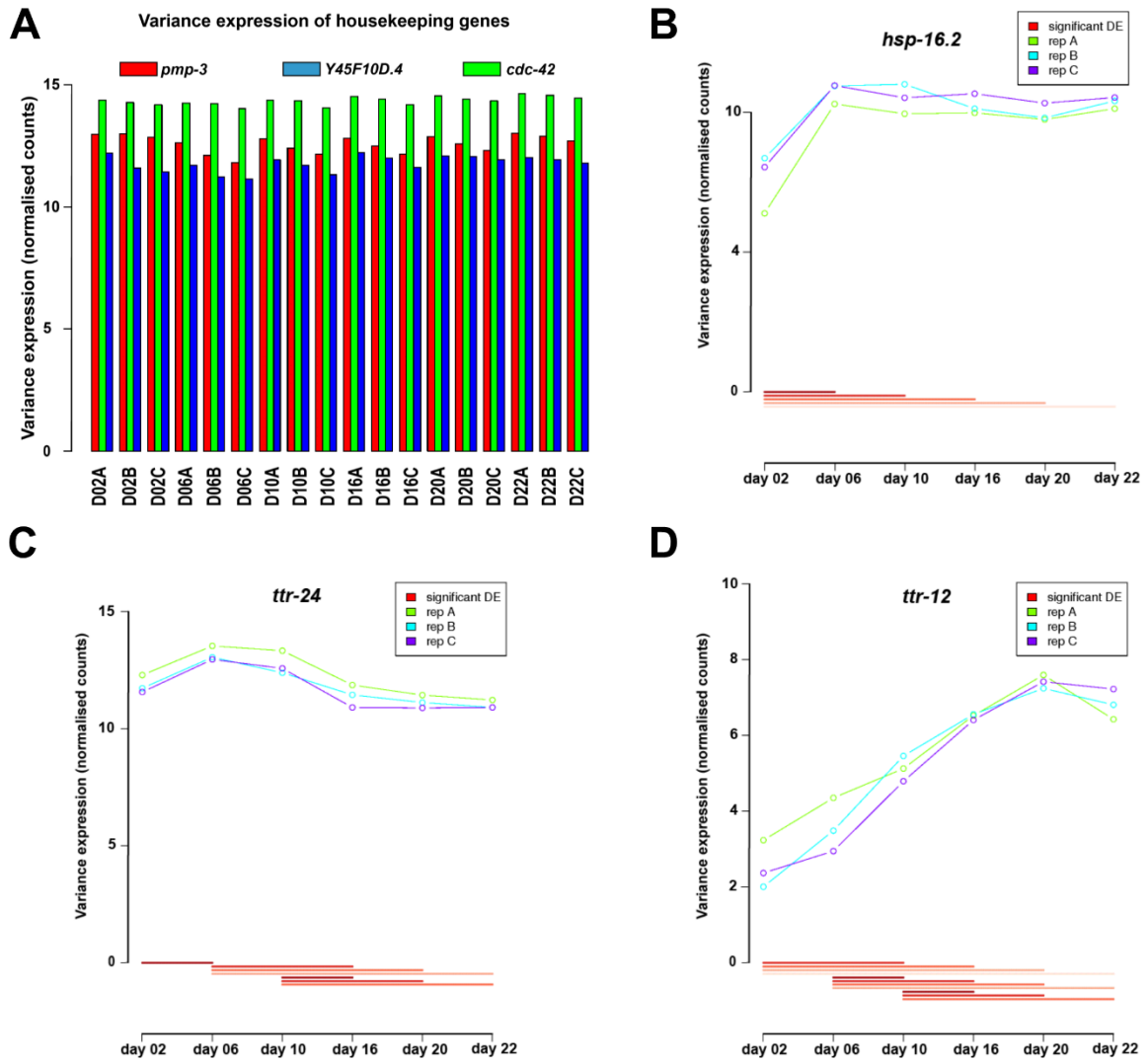


**Figure 8:** ***Comparative validation of RNAseq data.*** Variance stablised expression is equivalent to $Log_2$ fold change in expression. All axes and labels have been redrawn for clarity. Red lines on expression graphs indicate a significant change in expression between different time-points. Comparison between each point is indicated by a different shade of red. (A) Variance stabilized expression of three age-stable housekeeping genes over life, *cdc-42, pmp-3,* and *Y45F10D.4*. (B) Expression of *hsp-16.2* over life displays a sharp peak in expression between day 2 and day 6, ~32 fold, followed by stable expression thereafter. (C) Expression of *ttr-24* over life shows a peaking pattern with maximal expression between day 6 and day 10 followed by a stable reduction at later ages. (D) Expression of *ttr-12* displays a highly variable change in expression between day 2 and day 20 ~60 fold, followed by a declining expression.

Unfortunately, interrogation of the 727 genes with the greatest expression change failed to identify any genes related to chromatin function. As such, a series of qPCR validations of the RNAseq results were performed to ensure the accuracy of the data. Examination of three age-stable housekeeping genes (*cdc-42, pum-3* and *Y45F10D.4*) showed a consistent expression across life,

indicating that the sequencing displayed results consistent with previous studies (Hoogewijs *et al.* 2008) (Figure 8A). In addition, the expression of genes previously identified from the over life protein MS were examined for consistency with the RNAseq data. Diverging from the observed pattern in the MS analysis (Figure 6C), *hsp-16.2* showed a sharp increase in expression from day 2 to day 6 (~32 fold: variance expression is equal to $Log_2$ fold change) but then stayed largely stable across life (Figure 8B). TTR-family genes displayed a significant increase in expression for 26 of the 59 members identified. Additionally, the majority displayed a peak in transcription between days 6 and 10. This is in slight contrast to the observed protein abundance peak at day 20 in the MS data (Figure 6A) and exemplified by the pattern of *ttr-24* (Figure 8C). The gross maximum change in expression for all TTR genes was < 10 fold, with the only exception being *ttr-12* (Figure 8D), which was not previously identified by protein MS.

The lack of any similar ageing gene expression analysis in a truly WT background (Golden & Melov 2007) precluded an easy comparison of our results with those of other studies. The closest study in design criteria, (Lund *et al.* 2002), identified 210 significantly changing genes across lifespan, under a highly strenuous selection criteria, of which only 165 now retain their Wormbase gene-ID. We compared these 165 genes to the 727 identified genes with high expression changes. The expression pattern change for 44 of the 165 genes matched to the RNAseq data and included *hsp-16.2, hsp-70, msp-19 -113, acdh-1,* and *ins-2* amongst others. The level of similarity between these datasets is consistent with that seen in other comparative analyses where the genetic background was different (Golden & Melov 2007). As a further validation, the expression levels of genes of interest were examined with qPCR to determine if their expression patterns were consistent with results already obtained through RNAseq and MS. Using *cdc-42* as a reference the stable expression of the other two housekeeping genes, *pum-3* and *Y45F10D.4*, was confirmed (Figure 9A). Additionally, the expression of *his-11* (encoding H2B) was probed to ensure there were no changes in expression that could have skewed the interpretation of histone mark abundance: if the amount of the core histone changed over life then the relative abundance of any mark would likely change too. Consistent with the MS data (not shown), the level of *his-11* expression remained stable over life (Figure 9A).

The fall in H2Bub levels, previously observed with western blots (Figure 5A), prompted an investigation of the expression of *rfp-1*, whose protein is homologous to human RNF20/40 and responsible for H2B mono-ubiquitination in humans (Zhu *et al.* 2005). Both the qPCR and RNAseq data found no significant change in *rfp-1* expression. Although a trough pattern for *rfp-1* expression was observed, which was reminiscent of that for the H2Bub mark, with a decrease in expression between days 6-20 (Figure 9A). Examining the expression of the coding genes for the proteins identified through MS: *hsp-16.2, sod-1, ttr-12,* and *ttr-24* found that their general expression patterns were consistent with their detected protein abundances (Figure 9B).

*Figure 9*

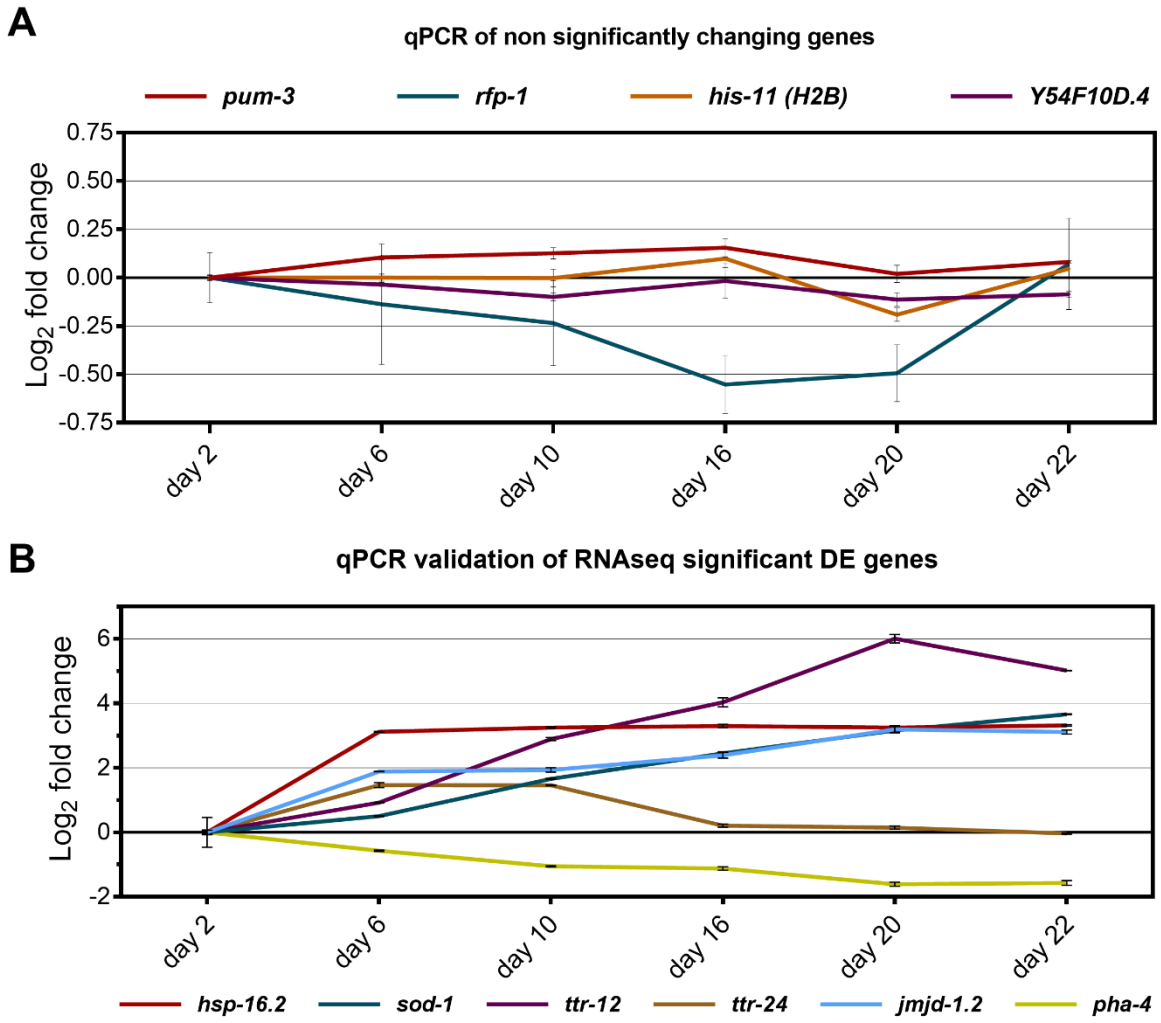


**Figure 9:** ***qPCR validation of RNAseq data.*** Gene expression changes were measured relative to *cdc-42* as age-stable control. $Log_2$ fold change is –ΔCT +/- SD for each time-point. (A) Relative expression of housekeeping genes, *pmp-3* and *Y45F10D.4* or the H2B histone gene *his-11*(H2B) do not change over life. Expression of *rpf-1* showed a non-significant change in expression, but with a pattern reminiscent of H2Bub levels. (B) Validation of a selection of genes previously identified by RNAseq or MS as changing over life. Expression profiles agreed with RNAseq data and the general identified patterns of protein behaviour. All genes depicted show significant changes in expression for all genes depicted $p<0.001$.

As genes influencing epigenetic states had not been identified amongst the genes with the greatest change in expression, a list of all chromatin interacting or influencing proteins was compiled and the expression profiles of their coding genes examined manually. This list contained 1048 genes belonging to 14 gene families know to influence epigenetic states. 322 genes from this list were differentially expressed between at least 2 time-points with the majority belonging to transcription factors (235: 22.4% of all tested genes) (Table 4). As an example, the expression of the FOXA transcription factor *pha-4* decreases over age (Figure 10A). GO-Term analysis of all identified transcription factors found that the majority were heavy metal ion binding (47%) followed by

homeobox-containing (17%). Also, significant changes in RING finger domain genes (50: 4.8% of all tested genes), chromatin remodelers (13: 1.2% of all tested genes) and histone interactors (22: 2.1% of all tested genes) were identified (Table 4).

*Table 4*

| Gene class | No. of DEGs |
|---|---|
| Transcription factors | 235 |
| RING finger encoding genes | 50 |
| Histone methyltransferases (HMTs)[#] | 9 |
| Mi2/NuRD* | 8 |
| Dosage compensation* | 7 |
| Histone demethylases (HDM) [#] | 3 |
| SWI/SNF complex[#] | 3 |
| Histone deacetylases (HDACs) [#] | 3 |
| Histone acetyltransferase (HAT) [#] | 2 |
| SET-1/COMPASS[#] | 1 |
| ISWI/NURF complex[#] | 1 |
| Protein degradation related (histone ubiquitination/sumoylation) | 0 |
| SWR1/SRCAP complex | 0 |
| NuRD/CHD complex | 0 |
| **Total** | **322** |

**Table 4:** ***Identified DEGs from epigenetic gene families.*** Number of identified significantly differentially expressed genes belonging to the 14-chromatin affecting gene families.

Of the 37 direct epigenetic modifiers identified (Supplemental Table 4) the most promising was *jmjd-1.2*, which increased in expression ~8 fold between day 2 and day 20 (Figure 10B). *jmjd-1.2* is a histone demethylase acting primarily to reduce H3K9me2 and H3K27me2 levels (Vandamme *et al.* 2015, Kleine-Kohlbrecher *et al.* 2010). Mutations in this gene lead to increased levels of the marks in protein lysates from mutant worms and RNAi inhibition from the L1 stage shortens worm's lifespan (Ni *et al.* 2012) and mice with higher expression early in life are longer-lived (Kleine-Kohlbrecher *et al.* 2010). The expression profile of *jmjd-1.2* inversely mirrored the level of H3K27me2, in both western blot and histone MS analysis (Figure 3C & Figure 4C). *jmjd-1.2* could, therefore, be an excellent AP candidate as it is known to be detrimental early in life but appears to become increasingly activated during ageing resulting in a likely fundamental global change in the chromatin state. Beyond *jmjd-1.2*, AP like expression patterns for both *swsn-6* and *hda-2* were also observed (Figure 10, C & D). *swsn-6* is a member of the SWI/SNF nucleosome remodelling family which has been associated with several ageing processes including transcription, regulation of chromosomal stability, and DNA repair (Ertl *et al.* 2016). Its expression profile showed a trough pattern dropping ~4 fold over life before rising again between day 20 and day 22 (Figure 10C). The peaking expression pattern of *hda-2*, a histone-deacetylase, suggested a possible role in the general global decrease in acetylation levels observed through western blots and MS (Figure 3B & Figure 10D). Though the total change in expression for *hda-2* over life was relatively low, ~2.5 fold, its published lifespan effects, along with those of *hda-6*, which displayed a similar pattern (Supplemental Table 4), warranted further investigation.

*Figure 10*

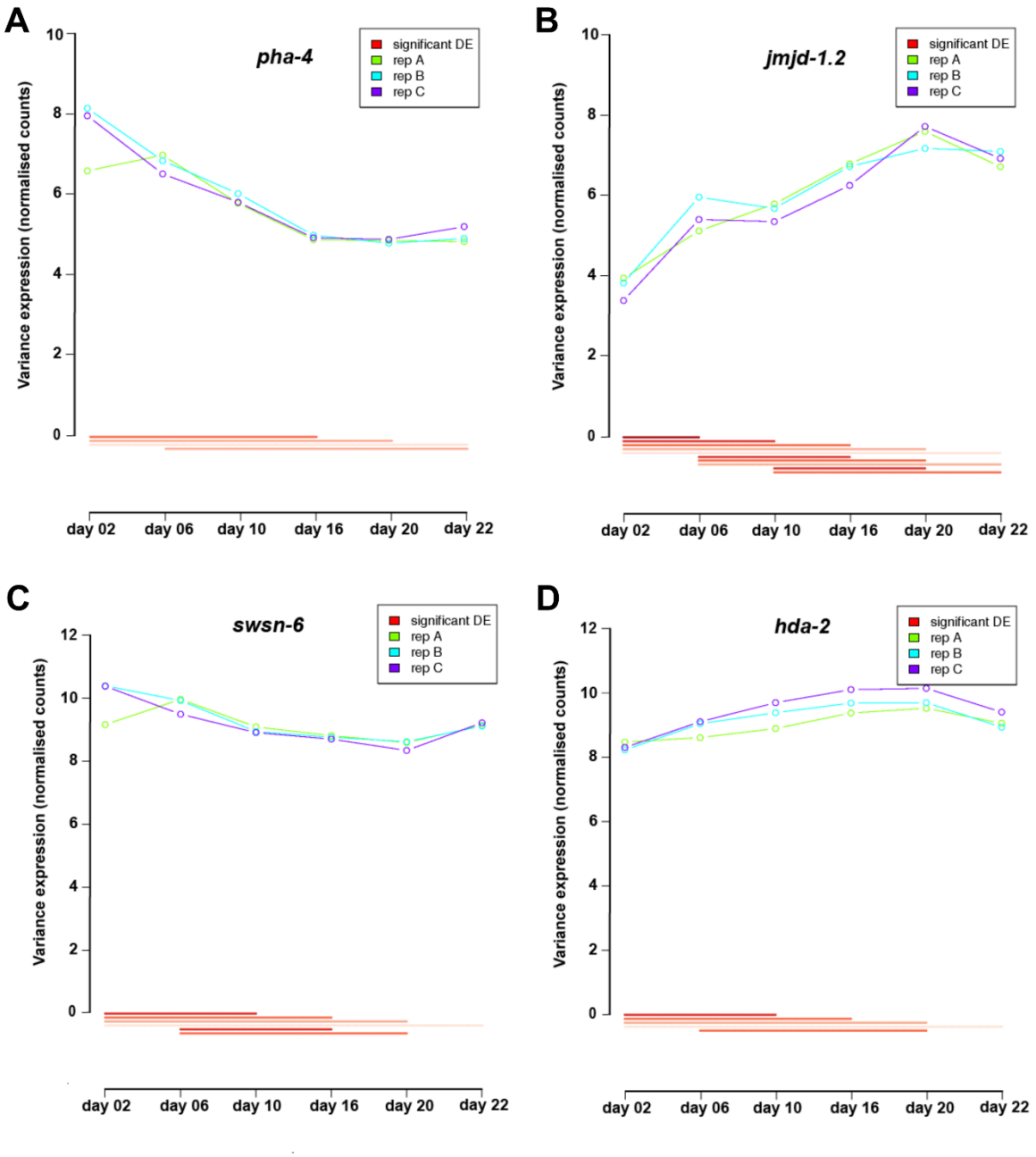


**Figure 10:** ***Potential epigenetic AP genes determined by gene expression profiles.*** Variance stablised expression is equivalent to $Log_2$ fold change in expression. All axes and labels have been redrawn for clarity. Red lines on expression graphs indicate a significant change in expression between different time-points. Comparison between each point is indicated by a different shade of red. (A) An example of a changing expression levels in transcription factors determined by RNAseq. The level of *pha-4* expression decreased by ~4 fold over life with the greatest change between day 2 and day 16. (B) Expression of *jmjd-1.2* shows a peaking pattern over life with ~9 fold change in expression between day 2 and day 20. (C) The SWI/SNF gene member *swsn-6* shows a mild trough expression pattern decreasing ~3.5 fold between day 2 and day 20 before rising again. (D) Expression of the histone deactylase gene *hda-2* shows a mild peaking expression pattern over life rising to a maximum value between days 16 and 20 before declining.

### 2.1.4 RNA interference in target gene expression

Having now used both MS and RNAseq methodologies to identify potential AP genes relevant to ageing, we next examined the effect of knocking down some of the most promising candidates late in life to observe their effects on lifespan. From all combined data, 19 genes were selected for testing. RNAi against these genes was initiated on day 9 in order to maximise the possibility of observing a benefit to lifespan. Included in this gene set were: the TTR genes *ttr-12* and *ttr-24*; *hsp-16.2; rfp-1,* which is required for H2B ubiquitination*;* members of the proteasome complex, *rpn-1, rpn-2* and *rpn-10*; *jmjd-1.2* (checked twice); another H3K27 demethylase *utx-1;* and several other epigenetic modifiers with expression patterns of interest including, *swsn-6, hda-2, hda-6*, *set-11, set-24*, *hat-1* and *taf-1* (Supplemental Table 5). Of the 19 genes investigated, no change in lifespan for 16 of the genes (Figure 11, A, B, & Supplemental Table 5) was observed but a significant reduction in lifespan for the three proteasome genes was detected (Supplemental Table 5). These results suggested that the current strategy for determining epigenetic AP genes was too limited and subject to cause and effect fallacies. As a direct consequence, the approach for identifying epigenetic AP genes was redesigned to focus on identifying AP longevity effects through direct RNAi gene inhibition in late-life.

*Figure 11*

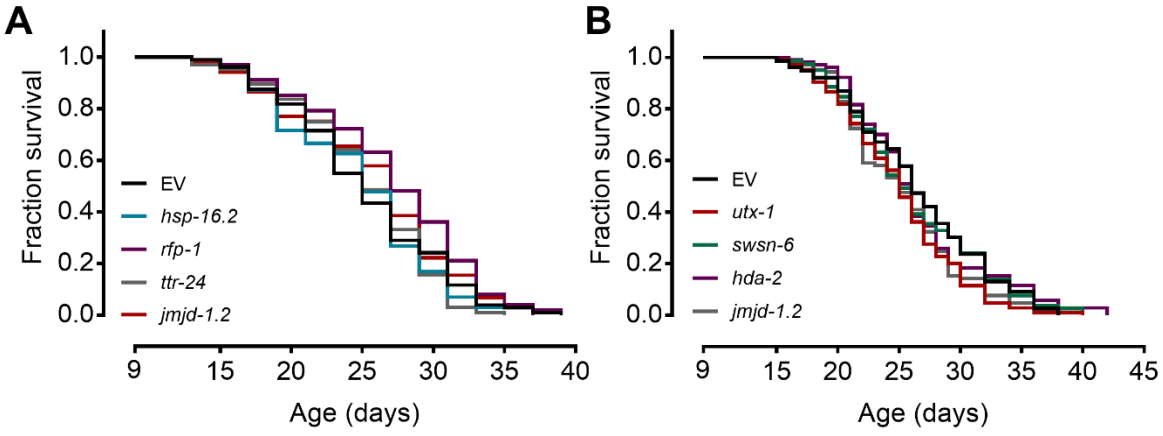


**Figure 11:** ***Potential AP genes identified with a top-down approach do not extend lifespan.*** (A) Day 9 RNAi knockdown of the stress responsive gene *hsp-16.2*, the H2B ubiquitin ligase *rfp-1,* the histone demethylase *jmjd-1.2*, and the transthyretin protein *ttr-24* failed to affect the lifespan of WT worms. (B) Inhibition of the histone demethylases *jmjd-1.2* and *utx-1*, the histone deacetylase *hda-2*, and the chromatin remodeler *swsn-6* had no effect on the lifespan of WT worms from day 9. Lifespan statistics are presented in Supplementary Table 5.

## 2.2 Experimental approach 2

### 2.2.1 RNAi screen design

The lack of success with the bottom-up approach in identifying AP genes prompted a change in the experimental strategy. As the main goal was to identify epigenetic genes with an effect on late-life longevity, it was decided to look for this phenotype directly through an RNAi screen rather than through changes in expression patterns. The advantage to this top-down approach was in gaining a direct readout on whether a gene affected the ageing process, with the disadvantage being that the number of genes investigated would be significantly restricted. Due to the focus on epigenetics and ageing, we decided to use a library of gene-regulatory factors involved in chromatin or transcriptional regulation. This library was obtained through a collaboration with the Tursun group at the Max Delbrück Center for Molecular Medicine in Berlin. The library contained 731 genes that are implicated or known to be involved in either gene or chromatin regulation. The target specificity of each RNAi in the library was confirmed through sequencing and new RNAi constructs created in the event of non-specific targeting or other errors. This library was supplemented with 54 additional genes based on previous data that were not part of the original library (Supplemental table 6). Of note and relevance for later, this included the transcription factor *pha-4*. One of the major issues with RNAi libraries is that they are regularly contaminated with other bacteria, many of which grow without problem in the presence of high ampicillin concentrations (to which the HT115 *E. coli* expressing the dsRNA of interest are resistant). To remove contamination as an issue, all bacterial clones in the library were serially passaged four times under tetracycline and ampicillin selection and a final single colony was picked and sequenced. With this method, the total contamination in the library was reduced from ~35% to effectively 0%.

Examining the effects of 800 RNAi knockdowns simultaneously with a measure of confidence required the observation of at least 3-4 replicates per gene with at least 20 worms per sample. This gave a total of ~3200 individual samples needing at least 100,000 worms as a starting culture. To work with such large numbers a system using 24 well agar plates was devised with 4 replicates of 6 different RNAi containing bacteria per plate, to which worms would be added in the post-reproductive period. To generate the strongest AP effect possible, the ideal time-point for RNAi knockdown was determined as day 9. This provided a balance between efficient gene inhibition, while worms still fed on the bacteria, and minimising the possibility of negative effects from early inhibition. Results from Thomas Wilhelm showed efficient gene knockdown of GFP with 3 days of feeding worms with *gfp* RNAi. This

effect could be observed up until day 10 after which complete knockdown of the protein took more time (Wilhelm 2017). Unexpectedly, there are still very rare, late reproductive events in *C. elegans* with occasional offspring seen still appearing at day 7-8 despite reproduction typically being complete by day 5. As such, a conservative starting point of day 9 represented the best balance for AP criteria. The identification of genes with lifespan-extending effects upon RNAi inhibition required that the number of worms in all wells was consistent (+/-1) to prevent false positives in scoring. Given the number of worms required for all plates, manual passaging was impractical for this purpose. This issue was overcome by designing a specialised protocol for the COPAS Biosorter s (a large particle Fluorescence-activated cell sorting [FACS] machine) to sort aged worm. To reduce the stress on worms subjected to sorting and to reduce the occurrence of duplicate events (these events were not sorted) the flow rate of the worms entering the acquisition chamber was reduced to less than 5 events/sec. Additionally, using the 1000 µm sorting chamber (FOCA) prevented adult worms becoming clogged in the machine. This, combined with the need to swap between plates and a sorting efficiency of 90%, resulted in a total estimated RNAi screen sorting time of 5hrs. Worms are normally sorted in M9 rather than in food, as aggregated bacterial clumps can be interpreted as signals similar to larvae or eggs. Concerned that 5hrs of continuous sorting without food would induce starvation and create variability in the response to RNAi, worms were therefore sorted in the presence of food (OD 1.5). This compromise resulted in an inability to reliably exclude events containing larvae or eggs without significantly increasing sorting time and the number of total worms needed. Sort efficiency in M9 was ~90% for a pure sort of adult worms but dropped to ~30% in food culture, increasing the estimated RNAi screen sorting time to 16hrs and requiring ~300,000 worms. Therefore, events below the threshold that would include food clumps, L1-L2 larvae and eggs were ignored. This allowed for the efficient sorting of adult worms with an error rate of 1/500 but required a *C. elegans* culture devoid of larvae or eggs to ensure reliable subsequent analysis.

### 2.2.2 Redesign of *C. elegans* large-scale liquid culture

One of the primary concerns for an RNAi screening approach was that the method of age-synchronous liquid culture employed to this point was simply not suitable for RNAi screening analysis. In the previous approach, WT worms for RNAi assays had to be grown manually on plates as the worms displayed a rapid die off and limited food uptake following transfer from the high rpm liquid culture to agar plates. Regular contamination and removal of dead worms using sucrose preparations caused stress on older worms and prohibited easy long-term culture. The high speed of the liquid culture was of particular concern as the shear forces involved often broke older, damaged worms apart. There was also a significant upregulation of mechanical stimulus genes in the early time-points of the RNA-seq

data (data not shown). There were concerns about the use of FuDR, as it is reported to affect lifespan (Aitlhadj & Stürzenbaum 2010) and could mask AP signals working through the germline (Ni *et al.* 2012). This meant that fully reproductively-viable worms would be cultured, which required a culture absent of any offspring. Finally, the RNAi screening protocol was limited to a single feeding of RNAi expressing bacteria to the worms, raising concerns that the strength or duration of the knockdown would be insufficient to allow for a robust detection of AP genes later in life.

To address these concerns a new liquid culture protocol that removed all of the outlined issues as significant factors was devised. First, the concern of ineffective RNAi knockdown was addressed by testing the efficiency of RNAi knockdown in *rrf-3* worms that are hypersensitive to RNAi (Simmer *et al.* 2002). For this strain, a single feeding duration of 3 days, starting at day 10 was found to be sufficient to ensure an efficient knockdown for the remainder of life (Wilhelm 2017). Additionally, the health and vitality of *rrf-3* worms appeared more robust than their WT counterparts, they were more motile after liquid culture and exhibited a slightly longer lifespan. As such, we used *rrf-3* mutant worms for the majority of subsequent experiments.

Second, by allowing the worms to reproduce without inhibition the requirement for FuDR was removed. Maintaining an age-synchronous population of *C. elegans* is not trivial due to their fast reproductive life cycle (3 days) and their ability to self-fertilise, with each worm producing 100-300 offspring. Any age-synchronous culture where even the smallest number of offspring are not removed (leak) quickly becomes dominated by second and third worm generations, thereby prohibiting experimental analysis. This obstacle has proven to be one of the most significant in *C. elegans* ageing research with the use of sterile strains, anti-reproduction drugs, or manual separation of parents and offspring required to maintain synchronisation. We developed a novel, simple method to overcome this limitation. When worms are allowed to settle in liquid it takes significantly longer for the majority of the juvenile worms to reach the bottom. From this observation repeated, time-limited, gravity sedimentation of mixed cultures of both adult and larval worms were tested. It was found that the length of time it takes an adult worm to sink is dependent on its age, with older worms sinking significantly quicker than younger worms (~10min for day 0-1 worms and ~4 min for day 7+ worms in a 50 ml Falcon). This results in a crude but effective way of separating parents from offspring. It was discovered that as the worms sink they create downdrafts in the liquid dragging larval worms with them, reducing the efficiency of the separation. Thus, to reduce the strength of the downdrafts, a wide bore container (50ml falcon tube) was used and the amount of sample sedimented was limited to < 1 worm / µl. Efficient removal of offspring required 6 to 10 rounds of sedimentation (depending on sample density), which by visual examination showed a high degree of success in larval removal with less than 1 larval worm per 1000 adults remaining post sedimentation.

Third, the need for sucrose cleaning of the worm samples was removed by creating stringent sterile culture protocols. Additionally, the feeding bacteria (HT115 EV) was liberated from any co-dependent bacteria not removed through standard colony selection via the selection of individual *E. coli* bacterial cells under a microscopic. These changes resulted in the majority of subsequent worm cultures remaining free from contamination for an indefinite duration, obviating the need for sucrose to remove contamination debris. Furthermore, it implied that the primary source of previous contamination was from the feeding bacteria. Along with debris, sucrose preparations also remove dead worms from solution. Dead worms cannot maintain their osmotic balance, absorb the sugar, become denser and sink into the sucrose layer at the bottom. To obtain pure samples of live worms at different time-points an alternative solution was required. We reasoned that dead worms might exhibit a naturally different density to living worms and could be separated using a non-toxic, non-osmotic density gradient such as Percoll. Through testing of different concentrations of Percoll it was discovered that a range of 34-40% was ideal for gravity separation of live and dead worms depending on the age of the culture. The specific gravity of worms is known to vary depending on age dropping 3-4 days after hatching and then rising until day 10 thereafter (Findeis *et al.* 1983). This trend matched our observations for the ideal Percoll percentage and the rate of natural sedimentation. By very careful layering of a worm pellet on top of a Percoll mixture, live worms will gradually settle to the bottom leaving egg clumps, dead worms and bagging worms trapped in the upper layers. The tendency of larval worms to hatch *in utero* (bagging), devouring the parent before eventually breaking free of the cuticle in the L3-L4 stage and then proceeding to lay eggs of their own, is particularly problematic for age-synchronous culture. This issue is not addressed by any current methodologies not involving sterilisation. The unexpected finding that Percoll facilitated the efficient removal of egg clumps and bagging worms proved crucial in ensuring a minimal amount of offspring leakage.

Finally, a switch from culturing worms in Erlenmeyer flasks at high rotation speeds, to a system using tissue culture flasks with gentle rotation (40-55 rpm) was implemented. One problem with liquid culture is that the bacteria in culture quickly use up all the available oxygen, suffocating the worms, unless subject to vigorous aeration. To overcome this issue, tissue culture flasks with a wide surface to volume ratio (75 $cm^2$ with 50 ml, 175 $cm^2$ with 100 ml, and 300 $cm^2$ with 200 ml) were employed, resulting in sufficient aeration at very low RPMs (40, 48, and 55 rpm respectively). Despite the large surface area available for oxygen diffusion in these flasks the requirement for aeration could not be completely removed. Due to the amount of offspring produced in the first two days of the worms' reproductive lives, and subsequent rapid food depletion, culture densities were limited to 0.5 worms/µl for the first 3 days and increased to 1 worm/µl thereafter. As with the previous liquid culture method, to avert accidental DR induction, each culture was started with a high food concentration (OD 3) and prevented from dropping below OD 1.5

*Figure 12*

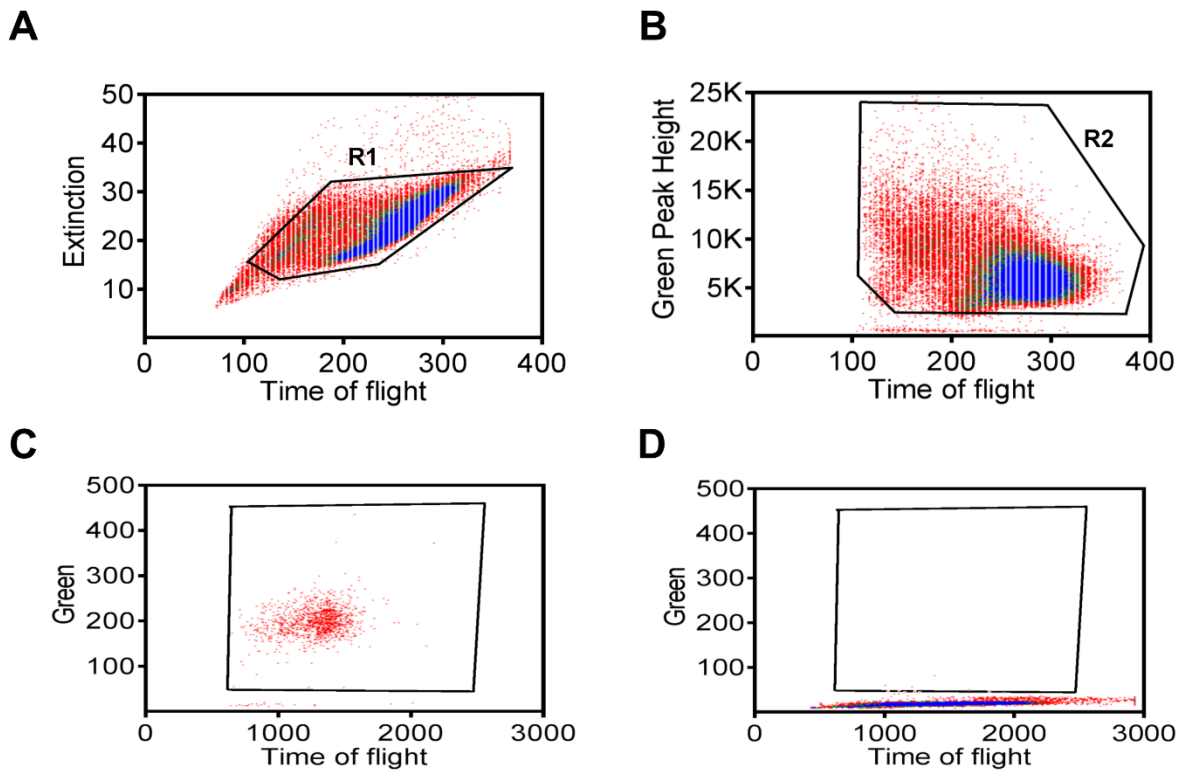


**Figure 11:** ***Validation of liquid culture used for RNAi screening and lifespans*****.** (A-D) Scatter plot of worm populations measured by the Biosorter. (A) A population of larval *myo-3p::GFP* mutants separated based on their opacity (extinction) and size (time of flight) were gated as R1. (B) The R1 population of worms show low total green fluorescence and are sorted from R2 based on their peak green intensity. (C) A control gate for young adult worms expressing GFP, which show strong green fluorescence. Any larvae not removed by sedimentation would appear here after two days. (D) Non-GFP worms synchronized with a liquid culture sedimentation protocol, spiked with >40,000 sorted green larvae every day for six days. By day 8, there are virtually no green adult worms present in the culture (>99.9% removal of larvae).

Validation of this novel method of age-synchronous liquid culture required a method to determine the level of offspring contamination present as the culture approached day 9. If at older ages there was a mix of different generations, from a failure to remove larvae or bagging worms, this would prevent accurate analysis and prevent a hands-off screening approach. As such, a simple test for the efficiency of the synchronisation protocol was devised. Worms highly expressing GFP (*myo-3::GFP*) were FACS sorted to obtain L1-L2 GFP expressing offspring using the Biosorter (Figure 12, A & B). Larvae were sorted to ensure that all expressed GFP as this transgene is occasionally silenced in offspring through RNAe (Leopold *et al.* 2015). More than 40,000 of these larvae were added twice daily to a non-GFP expressing age-synchronous liquid culture of 50,000 worms. The now spiked age-synchronous culture was then immediately subjected to the sedimentation protocol outlined above. This procedure was repeated 16 times over 8 days and the final population was examined on day 8 for

GFP expressing adults. Had any of the GFP larvae not been removed from the culture they would have subsequently developed into highly GFP expressing adult worms within 2-3 days. The entire age-synchronous culture was sorted and examined for GFP expression at day 8, along with day 0 – day 2 adult GFP worms as a control. The GFP adults were readily detectable in the control sample (Figure 12C) but only four worms minimally expressing GFP were detected in the sedimented age-synchronous culture (Figure 12D). In total this represented a >99.99% (4 out of ~640,000) removal of offspring from the culture.

### 2.2.3 RNAi screening results

Using the now validated liquid culture system, we proceeded to screen for AP genes starting with day 9 *rrf-3* worms. Worms were sorted onto the respective RNAi bacteria and observed periodically to determine when the majority of control worms appeared dead (day 32). A scoring system based on that of Curran and Ruvkun (Curran & Ruvkun 2007) was used whereby each well was flushed with M9 buffer resulting in visible movement for live worms, which were then counted and compared to the control conditions. Candidate longevity genes were identified by a significant increase ($p<0.01$) in the mean percentage of live worms compared to control samples. Of note, approximately 10% of the total wells were unable to be counted due to fungal contamination. From this analysis, a total of 36 candidate longevity genes (4.6% of the total library) were identified (Table 5), of which 31 have not been linked to lifespan previously (Figure 13 & Table 5). The list of observed phenotypes for each gene identified were examined using WormBase (WS258) to identify phenotypes of early life detrimental effects following mutation or knockdown, indicating good candidate AP longevity genes. Of the 31 novel longevity genes, 19 had described phenotypes associated with lethality, reproductive defects, developmental defects, small size and reduced health (Table 5). Apart from this trend, the only other observed enrichment within the identified genes was for the RNAi pathway. Six of the AP gene candidates identified are involved in aspects of RNA silencing: *eri-1, ego-1, drh-3, hrde-1, nrde-1,* and *rde-1*, suggesting a role for this pathway in the AP modulation of lifespan (Table 5).

*Table 5*

| RNAi Treatment | Mean % Worms Alive | n | p-Value | RNAi Treatment | Mean % Worms Alive | n | p-Value |
|---|---|---|---|---|---|---|---|
| **EV** | 11.98% | 93/120 | | ***plk-1*** [b] | 36.47% | 5/8 | 0.0001 |
| **GFP** | 16.22% | 86/96 | 0.0123 | ***# ego-1*** [b] | 36.05% | 4/4 | 0.0001 |
| ***age-1*** [a] | 52.2% | 3/4 | 0.0001 | ***cdl-1*** [b] | 35.84% | 4/4 | 0.0001 |
| ***epc-1*** [a, b] | 48.45% | 4/4 | 0.0001 | ***zfp-1*** [b] | 35.26% | 6/8 | 0.0001 |
| ***# pha-4*** [b] | 48.18% | 4/4 | 0.0001 | ***prg-2*** [b] | 35.00% | 2/4 | 0.0022 |
| ***# dot-1.1*** [b] | 47.96% | 4/4 | 0.0001 | ***ZC155.2*** | 34.88% | 4/4 | 0.0001 |
| ***akt-1*** [a] | 46.64% | 4/4 | 0.0001 | ***B0019.2*** | 34.07% | 5/8 | 0.0001 |
| ***glct-3*** | 46.48% | 3/4 | 0.0001 | ***hrde-1*** | 33.83% | 3/4 | 0.0002 |
| ***dcp-66*** [b] | 41.76% | 4/4 | 0.0001 | ***F40E12.2*** | 32.29% | 3/4 | 0.0007 |
| ***ntl-9*** | 40.00% | 2/4 | 0.0001 | ***msp-56*** | 31.94% | 4/4 | 0.0001 |
| ***T02C1.1*** | 39.26% | 3/4 | 0.0001 | ***nrde-1*** | 31.65% | 4/4 | 0.0001 |
| ***drh-3*** [b] | 38.57% | 3/4 | 0.0001 | ***mec-3*** | 31.47% | 3/4 | 0.0013 |
| ***# eri-1*** [b] | 38.15% | 3/4 | 0.0001 | ***sid-2*** [a] | 31.07% | 4/4 | 0.0002 |
| ***F54F2.9*** [b] | 38.03% | 4/4 | 0.0001 | ***# cyk-3*** [b] | 29.88% | 3/4 | 0.0048 |
| ***C17E4.6*** [b] | 37.92% | 4/4 | 0.0001 | ***spt-4*** [a] | 28.80% | 4/4 | 0.0015 |
| ***gna-2*** [b] | 37.90% | 4/4 | 0.0001 | ***dro-1*** [b] | 28.66% | 4/4 | 0.0018 |
| ***hda-6*** | 37.56% | 3/4 | 0.0001 | ***msp-76*** | 28.60% | 4/4 | 0.0019 |
| ***lin-29*** [b] | 36.67% | 3/4 | 0.0001 | ***taf-4*** [b] | 28.36% | 4/4 | 0.0024 |
| ***ruvb-1*** [b] | 36.56% | 4/4 | 0.0001 | ***let-716*** [b] | 27.74% | 4/4 | 0.0041 |

**Table 5:** ***Candidate genes identified from the RNAi screen.*** Genes whose RNAi mediated knockdown, at day 9, resulted in more alive *rrf-3* worms compared to control. RNAi was initiated at day 9. n: number of counted wells/number of total wells. Wells were not counted if contamination or offspring were present. EV is empty vector control. GFP was used as a non-targeting control. #: indicates gene knockdowns depicted in Fig. 14. a: indicates genes with known pro-longevity effects. b: indicates likely AP genes with negative fitness effects when inhibited/mutated early in life.

*Figure 13*

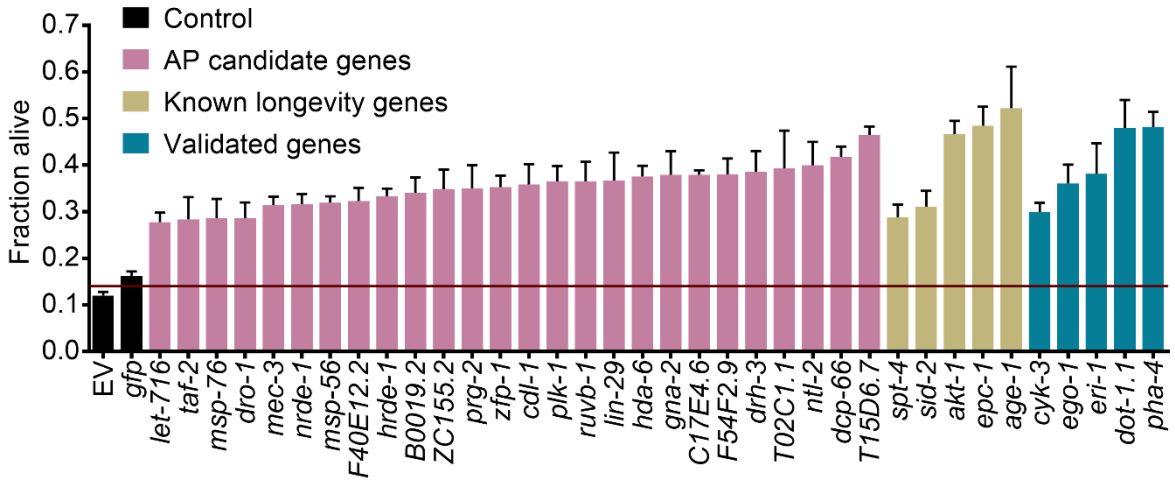


**Figure 11:** ***AP RNAi screening identifies both novel and previously known longevity genes.*** Potential longevity genes identified from the AP RNAi screen. The percentage of *rrf-3* worms alive at day 30 is shown. Controls: Empty vector (EV) and non-targeting GFP (black), novel longevity genes (purple), known longevity genes (khaki) and our top five candidate genes (blue). Red line indicates the threshold for consideration as a longevity candidate. Data represents the average of 4 replicates +/- the SEM. Statistics are presented in Table 5.

The identification of genes already known to extend lifespan such as *age-1* and *akt-1*, both functioning in the IIS pathway (Vanfleteren & Braeckman 1999), strongly suggested that our screening method was capable of identifying genuine longevity genes. As a further validation, the five most interesting genes from the list of AP candidates were selected for standard lifespan assays. These genes included two members of the RNAi pathway *eri-1* and *ego-1*, the histone demethylase *dot-1.1*, the transcription factor *pha-4*, and the ubiquitin hydrolase *cyk-3*. Standard lifespan assays involve the transfer of live worms to fresh RNAi containing bacteria every 2 days combined with live dead scoring. As treatments begin beyond the first day of adulthood the effects on lifespan were only measured from the first day of treatment (typically day 9). Results are then expressed as a change in the average lifespan for the treatment duration or mean treated lifespan (MTL) rather than as a percentage of the entire worm lifespan. We determined significance between two lifespan curves through use of the Log-Rank (Mantel Cox) test (Mantel 1966) that compares estimates of the hazard functions of two groups at each observed event time. This analysis is standard for survival comparisons (Lionaki & Tavernarakis 2013a). Lifespan assays for our five candidate AP genes showed a significant ($p<0.01$) extension of MTL following day 9 knockdown for all five genes (Figure 14). The degree of lifespan extension did vary across different biological replicates (*ego-1*: 12-26%, *eri-1*: 11-27%, *dot-1.1*: 10-19%, *cyk-3*: 15%, *and pha-4*: 17-33%) (Supplemental Table 7) and appeared to correlate with general worm health at the start of the experiment. In experiments containing more dead worms than average following Percoll treatment, there was, generally, a corresponding reduction in the percentage of MTL increase for all genes tested. This suggests that the health of the worms in liquid culture is vital for obtaining a robust late-life longevity increase.

*Figure 14*

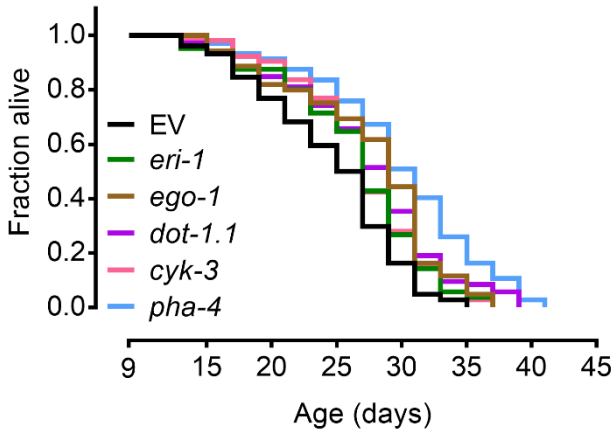


**Figure 14:** ***A post-reproductive extension of longevity by five AP genes.*** Day 9 RNAi against our top five candidate genes: *eri-1*, *dot-1.1*, *cyk-3*, *ego-1*, and *pha-4* extends mean treated lifespan (MTL) for all treatments in *rrf-3* mutant worms. Days represent days post first egg lays. Lifespan statistics are presented in Supplemental Table 7.

### 2.2.4 Investigating the pleiotropic nature of *pha-4*

The forkhead box A (FOXA) transcription factor *pha-4* is essential to development, functioning in organ specification and acting particularly as a master regulator of pharynx development during embryogenesis (Horner *et al.* 1998, Mango *et al.* 1994). CHIP sequencing of PHA-4 during embryogenesis and under starvation conditions in the L1 stage identified over 4000 unique binding sites for the transcription factor, with limited overlap in bound genes between the two conditions tested. Genes identified in the starvation condition included those regulating autophagy (Zhong *et al.* 2010). In addition, mutation or inhibition of *pha-4* in the embryonic stage is lethal due to a failure to develop a pharynx (Kiefer *et al.* 2007) and L1 RNAi inhibition also shortens lifespan and reduces reproductive capacity (Panowski *et al.* 2007, Sheaffer *et al.* 2008). The crucial nature of *pha-4* to early life fitness and longevity, combined with its ability to differentially regulate a large number of genes in different conditions, suggested that *pha-4* could be a model AP gene, switching its role from beneficial to detrimental later in life. As such, of the five AP genes validated from the screening results, the AP nature of *pha-4* was selected as the most promising for further investigation.

As *pha-4* is a transcription factor, it must act by modulating of the expression of other genes in order to exert an AP effect. Examination of the major transcriptional pathways modulated by *pha-4* identified autophagy as an interesting, potential driver behind *pha-4's* AP mechanics. *pha-4* is specifically required for the induction of autophagy following caloric restriction or removal of the germline (Panowski *et al.* 2007) and has been demonstrated to regulate the expression of the key autophagy genes *unc-51* and *bec-1* in young worms (Lapierre *et al.* 2011, Zhong *et al.* 2010). As such, if *pha-4* exerts its AP effects through the regulation of autophagy, then this regulation would still need to be active in later ages. Quantitative PCR (qPCR) analysis validated this assumption wherein a significant reduction in *unc-51* mRNA levels by day 12 was observed following *pha-4* inhibition from day 9 with further, reduced expression by day 15 (Figure 15A). The expression of *bec-1* was not affected at day 12 but was reduced to levels comparable with *unc-51* by day 15 (Figure 15A). UNC-51 is a serine/threonine kinase orthologous to the vertebrate ULK proteins and phosphorylates BEC-1 to initiate the autophagic cascade (Russell *et al.* 2013). The delayed reaction of *bec-1* to *pha-4* inhibition suggests a possible feedback loop whereby *bec-1* is transcribed, in part, in response to UNC-51 levels. To confirm a functional reduction in the levels of autophagy in response to *pha-4* inhibition we used an LGG-1::GFP reporter strain hypersensitive to RNAi. LGG-1 is a ubiquitin-like protein that is conjugated to phosphatidylethanolamine (PE) and is added to the growing autophagosome as it envelops its cargo (Kirisako *et al.* 2000). As a consequence, LGG-1 is the predominant marker used for the detection of autophagosomes (Zhang *et al.* 2015). Using a quantitative automated counting system designed specifically for use in old worms (Supplemental Figure 3), changes in the number of autophagic foci at day 15 following day 9 RNAi inactivation of both *pha-4* and *bec-1* were measured.

Inhibition of *pha-4* reduced total autophagosome numbers by 62%, comparing well with the 74% reduction seen upon *bec-1* inhibition (Figure 15, B & C). This, along with the genetic regulation of *bec-1* and *unc-51* demonstrates that PHA-4 is still critical for autophagy induction in late-life.

*Figure 15*

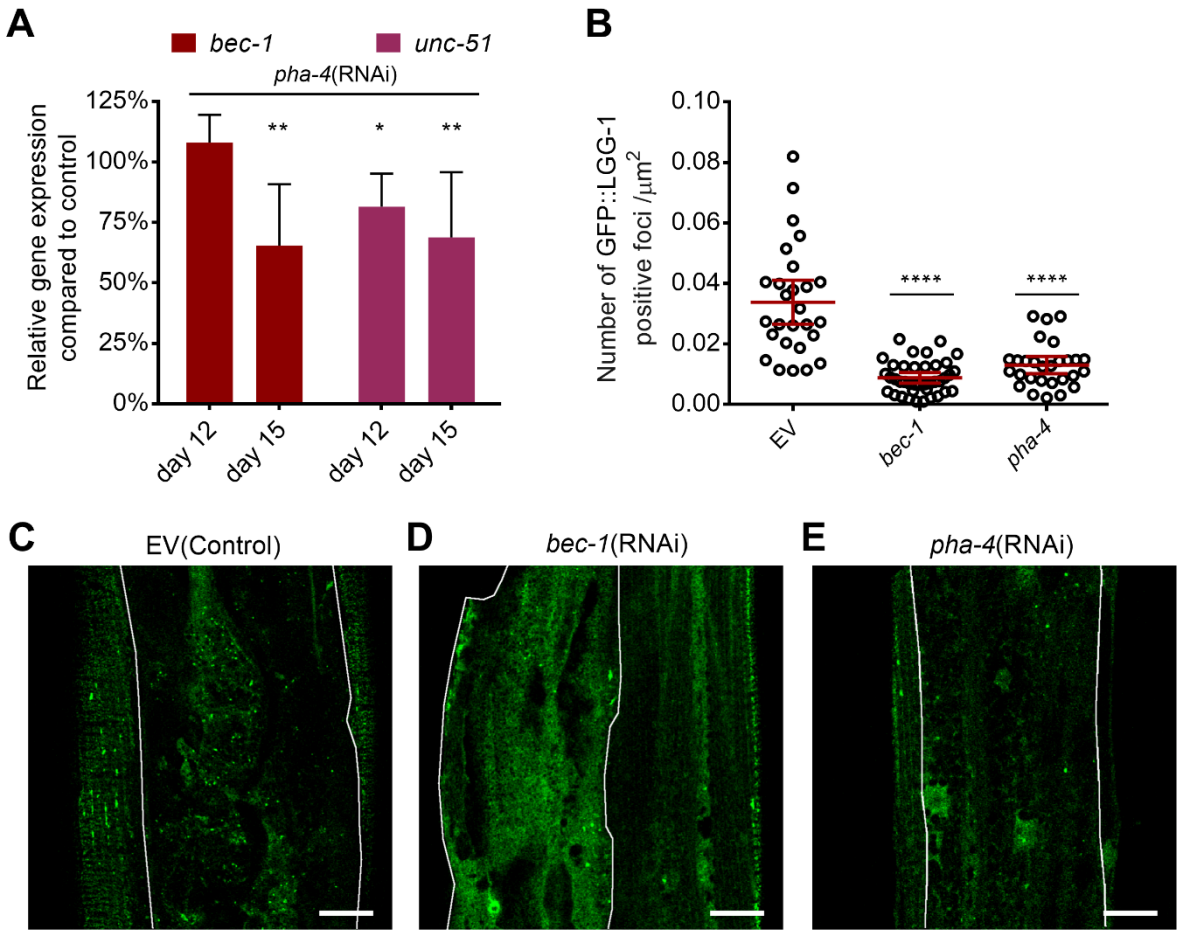


**Figure 15: *pha-4 still regulates autophagy late in life*.** (A) qPCR of *bec-1* and *unc-51* relative mRNA levels at day 12 and day 15 following *pha-4* knockdown at day 9. Data from at least four independent experiments +/- SD (* = p<0.05, ** = p<0.01). (B) Day 15 quantification of GFP::LGG-1 positive foci in hypodermal cells of *rrf-3; lgg-1p::GFP::lgg-1* worms. Data is depicted as a scatter plot with mean and 95% confidence intervals. RNAi against *pha-4* and *bec-1* was initiated at day 9. GFP::LGG-1 was quantified from images at 100x magnification (N=50) (**** = p<0.0001). (C-E) Day 15 representative microscopy images of GFP::LGG-1 foci quantity following control (C) *pha-4* (D) or *bec-1* (E) RNAi inhibition at day 9. The region bounded by the white line denotes the hypodermis. Magnification 100x. Scale bar 15 µm.

Before investigating the effects of autophagy gene inhibition on lifespan, a confirmation of the efficiency of RNAi knockdown in late-life was performed to exclude partial inhibition as a causative factor. qPCR analysis in *rrf-3* mutants showed a significant knockdown for both *pha-4* and *bec-1* from day 9, with a greater than 60% reduction in expression within 3 days (Figure 16A).

*Figure 16*

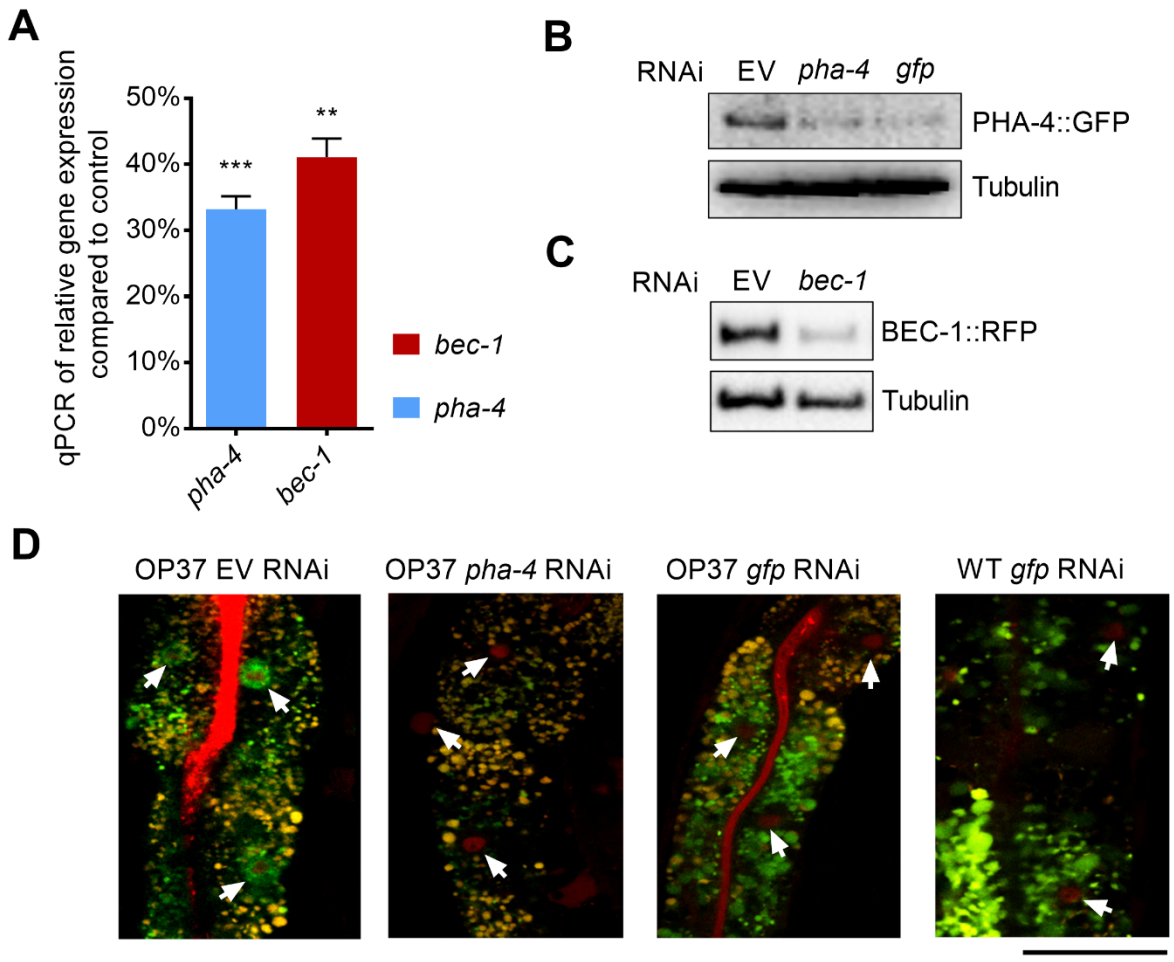


**Figure 16: *Validation of RNAi knockdown*.** (A) qPCR of *pha-4* and *bec-1* relative mRNA levels following RNAi inhibition of each gene at day 9. Data from at least three independent experiments +/- SD (** = p<0.01, *** = p<0.001) (B) Representative western blot of PHA-4::GFP levels following RNAi inhibition of *pha-4* or *gfp* in *pha-4::gfp* worms. RNAi was initiated at day 9 and samples were measured at day 15 using an antibody against GFP. (C) Representative western blot of BEC-1::RFP levels following RNAi inhibition of *bec-1* in *bec-1p::BEC-1::RFP* worms. RNAi was initiated at day 9. Samples were measured at day 15 using an antibody against RFP. (D) Microscopy of the PHA-4::GFP strain OP37 and WT worms at day 12. RNAi treatments for *pha-4*, *gfp* and EV were initiated at day 9. GFP signal is shown in the green channel and propidium iodide (PI) staining for the nuclei is shown in red. White arrows indicate nuclei. The PHA-4::GFP signal is very low, thus when imaging autofluorescence dominates. WT *gfp* RNAi knockdown shows that excessive signal in the green channel is due to this autofluorescence. Scale bar, 40 µm.

To examine the efficiency of the knockdown on the protein level two strains that expressed PHA-4::GFP (strain OP37) and BEC-1::RFP were utilised. Due to the lack of specific antibodies to either protein in *C. elegans,* antibodies to both GFP and RFP were used as proxy readouts for total protein level. Analysis showed a ~90% reduction for both proteins by day 15 following day 9 inhibition (Figure 16, B & C). Additionally, this knockdown was confirmed by microscopy with an absence of PHA-4::GFP in the nucleus of gut cells by day 15 following inhibition (Figure 16D). The low levels of PHA-4::GFP

expression in late-life, combined with high auto-fluorescence precluded reliable analysis of PHA-4 tissue distribution in old worms. Additionally, BEC-1::RFP inhibition could not be observed in the same manner as the RFP signal is generally diffuse, forming small autophagic punctate upon induction that are largely hidden by background auto-fluorescence.

With the establishment of a link from *pha-4* to *bec-1* and validation of the efficiency of late-life RNAi, the nature of *pha-4* and possibly *bec-1*'s role in ageing were subsequently examined. Reconfirming that day 9 inhibition of *pha-4* enhanced longevity showed a median MTL extension of 24% and maximum of 33% for all experiments (Figure 17A & Supplemental Table 7). As *bec-1* acts downstream of *pha-4* in the autophagic pathway, the effect of day 9 *bec-1* inhibition on lifespan was examined with the expectation that any lifespan extension would be of a similar magnitude to that seen for *pha-4* inhibition. Surprisingly, therefore, *bec-1* inhibition extended lifespan by twice that of *pha-4*, up to 63% with a median MTL of 52% over all experiments (Figure 17A & Supplemental Table 7). This unexpected result prompted further validation of the reliability of the methodology employed. First, the potential of an artificial induction of lifespan extension through culturing worms in liquid culture was examined. Using a plate based lifespan assay with *rrf-3* worms never exposed to liquid culture, MTL from day 9 following both *bec-1* and *pha-4* inhibition was reliably extended, though to a slightly lesser extent than seen from liquid culture (Figure 17B & Supplemental Table 7). This likely reflects the difference in health and feeding capacity between liquid and plate cultures observed, worms from liquid culture are typically healthier and more willing to eat at day 9 than their plate-reared counterparts. Next, to rule out effects specific to the *rrf-3* mutant strain background, another RNAi hypersensitive strain *ergo-1(tm1860)* (Yigit *et al.* 2006) and WT worms were used for post-reproductive lifespan assays. The *ergo-1* mutant worms showed a strong lifespan extension following *bec-1* inhibition (~40%), which was nearly double that seen for *pha-4* (~22%) (Figure 17C). The results were slightly weaker than seen for *rrf-3* mutants probably reflecting differences in the strength of the RNAi enhancement between the two strains (Zhuang & Hunter 2011). Notably, in the WT background, lifespan extension could be achieved with day 9 *bec-1* inhibition but not *pha-4* inhibition (Figure 17D). Additionally, the strength of the *bec-1* mediated MTL extension was reduced to ~25% (Supplemental Table 8). To exclude the possibility that *pha-4* RNAi was ineffective in WT worms RNAi was initiated from L1 and day 0. This resulted in a strong MTL reduction for both genes agreeing with published data (Meléndez *et al.* 2003, Sheaffer *et al.* 2008) (Figure 17E). In addition, examination of the strength of the RNAi knockdown for both *pha-4* and *bec-1* by qPCR in WT worms showed delayed but effective RNAi inhibition for both genes when compared to *rrf-3* mutants (Figure 17F). *rrf-3* mutants displayed maximum inhibition by day 12 (3 days post RNAi initiation), whereas inhibition in WT worms did not reach the same levels until day 15 (6 days post RNAi initiation). This likely explained some but not all of the observed reduction in lifespan extension between the two strains.

*Figure 17*

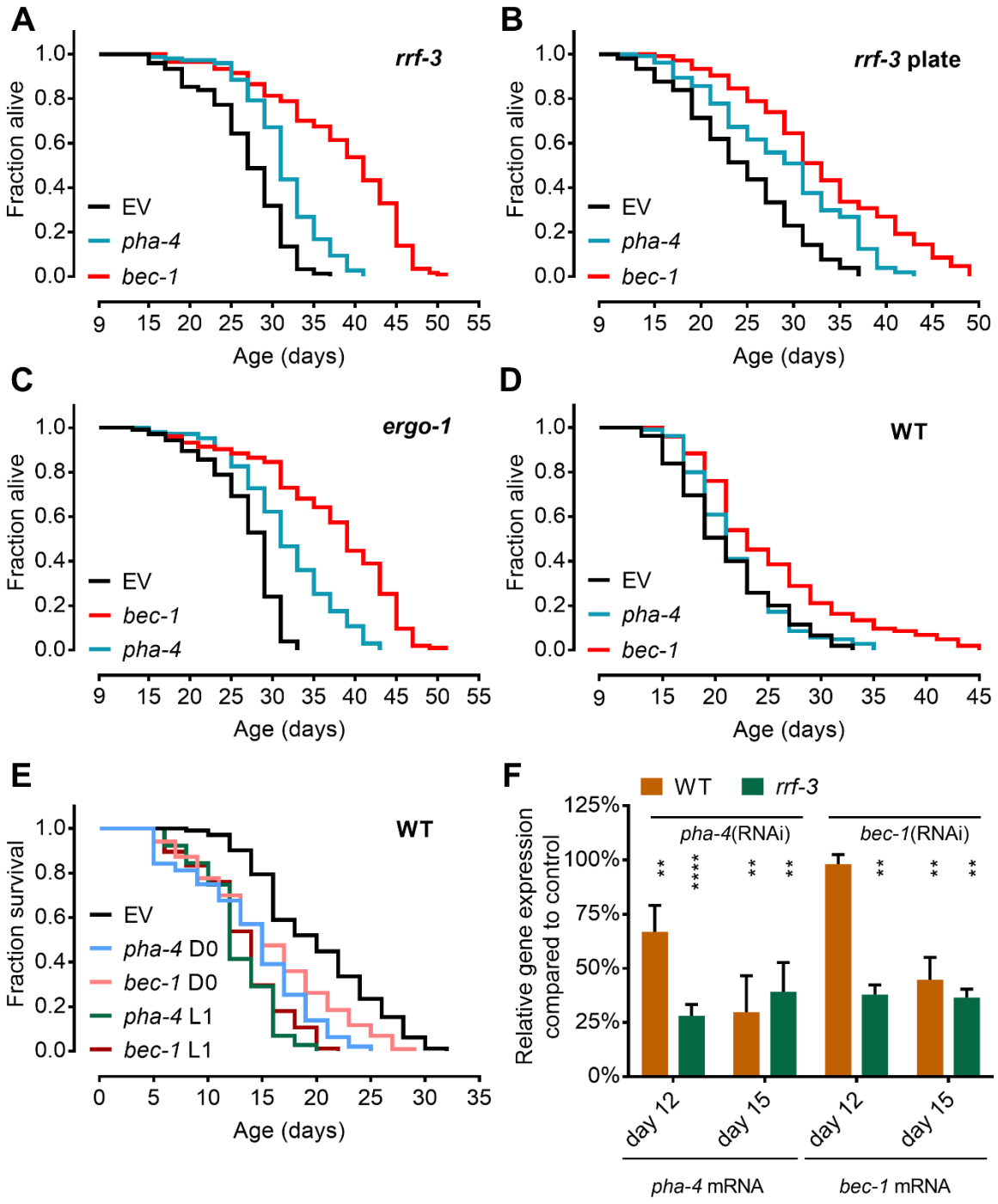


**Figure 17: *Lifespan extension is independent on the rrf-3* strain.** (A) Day 9 RNAi against *bec-1* extends MTL by twice that of *pha-4*. (B) *rrf-3* worms maintained only on NGM agar plates have a comparable MTL extension to worms maintained in liquid culture (C) Inhibition of *pha-4* and *bec-1* from day 9 in the RNAi hypersensitive strain *ergo-1* extends lifespan comparably to in *rrf-3*. (D) Day 9 *bec-1* RNAi extends MTL in WT worms but *pha-4* inhibition does not. (E) Inhibition of *pha-4* and *bec-1* shortens the lifespan of WT worms when initiated before adulthood. (F) qPCR showing the relative levels of *pha-4* and *bec-1* mRNA measured at both day 12 and day 15 in WT(N2) and *rrf-3* worms. RNAi was initiated at day 9. Data represents that of at least four different experiments +/- SD. (** = p<0.01, **** = p<0.0001). Lifespan statistics are presented in Supplementary Table 7 & 8.

The discrepancy in the strength of post-reproductive lifespan extension between *pha-4* and *bec-1* inhibition and the absence of an extension for *pha-4* in the WT background remained unresolved. We speculated that as PHA targets a broad spectrum of genes, some of which are fundamental processes (Zhong *et al.* 2010), detrimental effects to health could arise from their dysregulation subsequent to *pha-4* inhibition late in life. This hypothesis was tested by simultaneously inhibiting both *pha-4* and *bec-1* in post-reproductive worms. The combined RNAi treatments resulted in a reduction of *bec-1* mediated lifespan extension by 15 percentage points to a median MTL of 32% in *rrf-3* mutants (Figure 18A & Supplemental Table 9). Similar results were observed in the WT background with a reduction from a ~25% MTL increase for *bec-1* alone to ~12% in the double knockdown condition (Figure 18B & Supplemental Table 9). The feeding of two bacteria simultaneously does not result in reduced RNAi efficiency, as confirmed by qPCR, nor does it affect the degree of lifespan increase compared to single feeding (Figure 18, C & D), suggesting the reduced lifespan effect comes solely from the combination of the two RNAis. Furthermore, examining the genes disrupted by *pha-4* and *bec-1* inhibition, through RNAseq analysis, revealed that inhibition of *pha-4* in early life resulted in 3,455 DEGs (FDR <1%), which is in agreement with other published observations (Zhong *et al.* 2010). This is in stark contrast to the mere 60 DEGs discovered following inhibition of *pha-4* from day 9. Five of these later-life genes were also disrupted upon *bec-1* knockdown (Figure 18E). Examination the 60 DEGs showed that 40 are down-regulated and 20 up-regulated following inhibition. GO-Term analysis found no significant gene classes or terms for the up-regulated genes but did identify the innate immune response as a significant term (FDR 0.0009) for down-regulated genes. All of the genes identified in this class are also downregulated following day 0 *pha-4* inhibition, *dod-19*, *gst-6*, *hpo-6*, *B0024.4*, *C49C3.9, F53C11.1, Y47H9C.1*. Together this suggests that inhibition of *pha-4* at all ages generally reduces the innate immune response, compromising health and longevity. It is therefore likely that this aspect of *pha-4* mediated dysregulation contributes to the contraction of lifespan gain through autophagy inhibition. This reasoning, applied to the WT condition, results in a total, negligible lifespan extension that is consequently not detected as significant (Figure 17D & Supplemental Table 8).

The five genes that overlap following both *pha-4* and *bec-1* day 9 inhibition were scrutinised for potential processes relevant to the mechanism of autophagy-mediated lifespan extension. *acs-2* encodes an acyl-CoA synthetase that converts fatty acid to Acyl-CoA for subsequent β-oxidation (Van Gilst *et al.* 2005a). *lec-2* is a β-galactosidase binding lectin involved in carbohydrate metabolism (Nemoto-Sasaki *et al.* 2008). *hrg-7 (asp-10*) is a predicted aspartic protease with homology to PGA4 a human pepsinogen (Fruton 2002). *C15B12.8* and *C53A3.2* are predicted to have oxidoreductase activity and phosphatase activity respectively based on homology to human proteins (Shaye & Greenwald 2011). Interestingly, comparing the results of *pha-4* inhibition at day 0 and day 9, three of the genes (*lec-2, C15B12.8,* and *C53A3.2*) show a reversal in the direction of expression suggesting an age-

dependent difference in *pha-4* regulation that could be contributing to improved longevity (Figure 18F). *acs-2* was previously identified as one of the most highly variable genes in the previous over-life RNAseq analysis, suggesting that reducing its expression and consequently lipid oxidation could be a control point for the AP action of both genes.

*Figure 18*

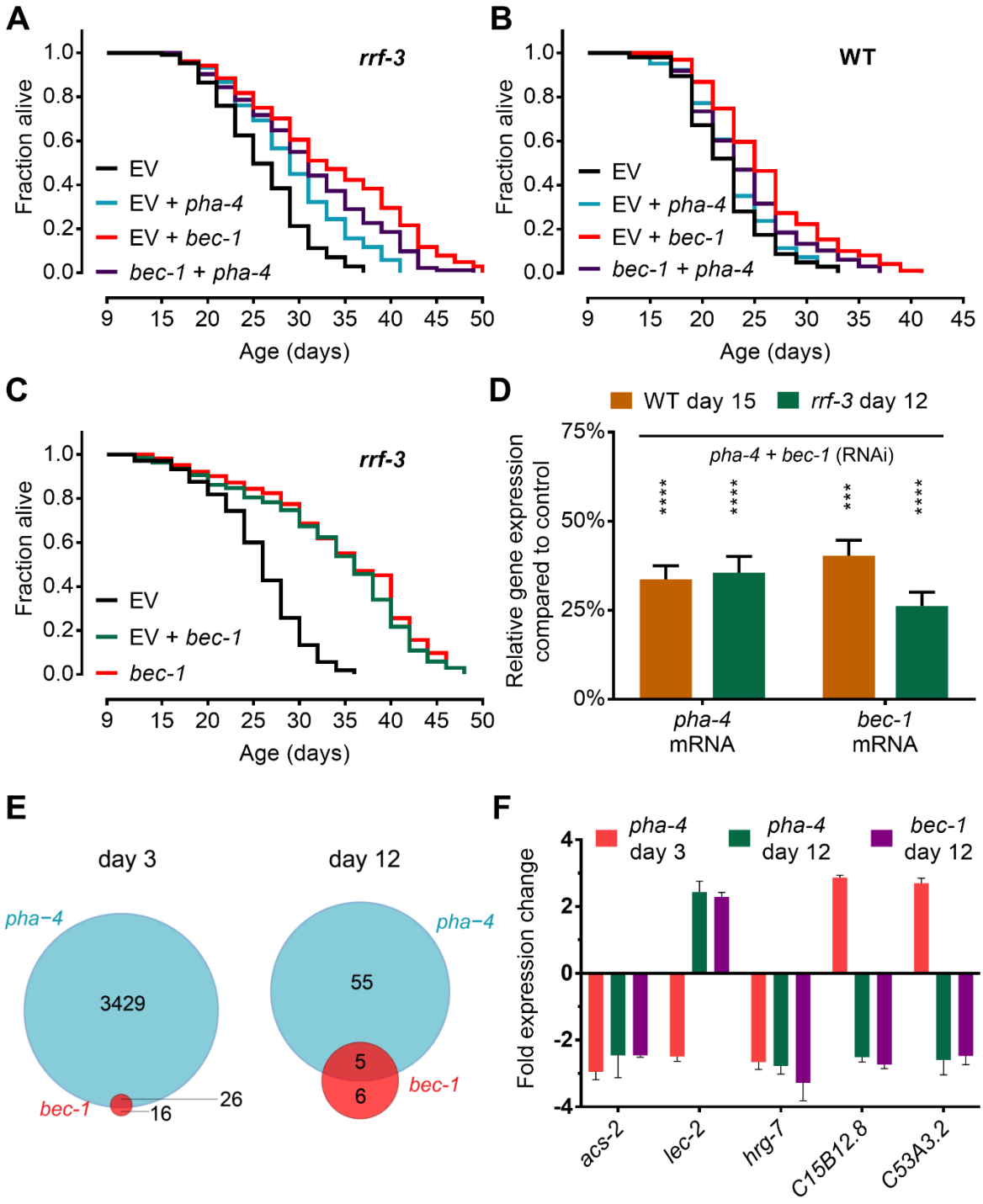

**Figure 18: *PHA-4 exerts pleiotropic effects beyond autophagy.*** (A) Inactivation of *pha-4* at day 9 in combination with *bec-1,* in *rrf-3* worms, reduces MTL extension compared to *bec-1* knockdown alone. (B) Double inactivation of *pha-4* and *bec-1* shortens the MTL extension of *bec-1* alone in WT worms (C) Feeding worms a dilution of RNAi bacteria with EV in a 50:50 ratio does not affect the MTL extension compared to undiluted feeding. (D) Feeding worms with two RNAi bacteria simultaneously still leads to a significant knockdown of gene targets as determined by qPCR. Relative mRNA levels of each targeted gene in *rrf-3* and *WT* worms were measured at day 12 and day 15 respectively. Data represents that of at least four different experiments +/- SD. (*** = p<0.001, **** = p<0.0001). (E) Venn diagrams depicting the number of differentially regulated genes following *pha-4* or *bec-1* knockdown at day 0 and day 9 as measured by RNAseq. Samples were measured for gene expression changes following 3 days of RNAi at day 3 and day 12 respectively. (F) Change in expression of the five genes overlapping between *pha-4* and *bec-1* knockdown at day 9. Fold expression change is measured compared to EV treatments for the same period. Lifespan statsistics are presented in Supplementary Table 9.

AP theory predicts that there will be different longevity effects dependent on the time of gene inactivation. As the balance for the natural action of a gene moves from beneficial to detrimental over life so should the effects of its inhibition inversely mirror this trend. We determined if the inhibition of *pha-4* and *bec-1* exhibited such an AP pattern via knockdown at different time-points across life. Following inhibition, there was a marked decrease in MTL from the larval stage that transitioned towards a neutral effect starting between day 0 and day 5, this was followed by MTL extension from day 9 onwards (Figure 19, Supplemental Figures, 4 & 5 & Supplemental Table 7). This pattern of behaviour visibly demonstrates the AP nature of both genes and represents the first clear identification of genes functioning as predicted by Williams over 60 years ago (Williams 1957).

*Figure 19*

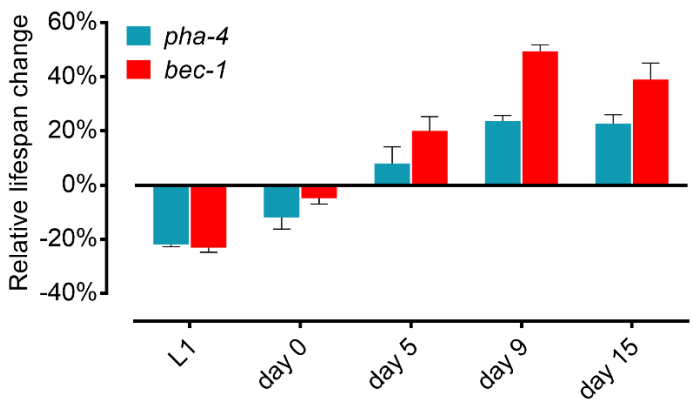


**Figure 19: *Both pha-4 and bec-1 show time dependent effects on longevity.*** Inhibition of *pha-4* and *bec-1* at different times across life shows differing effects depending on the RNAi initiation time point. Depicted is the relative percentage change in MTL +/- SEM compared to control, averaged for all experiments at each time-points. Lifespan statistics are presented in Supplemental Table 7.

### 2.2.5 Effects of AP gene inhibition on healthspan

Extending lifespan can come with costs to the functionality of the organism as a whole. Investigations into four of the main longevity regulating genes (*daf-2*, *ife-2*, *eat-2*, and *clk-1*) in *C. elegans* show worms whose life is extended through manipulation of these pathways have increased periods of frailty compared to WT (Bansal *et al.* 2015). This means despite living longer, the percentage of life spent with reduced capacity is also greatly increased, an obviously undesirable outcome. Therefore, it was investigated if the inhibition of both *pha-4* and *bec-1* translated the increase in lifespan into a similar improvement in functional aspects of health. One of the primary visual indications of ageing observed in worms is a structural decline in the pharynx, which can be delayed in longevity mutants such as *eat-2* and *glp-1* (Chow *et al.* 2006, Zhao *et al.* 2017). As such, the structural integrity of the pharynx in 20 day old worms following *pha-4* and *bec-1* inhibition from day 9 was examined. Using Differential-interference contrast (DIC) imaging, changes to the corpus, isthmus and terminal bulb were scored manually and pharynx integrity rated on a three-point scale; 1 - minimally, 2 - somewhat, or 3 - most deteriorated. From this analysis, it was determined that the inhibition of both *pha-4* and *bec-1* significantly preserved pharynx ultrastructure. More than twice the amount worms for both treatment conditions were characterised as minimally degraded compared to untreated worms at day 20 (Figure 20A). The age-associated decline in muscle integrity (sarcopenia) is a common trend for many species as they age (Marzetti *et al.* 2017). The degree of muscle integrity declines with age in *C. elegans* and defects in muscle capacity are a better predictor of subsequent longevity than chronological age (Glenn *et al.* 2004, Herndon *et al.* 2002). To examine this ageing hallmark, phalloidin staining of muscle fibers was used at day 20 following day 9 inhibition of *bec-1* and *pha-4*. Similar to analysis of the pharynx, muscle integrity was classified from 1 – 5 on a scale from least to most degenerated. The inhibition of both *pha-4* and *bec-1* again doubled the number of worms retaining muscles ranked in classes 1-2 by day 20 and exhibited no class 5 muscles in comparison to 10% in untreated worms (Figure 20B). Similarly, the rate of pharyngeal pumping and body movement (as counted by body bends) are both positively correlated with lifespan, are improved in lifespan extending mutants, and predict subsequent lifespan better than chronological age (Huang *et al.* 2004). Examining these trends at day 20 following *bec-1* inhibition showed a significant ($p<0.0001$) improvement in both factors compared to control (Figure 20, C & D).

*Figure 20*

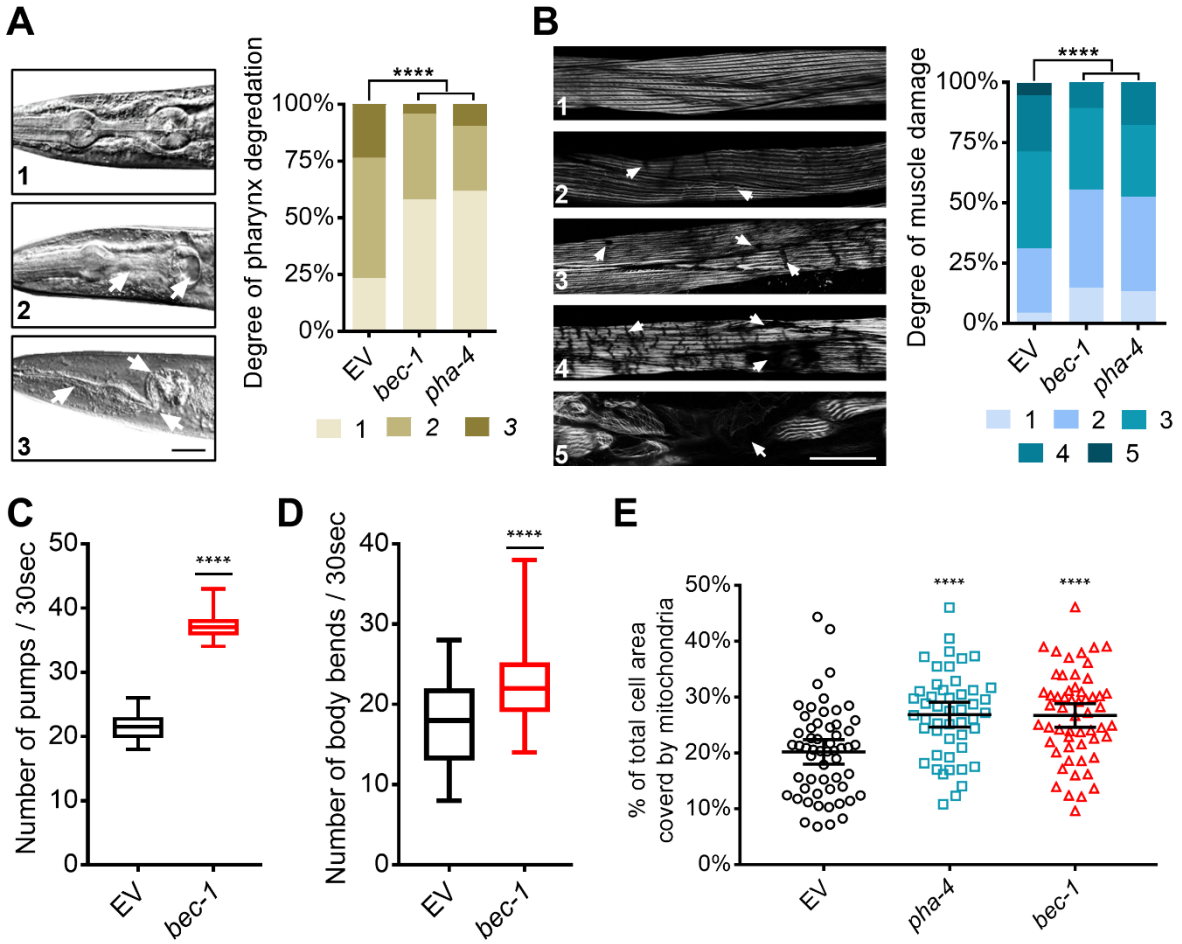


**Figure 20:** ***Inhibition of autophagy improves health.*** (A) Day 9 RNAi treated worms were scored at day 20 for pharynx degradation on a three point scale (N=90). Damage of the corpus, isthmus and terminal bulb (white arrows). Scale bar, 30 µm, magnification 63x (**** = p<0.0001). (B) Day 9 RNAi treated worms were scored at day 20 for phalloidin stained muscle damage on a five point scale (N=120). Sites of muscle damage (white arrows). Scale bar, 30 µm, magnification 63x, (**** = p<0.0001). (C) Day 20 quantification of body movement, measured by body bend counts, in *rrf-3* worms. Data is depicted as a box plot with min to max values. RNAi against *bec-1* was initiated at day 9 (N=50) (**** = p<0.0001). (D) Day 20 quantification of pharynx pumping rate in *rrf-3* worms. Data is depicted as a box plot with min to max values. RNAi against *bec-1* was initiated at day 9 (N=50) (**** = p<0.0001). Panels, C & D were produced by Johannes Geisinger. (E) Automatic quantification of mitochondrial area in body wall cells of *rrf-3* worms treated with control (EV), *pha-4* and *bec-1* RNAi from day 9. Mitochondria were visualised with use of Mitotracker staining and worms were examined for mitochondrial area at day 20. Data is depicted as a scatter plot with mean and 95% confidence intervals. (N=60) (**** = p<0.0001)

As *C. elegans* age they display a marked change in structure of their mitochondria in body wall muscles. The normally tubular mitochondrial network becomes fragmented with increasing age, which is accompanied by a decrease in total mitochondrial volume. Both phenotypes are modular, appearing faster and slower under lifespan shortening and extending regimes respectively (Regmi *et al.* 2014). Following inhibition of *pha-4* and *bec-1* from day 9, the total area covered by mitochondria was

significantly higher in the knockdown conditions compared to controls ($p<0.0001$). This suggests autophagy inhibition results in the preservation of a more youthful mitochondrial network (Figure 20E). Generally, as an organism ages, the rate of mitochondrial activity will decline as well (Sun *et al.* 2016). However, mutations in several key mitochondrial genes increase lifespan by decreasing respiration and electron transport chain activity (Wolff & Dillin 2006), suggesting decreased ROS production is a critical factor for ageing. The effect of *bec-1* inhibition on mitochondrial activity was examined with use of the vital dye Mitosensor, which accumulates in viable mitochondria-dependent on their activity. This revealed that following knockdown of *bec-1* from day 9 mitochondrial activity is more repressed at day 20 in knockdown worms than in the controls (Figure 21). Notably, the level of mitochondrial activity detected was either low, medium or high across the whole worm. Inhibition of *bec-1* resulted in more worms exhibiting a lower activity trend with none having the high activity seen in the control (Figure 20F, last row in the EV condition). Together, these results demonstrate a reduction in ageing hallmarks and improvements in healthspan following autophagy inhibition.

*Figure 21*

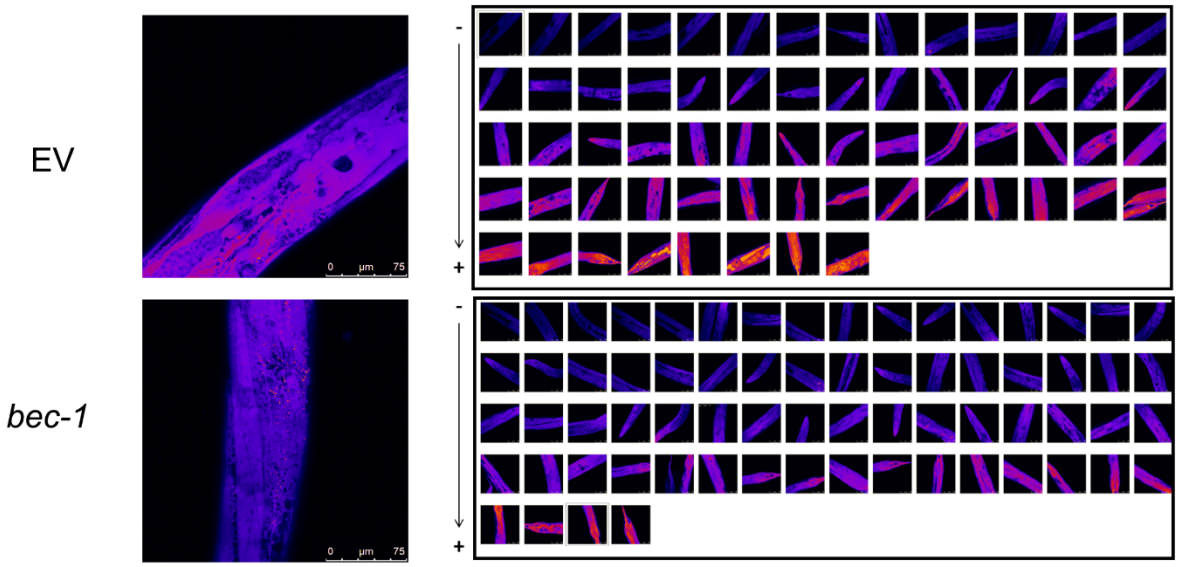


**Figure 21:** ***Inhibition of bec-1 in late-life reduces mitochondrial activity*****.** Heatmap of mitochondrial activity as measured by Mitosensor in *rrf-3* worms, Mitosensor stains more vividly in active mitochondria. Worms were treated with control (EV) and *bec-1* RNAi at day 9 and examined for mitochondrial activity at day 20. (N=70). Scale bar for larger images 75 µm. Images in panel are all worms successfully imaged for each treatment arranged in order of total Mitosensor fluorescence. Lowly fluorescing worms have reduced mitochondrial activity.

### 2.2.6 Mechanisms of longevity extension through autophagy inhibition

The transcriptional control of *bec-1* and *unc*-51 in late-life by PHA-4 combined with the robust lifespan increase (Figure 15A & 17A), strongly implied that inhibition of the autophagy process is at the root of the longevity effects. However, autophagy and the genes controlling its regulation intersect with other cellular mechanisms including the ubiquitin-proteasome system (UPS), apoptosis and necrosis (Nikoletopoulou *et al.* 2013, Lilienbaum 2013). As such, the contribution of these pathways to *bec-1* mediated longevity was examined. Initially, it was confirmed that the observed lifespan effects following late-life *bec-1* knockdown (through either RNAi or *pha-4* inhibition) are definitively working through the autophagic pathway. BEC-1 acts in two distinct pathways complexing with either CED-9 (the *C. elegans* orthologue of Bcl-2) in the caspase-mediated apoptotic pathway, or with VPS-34 and EPG-8 to promote autophagic nucleation (Yang & Zhang 2011, Takacs-Vellai *et al.* 2005). First it was clarified that both members of the autophagy nucleation complex, *vps-34* and *epg-8* also extended lifespan when inhibited from day 9 (Figure 22A). Notably, this extension matched that seen for *bec-1* inhibition, strongly suggesting a commonality of lifespan function for the entire complex. Secondly, it was investigated whether these genes exhibited AP behaviour by inhibiting them from the first day of adulthood. As seen with *bec-1* inhibition, both genes shortened lifespan significantly when inactivated in early life, which indicated that AP is a common feature of the autophagy nucleation complex (Figure 22B). When BEC-1 is inhibited this triggers CED-4 mediated apoptotic cell death (Takacs-Vellai *et al.* 2005), thus the apoptotic role of BEC-1 was investigated. Surprisingly, examination of the amount of apoptosis present in the germline in day 16 worms, following *bec-1* inhibition from day 9, did not reveal any significant increase in apoptosis levels (Figure 22C). If the inhibition of *bec-1* in late-life triggered an apoptosis mediated increase in lifespan, then a combinatorial suppression of both BEC-1 and CED-4 would reduce the observed lifespan. Simultaneous inhibition of both genes did not reduce *bec-1* mediated MTL extension and inhibition of apoptosis alone, through *ced-4*, also had no effect on lifespan (Figure 22D), demonstrating an apoptosis independent longevity extension. Autophagy has a rather complex interplay with necrosis but the ability of autophagy to functionally suppress different forms of necrotic cell death is regarded as one of its most critical pro-survival functions (Nikoletopoulou *et al.* 2013). Therefore, the effect of *bec-1* inhibition on necroptosis (programmed necrotic cell death) was examined using propidium iodide staining. Surprisingly, this showed that *bec-1* inhibition resulted in a sharp decrease rather than an increase in necroptotic bodies within the head region of the worm (Figure 22E). Interestingly, *vps-34* inhibition showed no effect for the same period (Figure 22E), suggesting that modulating necrosis is specific to *bec-1* inhibition and unlikely to be a factor in late-life autophagy mediated lifespan extension.

*Figure 22*

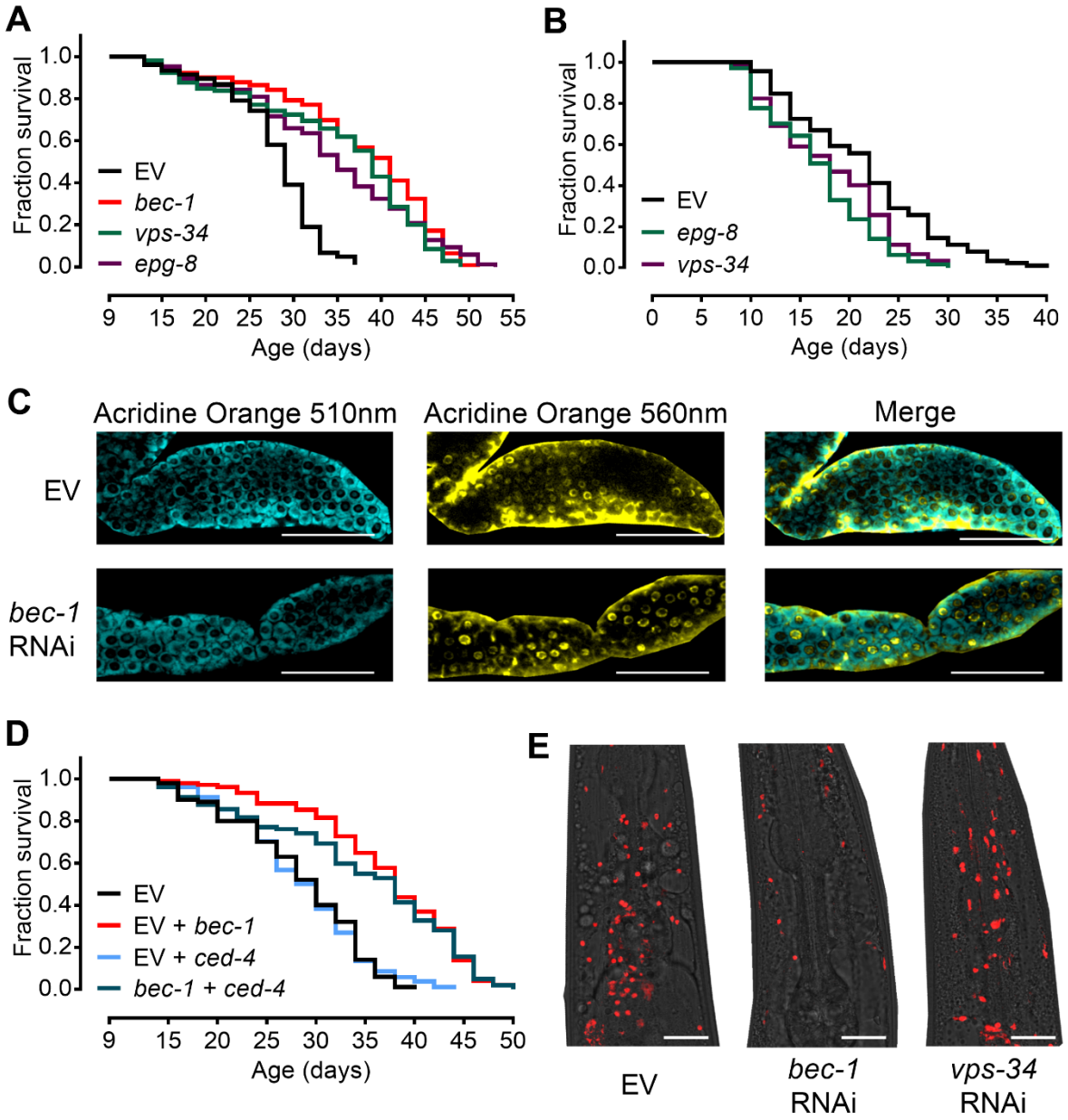


**Figure 22:** ***Inhibition of bec-1 extends lifespan through autophagy.*** (A) *bec-1*, *vps-34* and *epg-8* equally extend the MTL of *rrf-3* worms when inactivated at day 9. (B) Day 0 RNAi against either *vps-34* or *epg-8* shortens MTL. Panels A & B were produced by Thomas Wilhelm. (C) Acridine Orange staining for apoptosis in the germline of *rrf-3* worms following *bec-1* inhibition from day 9. Images were taken at day 15 and show uniform cytoplasmic staining at 510nm and active nuclear apoptotic cell staining at 560nm. Apoptotic cell number does not increase following *bec-1* inhibition. (D) *bec-1* RNAi treatment from day 9 shows no change in effect on MTL with simultaneous *ced-4* RNAi treatment at day 9 in *rrf-3* worms. Inhibition of *ced-4* alone has no effect on lifespan. (E) Propidium Iodide statining of necrotic cells in the head region of *rrf-3* worms. Stained cells are likely neurons. RNAi treatments were initiated at day 9 and images taken at day 15. Inhibition of *bec-1* but not *vps-34* reduced the amount of necrotic staining.

*Figure 23*

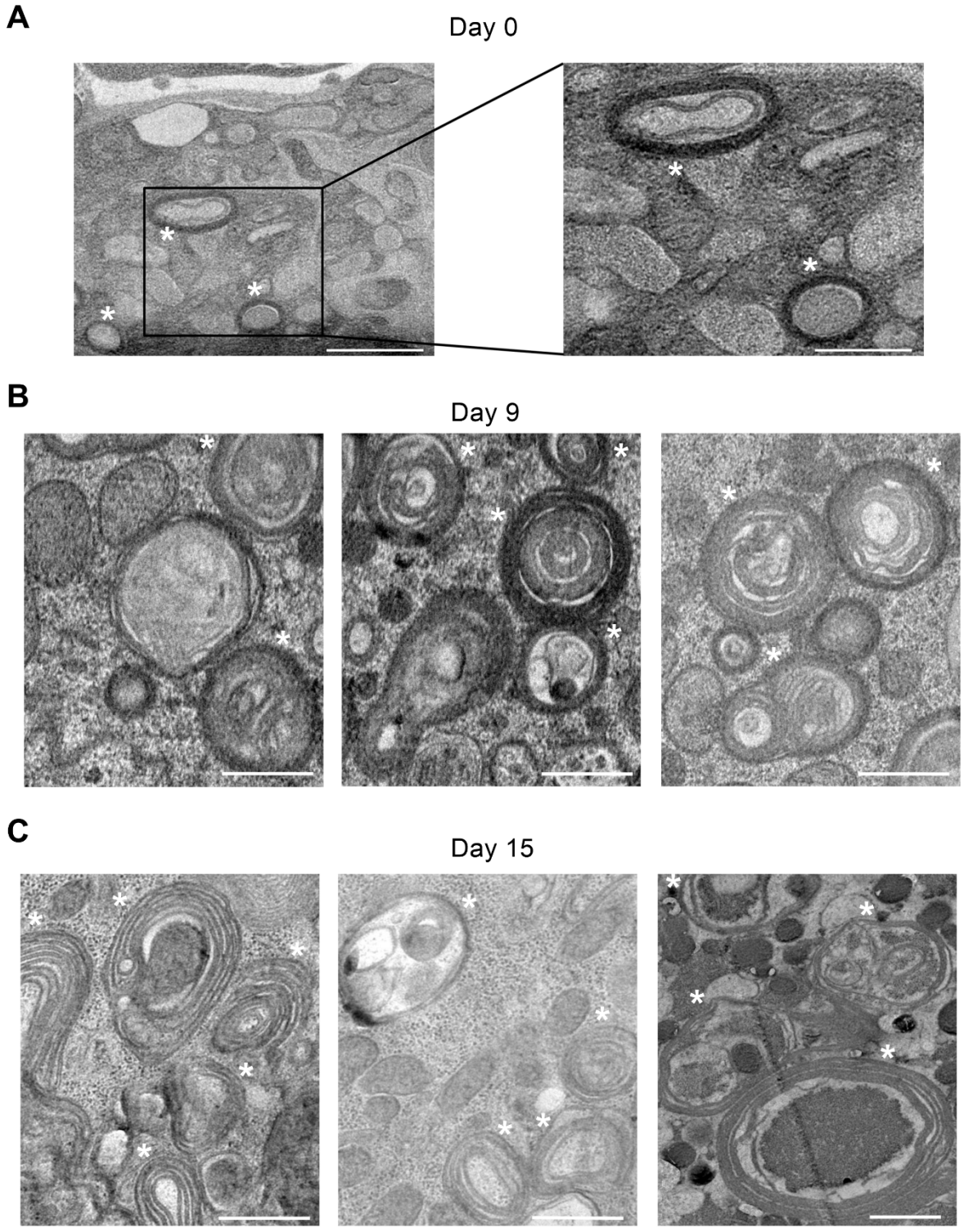


**Figure 23:** ***Electron microscopy of autophagic vesicles over life.*** Autophagic vesicles were identified in in the hypodermis of *rrf-3* worms and are indicated by white stars. (A) Autophagic vesicles are normal double membraned structures at day 0. Right panel 2x zoom. Scale bars 1 µm and 0.5 µm respectively. (B) Autophagosomes being to thicken and add extra membrane layers at day 9. Scale bar 0.5 µm (C) At day 15 autophagosomes are highly disrupted with numerous membrane layers and are filled with dark undigested material (panels 1 & 2). Scale bar 0.5 µm. Each image is from an independent worm (N=12).

To examine what is happening with autophagy in older worms that makes it so detrimental in late-life, we collaborated with Maria Kokkinopoulou (MPI-P, Mainz) and used transmission electron microscopy to observe changes in autophagesomal structures over the worm's life. The autophagosome when formed is identified in electron microscopy as double membrane-bound vesicles containing cytoplasmic material or organelles (Eskelinen 2008). Autophagosomes were identified from thin tissue section of worms across three time-points of life, day 0, day 9 and day 15. As worms age they displayed a marked increase in the number of disrupted autophagosomes, as indicated by increasing layers of membrane wrapping around each structure as time progressed (Figure 23, A-C). Such multi-layered membranes have previously been reported in autophagy mutants (Tian *et al.* 2010). These autophagosome were often packed with undigested material and high densities of lipofuscin (Figure 23B (second panel) & 23C (second panel)). In contrast, worms where either *pha-4* or *bec-1* were inhibited did not display any of these multilaminar structures (Figure 24, A & B). This strongly suggests that as ageing progresses autophagy becomes non-functional and degenerative resulting in a shorter life and its inhibition alleviates degenerative autophagosome formation.

*Figure 24*

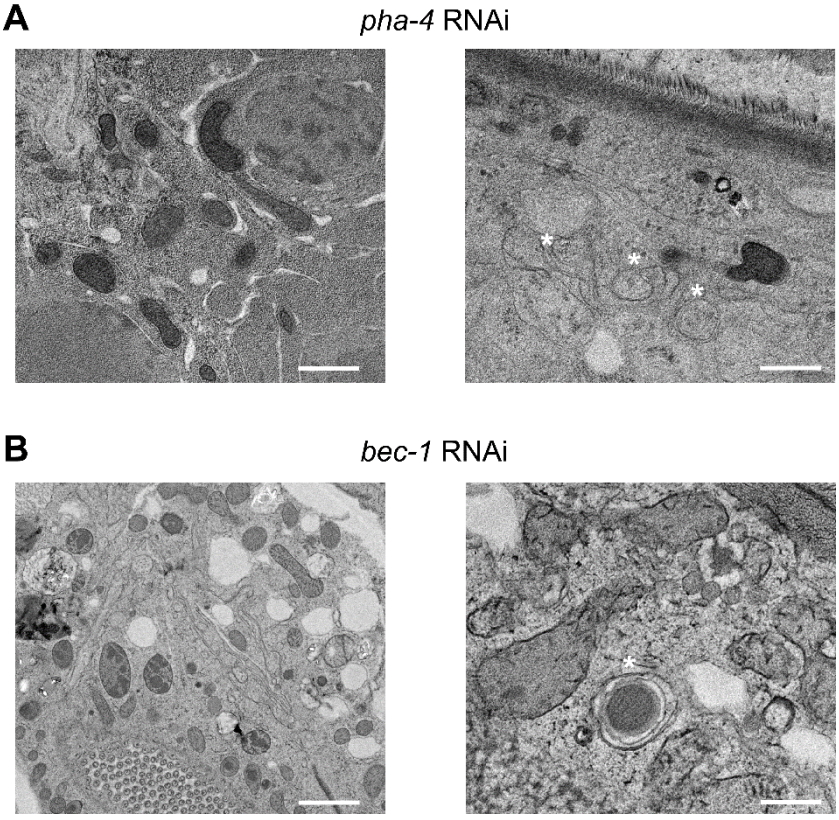


**Figure 24:** ***Inhibition of pha-4 and bec-1 removes damaged autophagic vesicles.*** Autophagic vesicles were identified in the hypodermis of *rrf-3* worms and are indicated by white stars. RNAi was initiated at day 9 and images taken at day 15. Inhibition of *pha-4* (A) or *bec-1* (B) prevents the formation of disrupted autophagosomes. The few autophagosomes that are detected appear to have a normal morphology. Scale bars 1 µm. Each image is from an independent worm (N=12).

*Figure 25*

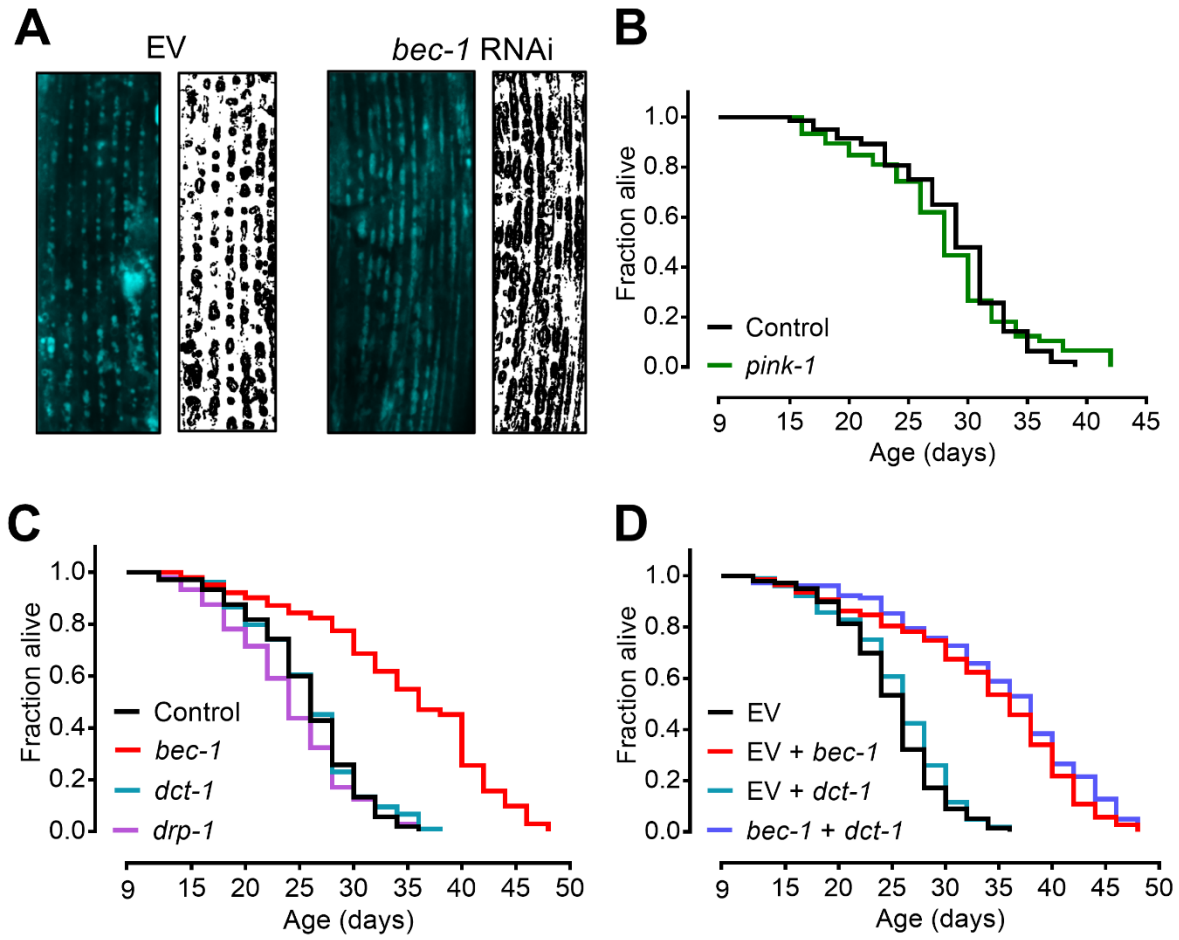


**Figure 25:** ***No role for mitophagy in bec-1 mediated lifespan extension.*** (A) Mitochondria stained with Mitotracker in body wall muscles of *rrf-3* worms. Fluorescent image is in colour with a bit map image in black & white. RNAi against *bec-1* was initiated at day 9 and images taken at day 20 (N=30). Knockdown of *bec-1* results in a retention of a tubular mitochondrial structure and shows less fragmented mitochondria. (B) Inhibition of the gene responsible for targeting mitochondria for degradation, *pink-1*, from day 9 does not affect MTL in *rrf-3* worms. (C) Inhibition of two critical mitophagy genes *dct-1* and *drp-1* do not extend MTL in *rrf-3* worms from day 9 whereas *bec-1* does. (D) Combinatorial suppression of *bec-1* and *dct-1* does not affect the MTL extension of *bec-1* alone in *rrf-3* worms. Lifespan statistics Supplemental Table 10.

Having established autophagy inhibition as the prime causal mechanism through which *bec-1* modulates late-life longevity, the contribution of mitophagy, as another principle form of autophagy, to lifespan was investigated. Previous observations showed a retention in mitochondrial mass following *pha-4* and *bec-1* inhibition (Figure 20E). Additionally, observations of the mitochondrial network structure showed the preservation of a more tubular network in body wall muscle at day 20 following *bec-1* inhibition at day 9 (Figure 25A). The preservation of this structure, while not a predictor of subsequent lifespan, is a hallmark of young, healthy worms (Regmi *et al.* 2014). These two findings could be due to a failure to recycle mitochondria through selective autophagic degradation (mitophagy). Mitochondria gradually accumulate with age in *C. elegans* (Artal-Sanz & Tavernarakis

2009) and the depletion of BEC-1 in young worms recapitulates this phenotype (Palikaras *et al.* 2015). Furthermore, while inhibition of mitophagy does not shorten WT lifespan it does reduce the lifespan gain in *daf-2* and *isp-1* longevity mutants (Palikaras *et al.* 2015). Therefore, it was investigated whether the mitochondrial phenotype and lifespan extension observed represented an unexpectedly beneficial retention of mitochondria in late-life. This was achieved through specific inhibition of key genes involved in mitophagy. Inhibition of the three core mitophagy genes *pink-1*, *dct-1* and *drp-1* (Palikaras *et al.* 2015) had no effect on lifespan in *rrf-3* mutant worms from day 9 (Figure 25, B & C). Similarly, when *bec-1* was inhibited in combination with *dct-1* there was no reduction in *bec-1* MTL extension (Figure 25D). Together this strongly suggests that mitophagy and increased mitochondrial retention are not causal for *bec-1* mediated longevity.

The ability to recycle proteins within a cell, thereby maintaining proteostasis, is normally vital for survival. As autophagy is one of the primary mechanisms by which this is achieved, it is puzzling how worms cope and survive better without this mechanism. Cells have another major protein degradation pathway, the ubiquitin-proteasome system (UPS) and cross-talk between the two systems has been observed (Wang *et al.* 2013). Therefore, the possibility that the inhibition of autophagy leads to an upregulation in the UPS system, potentially leading to beneficial lifespan effects, was examined. The 20S catalytic core of the proteasome contains six active sites that cleave between different amino acid residues dependent on their type. There are two chymotrypsin-like, two trypsin-like and two caspase-like that cleave hydrophobic, basic and acidic residues respectively (Coux *et al.* 1996). Using a fluorescent AMC assay that measures the activities of the different cleavage mechanisms, it was shown that there was no change in proteasome activity for any of these sites following *bec-1* inhibition (Figure 26, A & B). Additionally, in comparison to specific inhibitions of the proteasome through the drug Bortezomib (Chen *et al.* 2011) or knockdown of PAS-4, a component of the 20s core subunit (Papaevgeniou & Chondrogianni 2014), no increase in the abundance of lysine 48-linked polyubiquitin chains following *bec-1* inhibition was observed (Figure 26, C & D). These chains target proteins for degradation in the proteasome and increase following proteasome dysfunction (Tenno *et al.* 2004). Together these results show that inhibition of *bec-1* has neither an activating nor a repressive effect on proteasome activity.

Failure of autophagy could also be compensated for by improved protein maintenance through better folding by small heat-shock proteins, obviating the need for their recycling. Similarly, increased defence against oxygen radicals could also protect proteins from degradation requiring reduced turnover via autophagy. Consequently, primary genes involved in the unfolded protein response and oxidative stress response (Manière *et al.* 2014) were examined by qPCR for changes in expression following day 9 inhibition of both *pha-4* and *bec-1*. Examining gene expression at day 15 following inhibition revealed no significant changes in any of the genes tested for either stress response

programme (Figure 26, E & F). This argues strongly against the involvement of either pathway in the longevity phenotype.

*Figure 26*

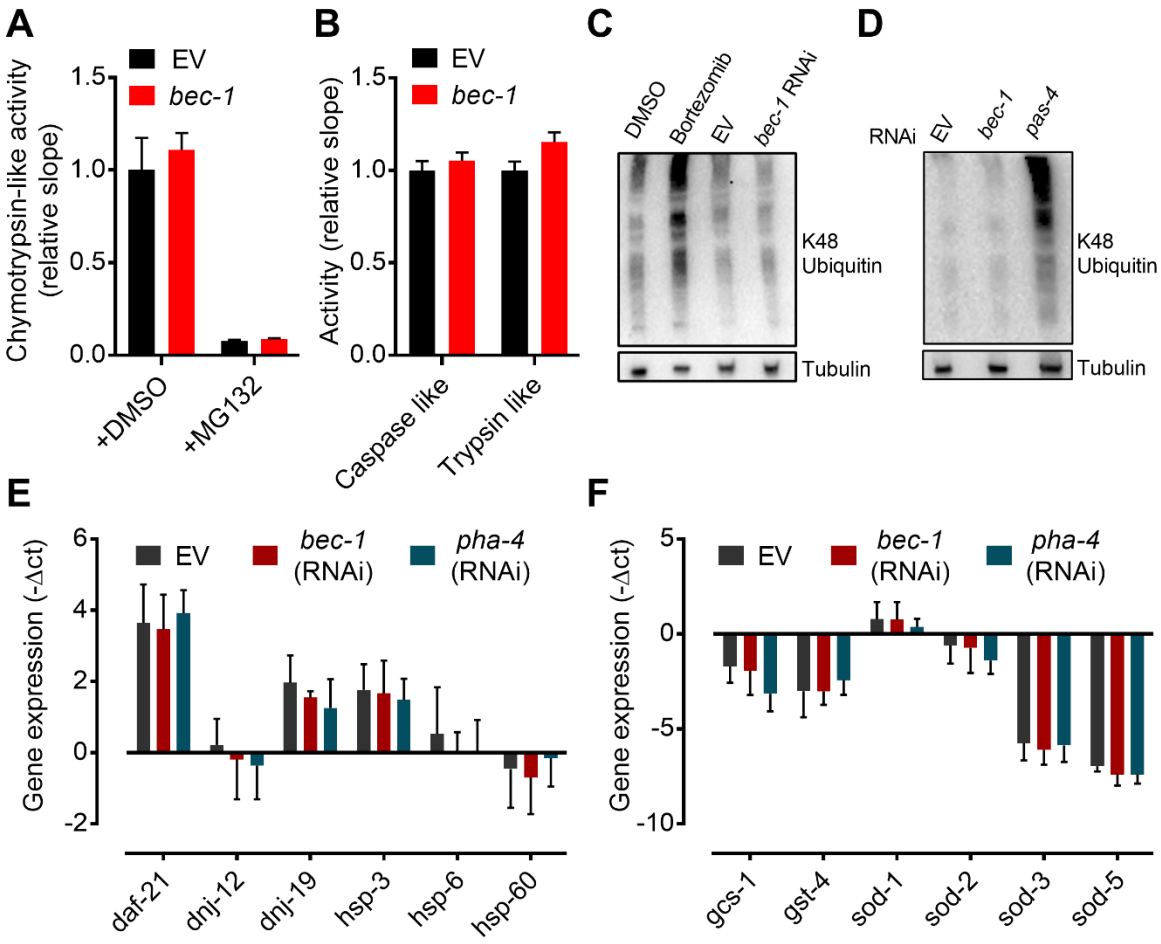


**Figure 26:** ***Other proteostasis mechanisms are not activated following late-life autophagy inhibition.*** (A) Proteasome function measured by chymotrypsin-like activity does not change upon *bec-1* knockdown in *rrf-3*, at day 9, when measured at day 15. MG-132 used as a control proteasome inhibitor, DMSO used as vehicle control. Measurements represent three individual experiments +/- SD. (B) Activity of caspase like or trypsin like cleavage is not changed following *bec-1* inhibition at day 9. Measurments taken at day 15 and represent three individual experiments +/- SD. (C) Representative western blot of K48 ubiquitin chains present in *rrf-3* worms at day 15. Worms were treated either: pharmacologically with Bortezomib (mAB) at day 9, DMSO used as vehicle control; or genetically with RNAi against *bec-1* or *pas-4* at day 9. Ubiquitin chains were detected using a specific K48 ubiquitin chain antibody. (F) Day 15 qPCR quantification of the mRNA levels of selected chaperone and (G) antioxidant genes. RNAi against *pha-4* or *bec-1* was initiated at day 9 in *rrf-3* worms. Data taken from at least three independent experiments +/- SD. Gene expression was determined relative to the housekeeping gene *cdc-42* (-ΔCt).

### 2.2.7 Crosstalk of autophagy inhibition with other longevity pathways

In *C. elegans*, the majority of genes identified as functioning in lifespan determination work through the now canonical lifespan pathways of the *daf-2*/*daf-16* axis, TOR signalling, germline signalling, mitochondrial regulation, and dietary restriction (DR) (Uno & Nishida 2016). Functional autophagy is a requirement for lifespan extension in each of these pathways with its inhibition precluding any longevity benefit (Madeo *et al.* 2015). Consequently, the inhibition of *bec-1* was used to investigate whether the AP nature of autophagy would result in a switch of this dependence paradigm in these longevity pathways late in life.

Lifespan-extending mutations have been identified in numerous genes encoding translation-related proteins where they act to reduce the rates of protein synthesis. These include translation kinases, translation initiation factors, and structural components of the ribosome and ribosomal RNA processing factories (Kennedy & Kaeberlein 2009). Indeed, autophagy and rates of protein synthesis are simultaneously coordinated through the actions of the TOR pathway (Antikainen *et al.* 2017). Therefore, potentially as a consequence of reduced autophagy, cells may modulate their levels of protein synthesis to maintain protein homeostasis. This link was investigated through examination of the transcriptional level of three genes essential for protein synthesis, *ife-2*, *ifg-1*, and *rsks-1*, following autophagy inhibition. Interestingly, there was a strong down-regulation of *rsks-1* expression following *bec-1* but not *pha-4* inhibition from day 9 (Figure 27A). The *rsks-1* gene encodes an S6 kinase (S6K) that is known to be positively regulated by TOR (*let-363*) and is required additively with IFG-1 for the normally high levels of protein synthesis and normal lifespan (Antikainen *et al.* 2017). Further investigation demonstrated that *rsks-1* expression is still significantly regulated through TOR in late-life but that its downregulation is not a feature common to autophagy reduction but to *bec-1* alone, as inhibition of *vsp-34* did not cause a similar decrease in expression (Figure 27B). Additionally, the degree of downregulation was weaker for *bec-1* than for *let-363* inhibition, suggesting *bec-1* may modulate *rsks-1* expression through a mild feedback loop with TOR activity. Reductions in S6K levels either directly or through TOR inhibition extend lifespan when initiated in youth (Pan *et al.* 2007). To probe for a possible contribution of *rsks-1* to *bec-1* longevity both genes were inhibited simultaneously to identify any epistatic effects on lifespan. There was no further extension of *bec-1* longevity when combined with *rsks-1* inhibition but, additionally, no change in MTL was observed following *rsks-1* inhibition alone from day 9 (Figure 27D). This does not preclude a contribution of reduced protein translation to *bec-1* lifespan extension but in combination with the failure of *pha-4* or *vps-34* to affect *rsks-1* expression, strongly suggests that any contribution is at best minimal.

*Figure 27*

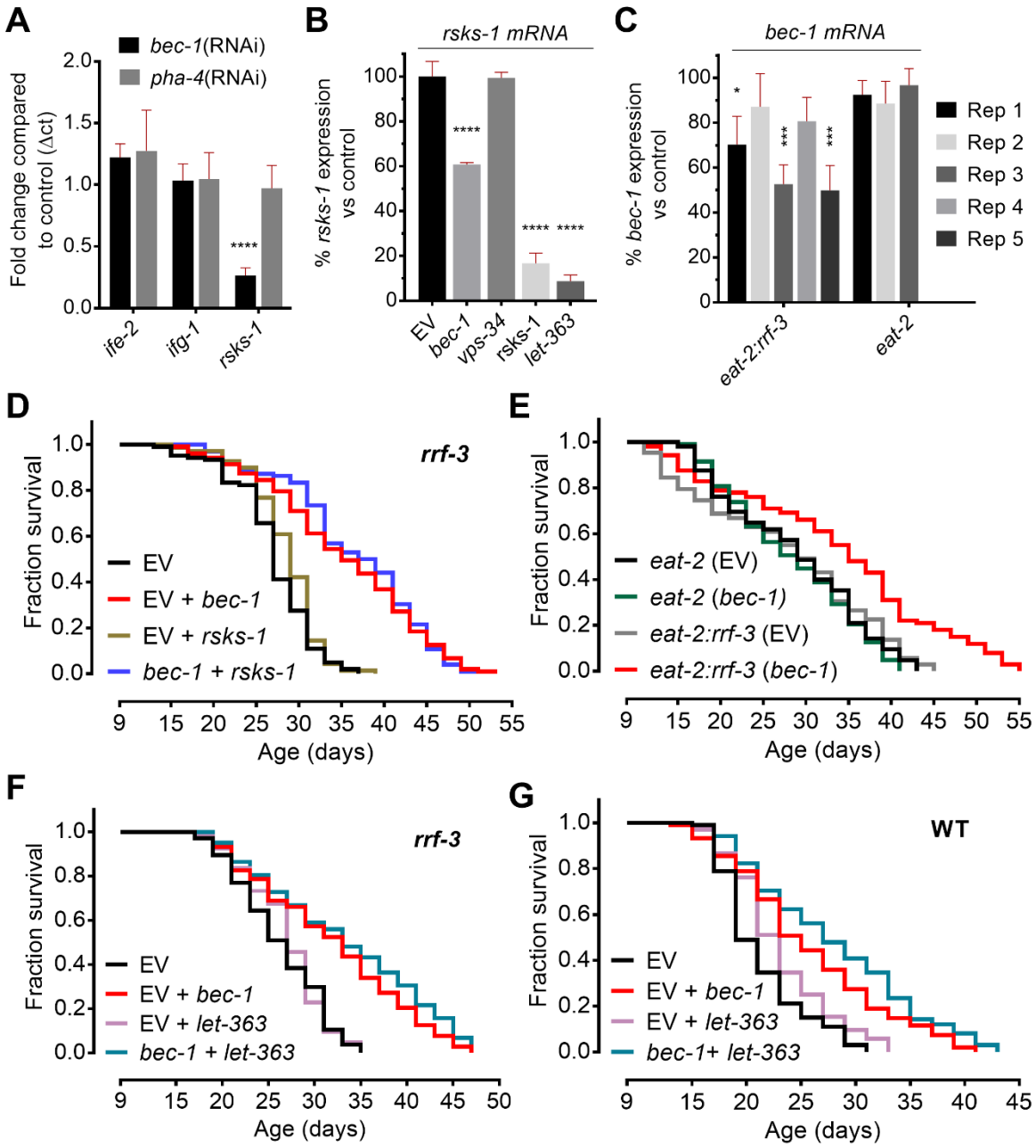


**Figure 27:** ***Interaction of protein synthesis and TOR with bec-1 longevity.*** (A) Inhibition of *bec-1* reduces the expression of the S6K gene *rsks-1* while *pha-4* does not. Other protein translation genes *ife-2* and *ifg-1* were not affected. (B) Inhibition of *vps-34* from day 9 also did not reduce *rsks-1* expression. The level of *bec-1* mediated expression change was significantly smaller than that followed by TOR or *rsks-1* inhibition. (C) Measurement of the degree of gene knockdown in *eat-2* and *eat-2:rrf-3* mutants following day 9 *bec-1* inhibition. No significant knockdown was achieved for normal *eat-2* mutants and there was limited success in the *eat-2:rrf-3* mutants. — For all qPCR measurements the relative mRNA levels of each targeted gene in *rrf-3* worms were measured at day 15. Data represents that of at least four different experiments +/- SD. (* = p<0.05, *** = p<0.001, **** = p<0.0001). (D) Combined RNAi inhibition of *bec-1* and *rsks-1* does not change the MTL of *bec-1* alone. (E) *bec-1* inhibition can extend the lifespan of *eat-2:rrf-3* worms from day 9. Graph is of replicate 3 from panel C. (F) Inhibition of *let-363* has no effect on MTL either alone or in combination with *bec-1* from day 9 in *rrf-3* worms. (G) Day 9 inhibition of *bec-1* further extended the MTL of *let-363* inhibition in WT worms from day 9. Lifespan statistics Supplemental Table 11.

For further investigation, we determined whether *bec-1* inhibition could still extend lifespan in worms undergoing DR which works at least in part through a downregulation of protein synthesis via nutrient deprived inhibition of the TOR pathway (Walker *et al.* 2005). One of the issues with this approach is that inducing DR through reduced feeding is precluded by the need for RNAi bacterial feeding to generate a late-life knockdown. Similarly, the use of mutants defective in pharyngeal pumping rates, such as *eat-2*, to reduced food intake also results in an inefficient knockdown from late-life (Figure 27C). As such, an *eat-2:rrf-3* mutant strain was used to overcome some of these issues, though it was observed that in this strain the level of gene knockdown still varied across experiments and influenced the strength of any longevity effect (Figure 27C & Supplemental Table 11). However, two of five experiments generated a sufficiently strong knockdown of *bec-1* from day 9 to observe a lifespan extension in the *eat-2* DR background (Figure 27E). Dietary restriction is known to work through the TOR/let-363 pathway in worms and inhibition of this pathway leads to an upregulation of autophagy and other stress response genes (Hansen *et al.* 2007). To examine if autophagy inhibition through *bec-1* functions through the same mechanism, a combined knockdown of *bec-1* and *let-363* was performed. Notably, in the *rrf-3* background, *let-363* inhibition failed to extend lifespan either alone or further extend lifespan in combination with *bec-1* (Figure 27F). Surprised at this result, the same combination in WT worms was tested and here a successful further extension of lifespan in both conditions was observed (Figure 27G). The inability to generate a lifespan extension with *let-363* in the *rrf-3* mutant background has previously been observed for young worms where there are opposing effects on lifespan through the two different TOR components TORC1 and TORC2 (Mizunuma *et al.* 2014). Thus, in spite of the dependence of the TOR pathways on autophagy early in life, it was demonstrated that the AP nature of autophagy inhibition remains true in late-life and strongly implies that inhibition of *bec-1* does, at best, minimally mediate its lifespan through TOR signalling.

Having observed an AP phenotype for autophagy in TOR-signalling, it was further examined if this held true for other longevity mutants similarly dependent on autophagy for their lifespan extension early in life, namely in *daf-2* and germline-less *glp-1* mutants. The germline is an important organ for the production of pro-ageing signals and its removal through mechanical or genetic means results in a ~60% increase in lifespan (Antebi 2013). Similar to other longevity pathways this extension is dependent on functional autophagy (Lapierre *et al.* 2013a, 2011). To investigate if *bec-1* inhibition could still extend late-life longevity in the absence of a germline a non-RNAi hypersensitive *glp-1* mutant strain was used, which prevents the development of the gonadal distal tip cells. In comparison to young worms (Lapierre *et al.* 2011), day 9 *bec-1* inhibition strongly increased longevity in *glp-1* mutant worms, by ~35% (Figure 28A). This agrees with the extension seen in WT worms and shows that autophagy is not dependent on and is dispensable for germline-mediated longevity in late-life.

*Figure 28*

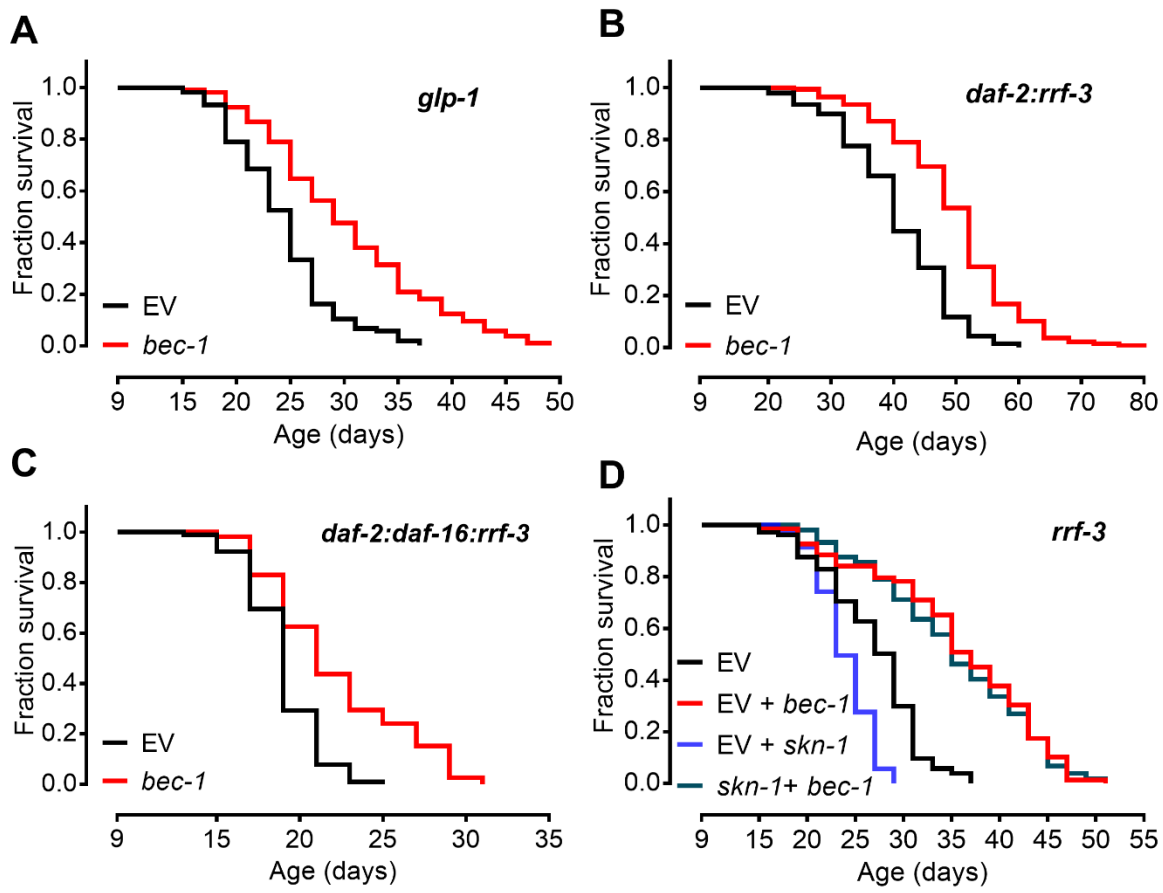


**Figure 28:** ***Inhibition of bec-1 extends lifespan in contexts normally dependent on autophagy.*** (A) Day 9 RNAi against *bec-1* extends the MTL of germline-less *glp-1* worms. (B) RNAi against *bec-1* extends the MTL of *rrf-3*:*daf-2* worms from day 9. (C) Inhibition of *bec-1* in *daf-16:daf-2: rrf-3* triple mutants extends MTL when initiated at day 9. (D) Day 9 knockdown of *skn-1* shortens MTL in *rrf-3* worms. However, combined inhibition with *bec-1* rescues this phenotype extending MTL to the same degree as *bec-1* inhibition alone. Lifespan statistics presented in Supplemental Table 12.

Much like the TOR pathway, mutations in the IIS pathway upregulate stress response genes and activate autophagy, though the IIS pathway works primarily through the action of the FoxO transcription factor *daf-16* (Russell & Kahn 2007). When autophagy is inhibited in young *daf-2* mutant worms the entire lifespan extension normally mediated by this mutation is abrogated (Lapierre *et al.* 2011, Hansen *et al.* 2008). In stark contrast to this, a significant (~25%) lifespan extension by *bec-1* inhibition from day 9 was discovered (Figure 28B). DAF-2 mediates its longevity effects through DAF-16, therefore to determine any contribution of *daf-16* activation in the mode of action for *bec-1* lifespan extension, *bec-1* was inhibited in *daf-2:daf-16:rrf-3* mutant worms. These worms have no DAF-2 signalling but cannot upregulate DAF-16 and therefore have normal lifespans. In agreement with that seen for *daf-2* alone, a ~25% increased in MTL following *bec-1* inhibition from day 9 was observed (Figure 28C). The degree of lifespan extension in both cases is not as strong as that seen for *bec-1* in

*rrf-3* mutant worms. This suggests that *bec-1* may be modulating some of its lifespan extending activities through reduced *daf-2* signalling independent of *daf-16* (Yu & Larsen 2001). One of the main routes of this *daf-16* independent signalling acts through SKN-1 in parallel to DAF-16 (Ewald *et al.* 2015). Interestingly, SKN-1 is also negatively regulated by the TOR pathway and in turn upregulates pro-survival stress response genes (Blackwell *et al.* 2015). Curious if *bec-1* inhibition could be upregulating SKN-1 and its transcriptional targets to increase lifespan, both genes were inhibited simultaneously from day 9. As reported in young worms (Okuyama *et al.* 2010, An & Blackwell 2003), inhibition of *skn-1* resulted in a shortening of lifespan from day 9. Surprisingly though, *skn-1* inhibition did not reduce the MTL following *bec-1* inhibition as would be expected for an epistatic interaction but instead *bec-1* inhibition appears to be able to compensate for the loss of *skn-1* (Figure 28D). This could suggest that inhibition of autophagy in late-life triggers some of the protective pro-longevity responses mediated normally by SKN-1 during ageing but does so downstream of SKN-1, perhaps regulating some of SKN-1's pro-survival response factors directly.

## 2.3 Results summary

In summary, attempts to identify AP epigenetic modifiers of lifespan through a bottom-up approach largely failed, although the roles of H2Bub and H3K27me2 warrant further investigation. The top-down RNAi screening approach proved to be highly successful, identifying 31 novel regulators of late-life longevity. Both *pha-4* and *bec-1* were found to function through autophagy to modulate-lifespan and their inhibition extended both health and longevity. The evidence suggests that autophagy becomes dysfunctional over age and its inhibition may alleviate this burden. Additionally, no evidence for an activation of other proteostasis mechanisms following autophagy inhibition was found nor evidence of apoptosis, necrosis or mitophagy being involved in the lifespan mechanism. Moreover, autophagy still exerts an AP effect in longevity mutants normally dependent on this process and does so largely independently of their pro-longevity signals. Indeed, beyond the removal of toxic autophagosome accumulation, autophagy may be mediating some of its longevity effects by negatively affecting a pro-longevity signal downstream of SKN-1.

# 3 Discussion

## 3.1 Producing age-synchronous worms for late-life analysis

When investigating the process of ageing there are two main approaches. Either one takes the youthful condition as a baseline, observing subsequent changes and identifying those that are causal, or one takes exceptionally old individuals living well beyond the median lifespan and interrogates what factors stand out that allowed these individuals to live for such a long time. Examining gene expression or phenotypic changes over time is an example of the first e.g. (Hill *et al.* 2000) while investigating the genome of centenarian humans an example of the second (Brooks-Wilson 2013). What is perhaps surprising about both approaches is there has been, prior to our investigation, little research performed in the period between these two points. Some of the notable exceptions to this are studies in middle-aged mice using a DR regime or parabiosis (fusing the blood circulation system) (Conese *et al.* 2017, Harrison *et al.* 2009). Given that the evolutionary biology theories of ageing have for 60 years predicted that the period between adult onset and late-old age is critical, this oversight is at first perplexing. As with many things, it ultimately comes down to availability and cost. Outside of humans, it is quite rare to have extremely old animals living in the wild. Even in managed facilities such as zoos, the ability to experiment on usually rare and endangered species is obviously limited. This has restricted the capacity of gerontologists to adequately investigate longevity in wild populations, though some efforts have been made, particularly in relation to mortality rates (Nussey *et al.* 2008). Availability issues are also problematic for laboratory research. Housing, feeding and maintaining lab animals into middle age are not insignificant. For mice, as an example, the cost of housing mice to week 6 vs week 12 can nearly double and for experiments, in middle ages (18–24 months, equivalent to 56–69 human years (Flurkey *et al.* 2007)) the cost is expected to rise to between 12-20 times (Jackson *et al.* 2017). These cost issues are one of the reasons that age of the mice studied is one of the most variable characteristics in disease research (Jackson *et al.* 2017).

Gerontologists have typically avoided these issues through the use of relatively inexpensive model organisms such as *C. elegans* and *Drosophila*. Nevertheless, the challenges in obtaining sufficient quantities of either species in an age-synchronised manner have resulted in the vast majority of experiments beginning their examinations in youth, rarely progressing to middle or older age with more than a very limited total sample number. In *C. elegans* gerontologists have typically used either temperature induced sterile strains e.g. Lund *et al.* 2002, or the reproductive inhibitor FuDR e.g. Kenyon *et al.* 1993, to maintain age-synchronicity. Sterile strains introduce their own variability when cross comparing effects between different experiments, as different sterile backgrounds are regularly used. Additionally, their use excludes the ability to investigate the role of germline to soma signalling,

which has become increasingly relevant to ageing (Antebi 2013). FuDR has been linked to direct effects on lifespan, on gene transcriptional programmes, and the up-regulation of heat-shock proteins and proteostasis (Brunquell *et al.* 2014, Feldman *et al.* 2014, Aitlhadj & Stürzenbaum 2010). Its use also introduces biases against identifying germline-dependent longevity regulators (Ni *et al.* 2012) making it unsuitable for longevity studies. These issues have prompted ageing researchers in *C. elegans* to shift away from such methods and to remove offspring through laborious manual means e.g. (Greer *et al.* 2010, Lapierre *et al.* 2011).

In our initial approach to creating aged *C. elegans* for analysis, we used both FuDR and large-scale liquid culture as devised by the Johnson lab (Fabian & Johnson 1994). The limitations of this system became quickly apparent. The system suffered from regular contamination and worms were subjected to high stress; osmotic from sucrose cleaning, mechanical from high shaking speeds, and environmental from bacteria and fungi in the culture. These factors undoubtedly influenced the results obtained with this method, including the upregulation of mechanical stress genes, upregulation of the innate immune response (Supplemental Table 3), and regulation of heat-shock proteins (Figure 6D). Through devising a system to overcome the issues and the limitations of the standard methods of large-scale *C. elegans* culture, we created a method that significantly outperforms current methodologies (Lionaki & Tavernarakis 2013b). When working with non-sterile worms the biggest challenge to maintaining an age-synchronous culture is the efficient removal of offspring. Previous reports have achieved results as low as 1% using filtration methods but then require manual attention soon after e.g. Maures *et al.* 2011. A recent development of a microfluidics platform for worm-lifespan determination has overcome these issues by providing a continuous flow that filters out younger worms, however *in utero* larval hatching still remains an issue (Xian *et al.* 2013). In contrast to previous methods, we show a greater than 99.99% removal of larval worms from liquid culture over an 8 day period (Figure 12, A-D). The use of gravity sedimentation in a non-osmotic and non-toxic Percoll solution is the essential element to the success of this novel method. Percoll has been used previously in combination with sucrose and centrifugation to remove debris from a worm culture (Fabian & Johnson 1994) and to estimate worm densities (Reina *et al.* 2013). In the developed liquid culture protocol, Percoll was discovered as an effective way to remove egg clumps and bagging worms, which together limited the number of subsequent larvae, thereby reducing food consumption, and prevented a single *in utero* hatch from rapidly de-synchronising the culture. Because this method still has a small error rate, the culture is consistently sedimented as long as it remains in liquid culture to avoid rare events from overwhelming a particularly long experiment. This novel technique enables the large-scale culture of millions of worms simultaneously with modest effort, allows for the use of WT strains without drugs, significantly reduces stress on the worms, and finally allows for the investigation of

ageing biology in the period that is ultimately relevant to AP and the development of ageing, namely mid-life.

## 3.2 Identifying epigenetic factors in longevity

As part of the top-down approach to identifying pleiotropic epigenetic factors in longevity, the change in abundance of numerous histone modifications over life were examined. In general, there was a decline in both activating and repressing modifications with age. Running counter to this trend was a detected increase in the transcriptional activation mark H3K4me3 by ~250% and increases in H3K18ac (~95%), H3K36me2 (~50%) and H4R3me2 (~110%) (Figure 3 & 4 & Table 1). While these trends were replicated between western blots and mass spectrometry, a few of the observed trends ran counter to published data for other species. The brains of aged senescent prone mice show a reduction in H3K36me3 levels and an increase in H3K27me3 levels (Wang *et al.* 2010a), counter to our observations. However, other studies on cell populations have shown decreases in H3K27me3 levels as the cells age (Bracken *et al.* 2007). This could suggest context specific results for this mark. H3K4me3 levels were shown to be slightly downregulated between young and old flies (Larson *et al.* 2012) in contradiction to the 2.5 fold rise seen here (Figure 3A). However increased H3K4me3 levels are linked to a pro-ageing phenotype (Greer *et al.* 2010) and increased/broader occupancy of H3K4me3 at promoters is seen in aged hematopoietic stem cells (Sun *et al.* 2014), suggesting a possible sign of faster ageing in the liquid culture. The degree of H3K4me3 methylation shown here may also be an artefact of the culturing conditions used to generate these samples. Additionally, this mark could not be identified by MS analysis preventing a secondary validation of this change.

Perhaps the most interesting change observed over life was the strong decline in H2Bub levels that was followed by a rise in late time-points (Figure 5A). This phenotype could be replicated to an even greater extent in long-lived *daf-2* mutant worms and was found to be downregulated upon dauer formation (Figure 5, A & B). H2Bub is linked to increased gene transcription and elongation through regulation of the SET-1 and DOT-1 histone methyltransferase complexes (Nguyen & Zhang 2011, Dover *et al.* 2002). DOT-1 specifically regulates H3K79me2 levels which were observed to decline in a pattern mirroring that of H2Bub (Figure 3A). Additionally, the RNAi screen identified *dot-1.1* as a functional AP gene, possibly suggesting an involvement of the H2Bub – H3K79me2 transcriptional control axis in late-life longevity.

Beyond H2Bub and *dot-1.1* we also identified a novel reduction in H3K27me2 levels over life in worms (Figure, 3C & 4C). This finding was of particular interest as *jmjd-1.2* was also identified as an AP candidate gene from analysis of RNAseq over life (Figure 10B). JMJD-1.2 is a histone demethylase that acts on both repressive histone marks H3K9me2 and H3K27me2 (Vandamme *et al.* 2015). As both

of these marks show decreased abundance with age, likely resulting in a relaxation of gene suppression, this suggests the possibility that during ageing the control of gene expression becomes less rigid. Thereby, genes that should remain inactive become activated leading to disruptions in the homeostasis of the whole organism. The idea of an age-related deregulation of gene transcriptional control being causal to ageing has existed for some time (Pal & Tyler 2016) and suppression of H3K4me3 or preservation of H3K27me3 have been proposed to extend longevity partially through a similar mechanism (Booth & Brunet 2016). A previous RNAi screen for actions of histone demethylases in longevity had identified a minor lifespan increase following *jmjd-1.2* inhibition from day 0 (Maures *et al.* 2011). Conversely, a separate study shows that high levels of *jmjd-1.2* correspond with longevity in mice and its inhibition shortened lifespan in electron transport chain mutants (Merkwirth *et al.* 2016). JMJD-1.2 activity is linked to the activation of mitochondrial stress response pathways and its upregulation in our data set could reflect increased mitochondrial stress in older age. Despite the promise of these findings, RNAi inhibition from day 9 in WT worms failed to produce any lifespan extension (Figure 11, A & B). It is possible that its failure to extend longevity was due to a weak lifespan extension effect that could not be fully realised in the WT background. A similar line of reasoning suggests that many of the genes considered as AP candidates may not have been detected in a WT background, owing to reduced knockdown efficiency decreasing already minor lifespan extensions. Of the 19 candidate longevity genes tested for longevity (Supplemental Table 5) 13 were also part of the RNAi screen and only one, *hda-6*, showed an increase in longevity in the RNAi hypersensitive *rrf-3* mutants (Figure 13 & Table 5). The six untested genes were *hsp-16.2, ttr-24, ttr-12, rfp-1, rpn-1,* and *rpn-2.* As the *rpn* genes shorten lifespan their effect would not have been detected by the screening methodology anyway. This strongly suggests that the bottom-up approach to identifying novel AP epigenetic regulators of longevity was identifying excessive false indicators leading to inaccurate assumptions. Nonetheless, the identification of *hda-6* suggests some success to this approach. Additionally, neither of the best AP candidates *jmjd-1.2* or *rfp-1* (which is the likely H2B ubiquitin ligase in *C. elegans*) were examined in the screen owing to contamination and an absence of targeting dsRNA bacteria respectively. As both histone marks function to control transcriptional rates, they would make excellent points of action for AP. Both H2Bub and H3K27me2 have been shown to have a vital role in neural development and neuronal health where their absence leads to neurodegeneration (Von Schimmelmann *et al.* 2016, Mohan *et al.* 2014, Subbanna *et al.* 2014, Weake *et al.* 2008). Given this, testing for late-life longevity extension in *rrf-3* mutants hypersensitive to RNAi particularly in neurons could still identify an AP function for both genes.

## 3.3 Screening for AP genes in the post-reproductive period

The period following the onset of reproduction is of utmost relevance to the ageing process. It is during this period that the actions of natural selection weaken and the first signs of age-related deterioration begin to appear (Milne 2006). Recent evidence shows that the disruption in protein homeostasis begins at day 7 in WT worms coinciding with a sharply reduced activation of the cytoprotective heat shock and unfolded protein response (Ben-Zvi *et al.* 2009). Additionally, the severely detrimental, age-related diseases; neurodegeneration, cancer, diabetes, cardiac disease, and stroke all have a strong genetic basis and primarily act in later life. Analysis of the 2q22.3 locus found a region that carries risk alleles for all these diseases but shows no evidence for negative selection despite the obvious detriment of these alleles (Kulminski *et al.* 2016). A Recent genome-wide association study (GWAS) in humans has also highlighted that the relative risk factor for an allele to a specific disease is often highly correlated with chronological age (Rodríguez *et al.* 2017), exactly as predicted by AP. Ageing pathways have the potential to act as control points for the treatment of these diseases (Partridge 2014) and they themselves show some signs of pleiotropic effects (Leroi *et al.* 2005). This means that treatment based on evidence from early life administration may not be effective or beneficial if administered later in life. Even more troubling is that many longevity pathways have to be inhibited in early life to have any subsequent longevity effect, which brings associated costs to development and fecundity with treatment e.g. *clk-1* mutations (Wong *et al.* 1995). For these reasons, it is increasingly imperative that a full understanding of any longevity intervention be assessed across life. However, despite this rationale and the potential abundance of pleiotropy in ageing, there has been little investigation into its relevance to ageing and age-related diseases.

This thesis and the associated publication (Wilhelm *et al.* 2017) mark a first foray into identifying AP ageing genes in late-life. Our screen of 800 chromatin and transcription-related factors returned an impressive 31 genes that have previously never been associated with longevity (Figure 13 & Table 5). While only five of those were formally validated through traditional lifespan assays (Figure 14), the success of that validation and the identification of early life fitness costs for 19 of the 31 genes strongly suggests that the majority identified will act as AP modulators of longevity. The success rate of this screen is comparable with that performed with lethal genes from the L4 period onwards, which identified 2.3% of the examined genes as exhibiting AP (Curran & Ruvkun 2007). If the success rate of these screens is applied conservatively to the whole genome (2.5%), it suggests 400 or more AP genes remain to be discovered. Combined with the need for ageing and disease treatments effective in mid to later life, the therapeutic potential of such AP gene identification should not be underestimated.

Beyond the identification of *pha-4* and *dot-1.1* (discussed below), another finding from the RNAi screen was the identification of six members of different RNAi silencing pathways *eri-1, ego-1, drh-3, hrde-1, nrde-1,* and *rde-1* (Figure 13 and Table 5). All of these genes, except for *eri-1,* are positive

regulators of RNA mediated transcription silencing (Fischer 2010). That these genes were identified as AP regulators of longevity is unexpected, as gene regulation through silencing, or transcription dampening by miRNAs and siRNAs is an essential feature of normal cellular transcriptional control. Indeed, the loss of transcriptional control is considered one of the defining features of ageing (Budovskaya *et al.* 2008). However, miRNAs can act as both positive and negative regulators of different pathways. Studies on miRNAs in ageing identified different miRNA classes that both promote and inhibit longevity (De Lencastre *et al.* 2010) and in fact identified more negative-regulators (i.e. longevity is extended upon their inhibition) than positive ones. It is therefore conceivable that the balance between the pro and anti-longevity effects of miRNA disruption shifts in late-life. This is more likely in older worms, as generally the inhibition of specific miRNAs produces no longevity phenotype and more so in later life (De Lencastre *et al.* 2010), thus further reducing any anti-longevity effects. Included in the group of negative longevity regulators is *mir-34,* which exhibits an adult-onset expression pattern in *Drosophila* brains and suppresses autophagy through *atg-9* (Yang *et al.* 2013). *mir-34* ectopic expression induced apoptosis, cell-cycle arrest and senescence (Hermeking 2010). Additionally, a *mir-34* loss-of-function mutation delayed age-related physiological decline, extended lifespan, and increased resistance to heat and oxidative stress, particularly in neurons. In work subsequent to that presented in this thesis, the suppression of autophagy in the neurons was found to be sufficient to generate the longevity phenotype (Byrne *et al.* 2017, Wilhelm *et al.* 2017). It, therefore, seems highly plausible that repression of *mir-34* activity could recapitulate *bec-1*'s late-life longevity via its modulation of autophagy in the brain, thus granting a longevity boost to late-life RNAi pathway inhibition. Another possibility suggests that the identification of members of the RNAi silencing pathway is an artefact due to the use of the enhanced RNAi strain *rrf-3*. The *rrf-3* mutation enhances somatic RNAi (Simmer *et al.* 2002) and it is possible that this action itself is lifespan shortening due to a too severe reduction in gene expression. As *rrf-3* mutants live longer than WT this implies that enhanced RNAi efficiency is itself AP, beneficial early in life but detrimental later. When combined with inhibition of RNA silencing pathways the total balance in gene regulation is then possibly restored towards a more normal level thus slightly boosting longevity. While this possibility is less likely it cannot be ruled out.

## 3.4 Inhibition of pha-4 and bec-1 extends lifespan through autophagy

At first glance, the autophagic pathway appears to be a poor candidate for an AP longevity mechanism. Autophagy acts as an essential catabolic process in development, growth and subsequent cellular homeostasis. It is intimately linked to ageing, required for almost all known lifespan extending processes and promotes neuronal preservation when functioning (Lapierre *et al.* 2013a, Wang *et al.*

2012b, Rubinsztein *et al.* 2011, Ross & Poirier 2004). None the less, multiple lines of recent evidence show that whether autophagy is beneficial or detrimental depends on its context and whether it is over- or under-activated (Thorburn 2014, Shintani 2004). Specific examples of this can be found across different organs and biological systems. In hypoxia-induced autophagy, excessive autophagy induction leads to reperfusion injury (Wang *et al.* 2017, Decuypere *et al.* 2015). In atherosclerosis, excessive autophagy leads to the death of smooth muscle and endothelial cells (Vindis 2015). In muscle homeostasis and muscular dystrophies, either excessive or insufficient autophagy is harmful to health and promotes disease (Sandri *et al.* 2013, Grumati & Bonaldo 2012). In diabetes, chronic activation of autophagy can lead to neuropathies and retinopathies (Di Rosa *et al.* 2016, Yerra *et al.* 2016). Finally, in neurodegeneration, where insufficient or excessive autophagy leads to a progressive decline (Jaeger & Coray 2009). What is clear from all these studies is that autophagy must remain functional and controlled to retain its status as a beneficial influence on health.

In the study here, we find that the post-reproductive inhibition of *pha-4* and *bec-1* extend lifespan and do so through reduced autophagy. Multiple lines of evidence point to this conclusion. As in young worms (Lapierre *et al.* 2011) both autophagy genes, *bec-1* and *unc-51* remain regulated by PHA-4 late in life (Figure 15A). Further, the formation of LGG-1::GFP foci was strongly reduced upon *pha-4* inactivation and was comparable in strength to *bec-1* inhibition (Figure 5, B & C). Inhibition of *pha-4* showed markedly similar phenotypic effects to those seen with *bec-1* (Figure 20, A-E) and both genes demonstrate a mirrored AP lifespan behaviour albeit with differing strengths (Figure 19). Inhibition of other members of the autophagy nucleation complex also extends lifespan in an AP manner (Figure 22, A & B) and the alternative BEC-1 pathways of apoptosis and necrosis induction do not appear to be relevant for the lifespan extension (Figure 22, C-E). Additionally, TEM images of autophagosomes over age show increased structural and functional disruption with the formation of multi-laminar vesicles packed with undigested material over age (Figure 23, A-C). Inhibition of *pha-4* and *bec-1* prevents the formation of these structures, and the few autophagosome detected had a normal appearance (Figure 24, A & B). The over-age disrupted phenotype is very similar to that seen for the *C. elegans* autophagy mutants *epg-3* and *epg-4* (Tian *et al.* 2010) and suggests that these multi-laminar vesicles can possibly no longer fuse with endosomes and lysosomes and thus are unable to degrade their cargo. Combined, this data indicates that both genes likely mediate their effects on lifespan through a common mechanism, namely autophagy, and strongly suggests that autophagy becomes increasingly dysfunctional with age and probably toxic. Its inhibition is therefore actually a net positive gain for overall health and longevity. Interestingly, the RNAi screen identified *hda-6* as a potential AP longevity gene (Figure 13 & Table 5). In a recently discovered role, HDAC6 controls the maturation of autophagosomes (Lee *et al.* 2010), possibly linking its longevity action to the proposed alleviation of toxic dysfunctional autophagy.

Additional evidence for dysfunctional autophagy comes from protein MS data. The identification of TTR proteins as rising in abundance over life indicates a dysfunction in autophagic degradation (Figure 6A). TTR proteins are neuroprotective for Alzheimer's disease when properly controlled by proteolytic mechanisms (Schwarzman *et al.* 1994). However, these proteins are subject to aggregation and can be disease-causing when left to accumulate (Johnson *et al.* 2005, Andrade 1952). Their rise in abundance over time, in comparison to their normally fine-tuned regulation, strongly suggests a shift towards an aggregation phenotype that could be neurotoxic. Additionally, the identification of a decline in activity in five VHA proteins over life implies a reduced acidification of lysosomes resulting in reduced autophagic degradation (Figure 6D). Further support for the argument of toxic autophagy came from research subsequent to the findings in this thesis (Byrne *et al.* 2017, Wilhelm *et al.* 2017). Here, it was shown that in later-life autophagy does become progressively defective in its degradative capacity, that the lifespan benefits of autophagy are limited to the stages up to the formation of the vesicle nucleation complex, and that the inhibition of autophagy extends lifespan through the neurons where it is neuroprotective (Wilhelm *et al.* 2017). In this context, it is clear that autophagy switches from beneficial to detrimental with age and exerts it AP phenotype through its actions in the neurons of *C. elegans.*

### 3.5 Autophagy inhibition and proteostasis

The question arises, without a recycling machinery how can animals compensate for accumulating proteins and dysfunctional byproducts. Alongside autophagy, a critical component of proteostasis in ageing is the ubiquitin-proteasome system (UPS). Numerous lines of evidence describe a decline in proteasome function in ageing cells, tissues and organisms (Saez & Vilchez 2014). Remarkably, studies in centenarians reported increased proteasome activity, which resembled that of their younger counterparts (Chondrogianni *et al.* 2000). Additionally increased proteasome capacity is observed in several exceptionally long-lived species including the naked mole rat (Perez *et al.* 2009). The selective degradation of proteins can proceed through either autophagy or the UPS with the distinction made by the type of ubiquitin chain marking proteins for recycling. K48 chains target proteins to the proteasome while K63 chains target them to autophagy (Dikic 2017). Recent evidence shows a crosstalk between both pathways whereby autophagy can be activated in response to proteasome inhibition (Lilienbaum 2013). However, no such mechanism has been found that allows for a compensatory activation of the proteasome when autophagy is blocked under normal cellular conditions (Wang & Wang 2015). Nevertheless, autophagy has been shown to regulate the turnover rates of proteasome subunits in plants and mammals (Cuervo *et al.* 1995, Wen & Klionsky 2016) and its blockage under stress conditions did induce proteasomal activity and the expression of proteasomal

subunits (Wang *et al.* 2013). As ageing is itself a stress condition, the possibility of compensation by the UPS system following autophagy inhibition was intriguing. Testing this, however, revealed that the levels of K48 ubiquitin chains do not change upon *bec-1* RNAi, nor do the activities of proteasomal cleavage, suggesting that the effects of BEC-1 depletion are not mediated by the UPS (Figure 26, A-D). As seen in these activity measurements the proteasome is still actively working in older age (day 15) and there were strong lifespan-shortening effects upon day 9 inactivation of UPS components (Supplemental Table 5). Furthermore, testing the mRNA expression of the core proteasome subunits *pbs-6* and *pas-4*, as well as the two proteasome regulatory subunits *rpn-12* and *rpt-3* did not indicate a significant change in the mRNA expression level of these proteasome subunits upon post-reproductive inactivation of *pha-4* or *bec-1* (Figure 29). This is in contrast to the rise in protein abundance over life seen for RPN-1, RPN-2 and RPN-12 by mass spectrometry (Figure 6C). The rise in abundance for these proteins was particularly pronounced in very late ages, day 20 onwards, possibly reflecting a failure of autophagy to degrade the proteasome, leading to increased UPS components in the cell without a corresponding increase in mRNA expression. Increased UPS activity with age has been observed previously in *C. elegans* (Vilchez *et al.* 2012) and could be reflecting this lack of turnover capacity from ineffective autophagy. Combining these two findings strongly indicates that day 9 inactivation of autophagy does not alter proteasome activity but that its degradation insufficiency in later-life may be increasing the quantity and therefore activity of the proteasome. Furthermore, it shows that the proteasome is more vital for health in older age than autophagy and suggests its activity may already be upregulated and be enough to compensate for autophagy's absence.

*Figure 29*

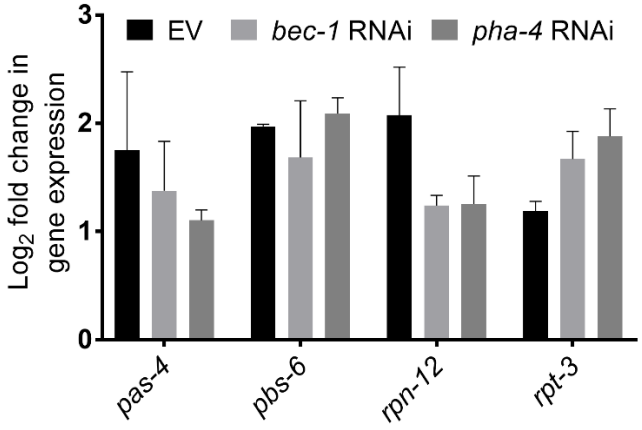


**Figure 29:** ***Proteasomal gene expression following autophagy inhibition.*** Two of the core proteasome genes *pas-4* and *pbs-6* and two regulatory subunit genes *rpn-12* and *rpt-3* do not show significant changes in expression following autophagy inhibition. RNAi was initiated at day 9 and samples tested at day 15. $Log_2$ values of gene expression compared to internal control +/- SD as measured by qPCR.

In experiments subsequent to this thesis, results showed that the autophagic flux is already impaired late in life (Wilhelm *et al.* 2017). As such, the situation is not worsened following inactivation of autophagy genes and proteostasis is not further impaired. This was reflected in the absence of

negative effects upon late-life inactivation of all tested autophagic flux genes, with the exception of *lgg-1*, on *C. elegans* lifespan (Wilhelm *et al.* 2017). This conclusion is further supported by TEM analysis of autophagic structures over life, which display an aberrant accumulation of multiple lipid layers in old age (Figure 23, B & C) similar to those seen in autophagy mutants (Tian *et al.* 2010). These are removed following *pha-4* or *bec-1* inhibition and some normal autophagosomes can be observed (Figure 24, A & B) possibly allowing for a minimal level of functional autophagy, which, therefore, remains cytoprotective. Alternatively or additionally, cells could respond with enhanced sequestration of damaged or misfolded proteins to reduce their cytotoxic effects (Walther *et al.* 2015, Escusa-Toret *et al.* 2013). These potential compensatory mechanisms represent promising directions for further research.

### 3.6 Late-life dysfunctional autophagy, adaptive or non-adaptive ageing?

Despite the success of AP theory in explaining the evolutionary origins of ageing, there is relatively limited evidence for genes behaving strictly according to its predictions. The majority of proposed AP genes do not exhibit obvious trade-offs with fertility in early life. A good example of this is p53, which is considered a model AP gene by averting cancer but also suppressing the division of stem cells, therefore preventing renewal and replacement of deteriorating tissues during ageing (Rodier *et al.* 2007). When deleted in mice, p53 allows for adult development but with significantly increased cancer risk (Donehower *et al.* 1992). While cancer and death are obviously detrimental to fitness, this effect arrives late enough to not necessarily effect immediate reproductive capacity, which is in conflict with AP predictions. Additionally, pleiotropic gene actions should exert direct pleiotropic effects and these effects should not be attributable to other genes that can modify its behaviour or activity, as is the case with p53 in mice where its activity is modulated by SIRT1 (Ungewitter & Scrable 2009). Even the AP genes identified in an L4 screen of otherwise lethal genes (Curran & Ruvkun 2007) do not fit Williams' predictions (Williams 1957). While obviously detrimental to fitness when inhibited, their detrimental effects appear while still under the full pressure of natural selection contravening the AP model. Williams' theory says that AP genes arise through the weakening force of natural selection over age, thus allowing a fitness trade-off to exist that is positively selected. Therefore, any gene that exhibits a positive lifespan effect when inhibited before reproductive onset cannot truly be considered as AP. In this context, the inhibition of autophagy does appear to be a genuine example of an AP process mediated by AP genes. Inhibition of autophagy genes prior to reproductive onset induces embryonic lethality or sterility and reduces lifespan (Figure 17E, 19 & 22B). There is a clear shift in the beneficial to detrimental relationship as observed by the time-dependent increase in longevity benefits for both *pha-4* and *bec-1* (Figure 19 & Supplemental Figure 4 & 5). This behaviour is exactly as

is predicted by AP theory for genes with continuous expression profiles (Leroi *et al.* 2005). However, analysis of the genomes of different organisms has, as yet, not discovered an AP signature for autophagy genes (Everman & Morgan 2018, Carter & Nguyen 2011, Qian *et al.* 2012). The question remains, how does autophagy go from beneficial to detrimental over life? In its general decline in functionality over time, autophagy appears to act in a manner similar to predictions of classical limitation theories of ageing. However, autophagy is, in essence, a repair process that should be maintained at optimal efficiency through evolutionary selection. That it is not suggests there could be a programmed element to its deterioration. Programmed ageing predicts that organisms will actively cause the deterioration of vital cellular processes (Goldsmith 2014, Longo *et al.* 2005). Autophagy would appear to be a good candidate for such programming as its failure is not actively compensated for by other biological processes (Lilienbaum 2013). Autophagic failure will be particularly detrimental to survival under stress conditions when resources are low, removing old less reproductively viable organisms from the population favouring the survival of the younger fitter members. In the theory of programmed ageing, such a scenario is exactly what gives rise to the evolution of a programmed ageing paradigm (Longo *et al.* 2005). Once established, such a programmed system would be hard to overcome through evolutionary adaptation. This is because autophagy requires multiple genetic components, most of which are essential for fitness, all acting in concert. Thus, adaptive changes would nearly always be detrimental in youth and selected against, precluding the removal of its detrimental effects in later life. The mechanism behind the damaging effects of late-life autophagy would be different for a programmed vs a non-programmed ageing system. A programmed system would work to actively create a declining efficiency of autophagy with age, possibly through modulation of the efficiency of lysosomal acidification genes. This would lead to death through the absence of the beneficial effects of autophagy. In a non-programmed (AP) system, autophagy would become destructive when over-activated by the increasing proteostasis demand with age resulting in the eventual collapse of the system, which is itself toxic. This would be actively rather than passively detrimental, accelerating the ageing process. In the programmed scenario then autophagic inhibition should not lead to an increase in lifespan but rather to an accelerated collapse in health. In an AP scenario, autophagic inhibition could relieve the system of the direct, detrimental burden of activated autophagy feeding into a dysfunctional system. In this and subsequent studies, it is the latter scenario that is observed (Figure 15B, 17A & 24, A & B) (Wilhelm *et al.* 2017), meaning autophagy is an AP mechanism of ageing and not a programmed one. That autophagy inhibition is a novel AP mechanism raises the question of whether a similar effect will be observed in other species, as AP effects are predicted to be more species-specific than programmed effects. That autophagy acts as a double-edged sword in neurodegeneration in numerous species (Shintani 2004, Thorburn 2014,) suggests this

is a strong possibility and that the findings of this thesis could be relevant for the treatment of neurodegenerative diseases in humans.

### 3.7 PHA-4 has pleiotropic effects beyond autophagy

Given that autophagy is the underlying mechanism behind *pha-4* and *bec-1* mediated longevity, the difference in the strength of the lifespan effects for both genes and the absence of a lifespan extension for *pha-4* in the WT background required explanation. Epistasis experiments revealed a reduction in *bec-1* mediated lifespan following combinatorial inhibition with *pha-4* from day 9 (Figure 18, A & B). If *pha-4* inhibition mediated its lifespan-extending effects through a process other than autophagy, we would observe additive lifespan effects upon simultaneous inactivation of *pha-4* and *bec-1* at day 9 (Gems *et al.* 2002). The shortening of the *bec-1* mediated lifespan effect instead implies that *pha-4* has effects beyond autophagy that are detrimental. As such, the late-life inhibition of *pha-4* results in competing effects on lifespan: beneficial effects through the disruption of *bec-1* and autophagy, and detrimental effects through the disruption of other non-AP pathways that remain essential throughout life. The strength of the positive lifespan effects then overcome negative lifespan effects, resulting in a net gain in longevity. Importantly though, as observed, the degree of lifespan extension for *pha-4* would not be the same as seen with direct inhibition of autophagy alone through *bec-1* (Figure 17A). We traced the origin of the negative effects of *pha-4* inhibition to its role as a transcription factor. PHA-4 acts on many multifaceted targets that likely mask the lifespan-promoting effects of autophagy inhibition. Examination of the changes in gene expression following of *pha-4* knockdown from day 9 revealed a marked shift in regulatory capacity between day 0 and day 9, from ~3,500 to 60 (Figure 18E). This result provides supportive evidence that *pha-4* is likely to be mediating its effect through autophagy, since the pool of affected targets is smaller thus making it much less likely that another pathway could be generating the same effect. However, the result also limits the possible candidates for *pha-4* negative late-life effects. Of the 60 identified *pha-4* targets in late-life, 7 genes functioning in the innate immune response were identified, 6 downregulated and 1 up. All downregulated genes, *dod-19*, *gst-6*, *hpo-6*, *B0024.4*, *C49C3.9, F53C11.1*, have been identified as PHA-4 transcriptional targets in early life (Zhong *et al.* 2010) and were down-regulated following day 0 inhibition in our data set. Inhibition of *bec-1* does not affect the same targets. The coordinated regulation of immune defences is obviously vital given the bacterial dense environment in which *C. elegans* lives and indeed immune responses are upregulated in long-lived worms (Kurz & Tan 2004). The action of PHA-4 in regulating these genes is, therefore, a strong candidate for the origin of the difference in lifespan between the two treatments. In further support the physical hallmarks of ageing were conserved between both gene inhibitions while their lifespans were not. This implies, that rather than a difference

in the rates of ageing there is instead a collapse in late-life worms, when *pha-4* is inhibited, as they succumb to bacterial infection more rapidly. Additional testing of a combined inhibition of *bec-1* with each of these immune genes would provide conclusive evidence for this explanation.

The pleiotropic actions of *pha-4* should also then explain the lack of an observable lifespan extension for *pha-4* inhibition in WT worms from day 9 (Figure 17D). The combination of positive and negative PHA-4 effects, allied with the overall reduction in RNAi mediated lifespan extension, as seen for *bec-1* which is reduced by more than half (Figure 17, A & D), results in a total, minimal change in the lifespan of WT worms following *pha-4* inhibition. The shorter natural lifespan of WT worms, in comparison to *rrf-3* mutants, combined with the reduced lifespan phenotypes in WT vs *rrf-3* mutants, leads to an alternative hypothesis i.e. that the observed *pha-4* effects could be *rrf-3* specific. Addressing this required the use of another RNAi sensitive strain, as other non-hypersensitive mutants would suffer from the same lack of effect as WT. RNAi sensitive strains display different RNAi efficiency in specific tissues, so to avoid confounding errors the *ergo-1* mutant strain was chosen as it best reflected the tissue efficacy of *rrf-3* (Zhuang & Hunter 2011). Notably, this strain demonstrated the ability of both *pha-4* and *bec-1* inhibition to extend lifespan in a comparable manner to that in *rrf-3* mutants (Figure 17C), largely ruling out specific effects in the *rrf-3* background. The reduction of *bec-1* lifespan in WT worms was initially assigned to a less effective global gene knockdown compared to the RNAi hypersensitive *rrf-3* mutant (Simmer *et al.* 2002). qPCR assessment of the comparative knockdown for both genes between WT and *rrf-3* mutants supported this conclusion, showing a reduced knockdown efficiency in WT worms three days after day 9 RNAi that only equalized to *rrf-3* levels by day 15 (Figure 17F). However, the increased global knockdown efficiency in *rrf-3* worms alone may not be sufficient to explain its much greater longevity gains. Investigations subsequent to this thesis lead to the identification of a dysfunctional late autophagy flux as causal in *pha-4* and *bec-1* mediated lifespan (Wilhelm *et al.* 2017). Numerous reports link various neurodegenerative diseases with blocked autolysosomal degradation (Nixon 2013, Wong & Cuervo 2010). Additionally, *rrf-3* and *ergo-1* mutant worms are particularly hypersensitive to RNAi in the nervous system in comparison to WT (Simmer *et al.* 2002, Zhuang & Hunter 2011). Based on these results and published studies, it was hypothesised that the observed longevity effects were neuronal in origin. Subsequent results indicated that the increased longevity in the *rrf-3* mutant mainly arises from its particular sensitivity to RNAi in the nervous system where the inhibition of vesicle nucleation via *bec-1* prevents age-associated neuronal degeneration, which translates into increased health- and lifespan (Byrne *et al.* 2017, Wilhelm *et al.* 2017). In total, these findings imply a reduced general efficacy of RNAi in WT worms that is particularly acute in the nervous system where autophagy inhibition functions to extend lifespan. PHA-4 is likely to have different detrimental effects in different tissues, likely explaining the specific

absence of an extension in the WT background as its neuronal benefits would be even further diminished in comparison to *rrf-3*.

### 3.8 Actions of autophagy inhibition through other longevity pathways

Following *bec-1* inhibition and thereby autophagy, the expression of only a few genes were affected and none have known roles in other lifespan extension pathways. Of the 60 genes dysregulated by day 9 *pha-4* inhibition, 10 (all down-regulated) are known to be modulated by the actions of other longevity pathways: *asp-3* inhibition reduces the lifespan of *eat-2* mutants; *cpr-2, mtl-1, mtl-2, dod-19,* and *dod-23* are all regulated by *daf-16*; *fat-5* and *hsp-16.41* are regulated by *daf-2*; and both *B0024.4* and *Y47H9C.1* are regulated by *skn-1*. While this did suggest that other known longevity mechanisms were not being invoked by autophagy inhibition, it is possible that more genes become dysregulated than were identified. This is due to the short time between RNAi inhibition and sample sequencing (3 days), which precluded the identification of less direct or slower changes in gene expression. Further insight into the connections between autophagy inhibition and know longevity pathways was therefore obtained through epistasis experiments.

Autophagy is required for longevity under DR, and it is well-established that *eat-2* mutants require *pha-4*, *bec-1* and *vps-34* for their long lifespan (Gelino *et al.* 2016, Hansen *et al.* 2008, Panowski *et al.* 2007). Attempts to modulate longevity with *bec-1* inhibition in standard *eat-2* mutants failed, likely due to an inability to generate a robust RNAi knockdown as a consequence of the reduced feeding observed for the strain (Lakowski & Hekimi 1998) (Figure 27E). We obtained an *eat-2;rrf-3* mutant from Malene Hansen (SBP, California) for lifespan experiments. This mutant, similar to mutants with *eat-2* alone, suffers from excessive bagging early in life and can only be grown on plates. Attempts to grow these strains in liquid culture resulted only in death, likely due to a pumping rate insufficient to suck bacteria from the liquid. Generating the worms required for late-life analysis proved extremely challenging. We succeeded in five independent experiments, however, these produced mixed results with three experiments showing no effect following *bec-1* knockdown and two producing only a modest effect of ~20% (Figure 27E & Supplemental Table 11). Testing the level of gene knockdown showed that the level of target gene transcription was both variable across experiments and lower than that seen in either *rrf-3* or WT animals (Figure 27E & 17F). It seems likely that the variation in lifespan and the lack of strong effects are linked to this reduced knockdown. Furthermore, *eat-2* mutants act as a DR mimic by causing a functional defect of the pharynx, reducing pharyngeal pumping rates, which limits food intake. DR is known to work through the TOR/*let-363* in worms (Hansen *et al.* 2007). Therefore, it would be expected that if *bec-1* inhibition acted through DR it would be unable to further extend the lifespan of worms treated with *let-363* RNAi. Despite the failure to obtain consistent

results with the *eat-2* mutant, there was a successful lifespan extension of both WT and *rrf-3* worms upon combinatorial RNAi targeting *bec-1* and *let-363* (Figure 27, F & G). Additionally, if knockdown of *bec-1* reduced feeding rates, causing a DR mimic, a reduction in pharynx pumping would be observed. Instead, *bec-1* inhibition increased pharynx pumping rates (Figure 20C) making it very unlikely that this treatment reduces caloric intake in the worms. Lifespan extension through DR is also dependent on SKN-1 (Park *et al.* 2010), which is superfluous to *bec-1* mediated lifespan extension strongly excluding DR as an activated mechanism (Figure 28D). While direct evidence for a consistent independence from the *eat-2* mutant background was not shown, our indirect evidence indicates that it is highly unlikely that inhibition of early autophagy either mimics or works through the DR pathway.

Along similar lines, TOR/LET-363 has a major role in autophagy regulation. Under conditions of nutrient deprivation or stress, TOR inhibition induces autophagy through the activation of the Ulk1 complex (Ganley *et al.* 2009) that in turn phosphorylates and activates BEC-1 and VPS-34 leading to the induction of vesicle nucleation (Russell *et al.* 2013). TOR mutation causes developmental arrests at the L3 stage but its later inhibition extends lifespan (Long *et al.* 2002, Vellai *et al.* 2003). Combinatorial inactivation of TOR/let-363 and *bec-1* at day 9 showed an additive effect on MTL in WT worms but not *rrf-3* mutants (Figure 27, F & G). The lack of lifespan extension for *let-363* inhibition in *rrf-3* worms has been explained in previous studies (Mizunuma *et al.* 2014). Here they showed that LET-363 forms two protein complexes TORC1 and TORC2 which have opposing effects on lifespan in neurons where TORC2 inhibition is lifespan shortening. As such following *let-363* RNAi in *rrf-3* mutants, there is an abrogation of the positive lifespan extension from inhibition of TOR outside of the nervous system. Despite this peculiarity, the ability of *bec-1* inhibition to extend lifespan in both strains strongly implies an independence of longevity effects from the TOR signalling pathway.

Attenuated protein synthesis is a conserved feature during the ageing of many organisms (Rattan 2010, Partridge & Gems 2002, Makrides 1983) but conversely, its inhibition can also extend longevity in worms and mammals (Pan *et al.* 2007, Selman *et al.* 2009), which is dependent on *daf-2*/*daf-16* or TOR inhibition (Tavernarakis 2008) and upregulation of SKN-1 (Wang *et al.* 2010b). Curiously, when examining the effects of *bec-1* inhibition on the expression of genes involved in translation a strong reduction in *rsks-1* (S6K) mRNA but not *ife-2* or *ifg-1* mRNA was observed (Figure 27A). Both *rsks-1 and ifg-1* are positively regulated by TOR signalling suggesting that *bec-1* inhibition is not working directly through TOR to reduce protein synthesis. Additionally, the inhibition of other autophagy genes *pha-4* or *vps-34* had no effect on *rsks-1* levels (Figure 27B). Also, if *bec-1* inhibition was working through TOR to reduce protein synthesis then combinatorial inhibition of *bec-1* and *let-363* in the WT background would not have been additive for lifespan. Independent of its effects on *bec-1* mediated longevity, this finding warrants further investigation to determine modes of BEC-1 activities outside of autophagy.

The removal of germline stem cells (GSC), either genetically or mechanically, significantly extends longevity in *C. elegans* (Arantes-Oliveira *et al.* 2002, Hsin & Kenyon 1999). As with other longevity strains, lifespan extension by GSC removal also depends on autophagy (Lapierre *et al.* 2011). For these lifespan assays, an *rrf-3* hypersensitive background could not be used. Attempts to create *glp-1;rrf-3* double mutants displayed an interesting synergistic lethality between the two mutations that resulted in an inability to obtain viable adults homozygous for both mutations. Despite these worms not being hypersensitive to RNAi, the strength of this effect was higher than that seen in WT worms with a median MTL of 35% (Figure 28A). As RNAi in the neurons of *glp-1* worms would be limited, combined with the reduction in *acs-2* following autophagy inhibition (Figure 18F), it was quite surprising to extend the late-life of GSC(-) *glp-1* mutant worms by such a degree. The strength of the lifespan extension, at a minimum, displays a decoupling of autophagy dependence in late-life in *glp-1* mutants. However, it could also imply a second additional role for autophagy that is particularly effective in a GSC(-) background (discussed in section 3.9).

Longevity extension in *daf-2* mutant worms is similarly dependent on autophagy and SKN-1 (Meléndez *et al.* 2003, Wang *et al.* 2008). Notably, *bec-1* inhibition only extended the life of this strain by a median MTL increase of 18%, despite the use of a *daf-2*:*rrf-3* double mutant to improve RNAi efficiency (Figure 28B). One possibility for this reduced lifespan extension is that since *daf-2* mutant worms are so long-lived, the beneficial vs detrimental trade-offs of autophagy inhibition are not optimal at day 9 in this strain. Indeed *bec-1* inhibition at day 20 improved the median MTL to 26% (data not shown). This extension is still well below that for *rrf-3* mutants and suggests a possible interaction of autophagy inhibition and the *daf-2* IIS pathway. Reduced *daf-2* signalling activates protective genes through the FOXO transcription factor *daf-16* (Ogg *et al.* 1997). Inhibition of *bec-1* in *daf-2:daf-16:rrf-3* triple mutants, which have a normal lifespan but cannot upregulate *daf-16* target genes, also showed an improved but truncated lifespan extension with a median MTL change of 28% (Figure 28C). This was somewhat surprising as the majority of *daf-2* lifespan extension processes require *daf-16.* However, the result implies a partial *daf-2, which* operates independently of *daf-16*. Interestingly, recent evidence shows that such a pathway exists and works via SKN-1 (Ewald *et al.* 2015).

## 3.9 Autophagy and metabolism, a new AP longevity link?

As autophagy is such a central process within the cell it might be expected that its inhibition would lead to radical changes in the overall biology of the organism in a compensatory response. It is therefore surprising that even in early life *bec-1* inhibition only results in a change in the expression levels of 42 genes, dropping to 11 in late-life (Figure 18E). The five genes dysregulated by both *pha-4* and *bec-1* in late-life are particularly interesting as potential causal factors triggered by inhibition of

the autophagic process. The gene *lec-2* changes from being downregulated to upregulated between early and late autophagy inhibition (Figure 18F). *lec-2* has β-galactoside-binding activity and is involved in sugar metabolism (Nemoto-Sasaki *et al.* 2008), suggesting a possible shift in energy requirements towards sugar metabolism following late-life autophagy inhibition. In the same vein, there is a continuous decrease in *acs-2* expression at both early and late time-points following autophagy inhibition. The *acs-2* gene encodes an acyl-CoA synthetase converting fatty acid to Acyl-CoA for subsequent β-oxidation in mitochondria (Van Gilst *et al.* 2005a). Over-life RNAseq analysis identified *acs-2* as one of the most highly variable genes changing more than 10 fold (Figure 30B). Similarly, in a subsequent sequencing analysis using the improved liquid culture protocols (separate from this thesis), *acs-2* was identified as ~4 fold upregulated between days 3 and 9 (Figure 30C). *acs-2* is activated by NHR-49 in mitochondria, where its activity promotes β-oxidation (Van Gilst *et al.* 2005b) and NHR-49 is significantly upregulated over life, ~2 fold (Figure 30, C & D). Mitochondrial activity was shown to be high in older worms and that this activity could be reduced through *bec-1* inhibition (Figure 21), likely through reduced *acs-2* activity.

Autophagy, in a process called lipophagy, is one of the primary mechanisms through which fat reserves are utilised for energy by the mitochondrial through NHR-49 and *acs-2* (Singh *et al.* 2009). Furthermore, autophagy is required to maintain high lipase activity in germline-deficient animals and in a reciprocal manner, lipases are required for autophagy induction (Lapierre *et al.* 2013b, 2011). Increased lipid storage along with autophagy is a requirement for the majority of longevity pathways in *C. elegans* (Hansen *et al.* 2013). Despite this and contrary to what might be expected, reduced autophagy does not lead to an accumulation of fat that but instead in, young worms, leads to a reduction in total fat content (Lapierre *et al.* 2013b). In similar results, we found a reduction in fat content in late-life following autophagy inhibition (Figure 30A). This combined with the identification of *acs-2* as down-regulated and *lec-2* as upregulated upon autophagy inhibition (Figure 18F) suggests a shift in metabolism away from the Krebs cycle and towards the glyoxylate cycle (Ruzanov *et al.* 2007). Such a shift directly contributes to the longevity of mitochondrial mutants (Apfeld *et al.* 2004) and suggests an intriguing possibility for the benefits of late-life autophagy inhibition acting via altered lipid metabolism following a reduction in fat content.

*Figure 30*

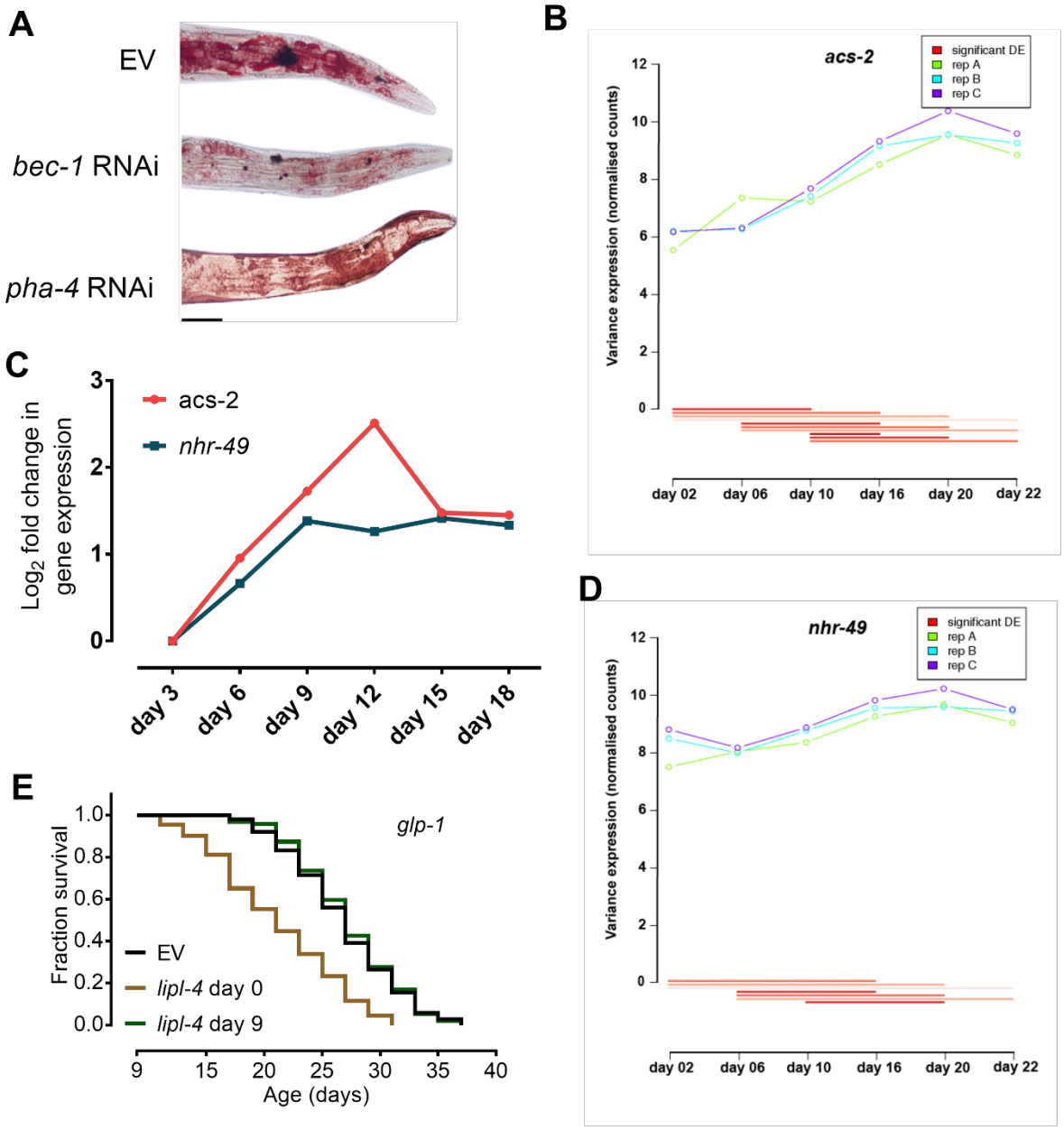


**Figure 30:** ***A link between fat metabolism and autophagy mediated lifespan extension.*** (A) Inhibition of *pha-4* or *bec-1* in late-life reduces fat accumulation as measured by Oil Red O staining for lipid contents at day 20. (B) The expression of *acs-2* is highly variable over time changing more than 10 fold between day 2 and day 20. (C) RNAseq using improved liquid culture protocols shows both *acs-2* and *nhr-49* increase in expression over time. Expression at all time-points is compared to day 3. (D) *nhr-49* expression changes around 2 fold over life as measured by RNAseq. (E) Day 9 inhibition of *lipl-4* has no effect on the MTL of *glp-1* mutant worms in comparison to day 0 inhibition, which shortens lifespan. — For B & C, axes and labels have been redrawn for clarity. Red lines on expression graphs indicate a significant change in expression between different time-points (FDR<1%). Comparison between each point is indicated by a different shade of red.

Worms produce lipoproteins (vitellogenins) in the intestine where they are then transferred to the gonad to support oocyte development (Brandt *et al.* 2005). However, once reproduction has ceased the levels of these proteins rise precipitously and they become localised throughout the worm and particularly in the body cavity (Zimmerman *et al.* 2015, Herndon et al. 2002 ). This deposition leads to a loss of tissue function in old worms and indeed, inhibition of specific vitellogenin genes *vit-2* or *vit-5* can extend lifespan (Murphy et al. 2003). Long-lived mutants deal with this problem in three main ways. First, the levels of lipoprotein production are reduced post-reproductive onset (Seah et al. 2016). Secondly, they upregulate autophagy levels to help with fat breakdown (Antebi 2013). This, however, places strain on the autophagic system and indeed overexpression of *vit* genes reduces the lifespan of long-lived mutants, likely by impairing autophagy (Seah *et al.* 2016). Third, they upregulate SKN-1, which acts to help stabilise the balance of lipid levels within the worm (Steinbaugh et al. 2015, Depina et al. 2011). SKN-1 is both activated by lipids and also regulates lipid metabolism gene expression, including *acs-2* (Steinbaugh *et al.* 2015), and like autophagy is required for numerous lifespan extension pathways (Blackwell *et al.* 2015). Inactivation or mutation of SKN-1 results in an accumulation of fats in WT and germline ablated worms (Steinbaugh *et al.* 2015). SKN-1 is itself affected by ageing, with changes in transcriptional targets and dysregulated activity seen over age (Rahman et al. 2013). When inhibited in late-life, *skn-1* loss was highly detrimental to the health and longevity of *rrf-3* worms (Figure 28D). This implies that in older age, the combination of ineffective autophagy and reduced SKN-1 activity combines to produce a toxic build-up of lipids in lysosomes that cannot be degraded (Ward *et al.* 2016). These lipids then act either to damage mitochondria via oxidative stress, resulting in mitochondrial destruction, or feed into a dysfunctional late-life lipophagic system where lipid droplets accumulate. Both of these mechanisms lead to neurodegeneration (Flint Beal 2008, Ward *et al.* 2016). Amazingly, this deficiency could be completely compensated for by simultaneous inhibition of *bec-1*. That *bec-1* extends *skn-1* inhibited lifespan suggests that inactivation of autophagy alleviates toxicity via a vital longevity process downstream of SKN-1. This process must compensate for an essential aspect of SKN-1 activity thereby negating the effects of loss of *skn-1* to the worm's health. The overlapping role of reducing levels of fat storage for the two pathways appears to be the most likely candidate.

SKN-1 functions to reduce fat content through the activation of lipase genes and the β-oxidation of lipids in the mitochondria along with NHR-49 and *acs-2* (Pathare *et al.* 2012, Goudeau *et al.* 2011). The SKN-1 program will be sub-optimal for health in advanced age due to increasing oxidative stress on mitochondria. Indeed, such stress promotes mitophagy as does increased SKN-1 activity (Palikaras *et al.* 2015) which in turn leads to mitochondrial loss and ageing. In the absence of functional autophagy, such a program must eventually collapse, becoming toxic from both lipid-filled

autophagosomes and impaired mitochondrial functionality. In contrast, directly inhibiting autophagy in late-life bypasses these issues by removing the build-up of fats in the first place, alleviating the strain on mitochondria and preventing toxic accumulation of lipid-filled lysosomes. Thus, autophagy inhibition would give a significantly greater benefit to longevity than that of increased SKN-1 activity as found in *daf-2*, *let-363* and *glp-1* longevity mutants (Blackwell *et al.* 2015). This is borne out by epistasis experiments in these mutants showing an increase in lifespan following *bec-1* inhibition (Figure 27F & 28, A & B). Notably, the degree of extension was smaller than that seen for *rrf-3* mutants suggesting that the increased *skn-1* activity in these strains is partially overlapping in function with autophagy inhibition. This results in some of the longevity benefits from late-life autophagy inhibition already being present through the SKN-1 pathway (approximately half of it). In both *daf-2:rrf-3* and *daf-2:daf-16:rrf-3* mutant worms this results in a ~26-28% MTL extension at the optimal time-points (Supplemental Table 12) and in combination with *let-363* it generates a ~35% MTL extension (Supplemental Table 11). The situation in *glp-1* worms is slightly more complicated as they are WT for RNAi sensitivity and we observed a ~35% MTL extension, which is highly comparable to that of WT worms. SKN-1 is activated in GSC(-) worms through lipid signalling in the germline via DA/DAF-12 (Antebi 2013). However, this form of signalling requires an intact somatic gonad. However, the gonad degrades with age undergoing hypertrophic and eventually disintegrating (Riesen *et al.* 2014, Luo *et al.* 2010, Garigan *et al.* 2002). This implies that in later-life lipid signalling and thus SKN-1 activity will be greatly reduced in GSC(-) worms. Indeed we find that in comparison to early life where *glp-1* longevity is strictly dependent on *lipl-4* levels (Lapierre *et al.* 2011) we find that this dependence is lost in later-life (Figure 30E), suggesting a decoupling of lipid metabolism and longevity in late-life. As such it seems reasonable to speculate that autophagy inhibition is more effective in this mutant because of declining SKN-1 activity in late-life.

While the theory of late-life fat content being detrimental to longevity needs significant further investigation. If found to be true, lipids could also be pleiotropic ageing factors and genes involved in their regulation may turn out to be a novel class of AP genes.

# 4 Conclusion

The concept for how a deleterious process such as ageing could arise through evolutionary processes has existed now for over 60 years (Williams 1957). Yet despite the success of the theory of antagonistic pleiotropy in providing an evolutionary framework for ageing, little or no evidence for genes that behave according to its predictions has been described. Part of the issue is the difficulty in creating and working with organisms in mid to late-life, where AP theory predicts the actions of such genes will be observed. In comparison to large-scale *C. elegans* culture methods that have predominated since the mid-90s (Fabian & Johnson 1994), we developed a novel method of large-scale, age-synchronised liquid culture that allows for the easy generation of mid to late-aged worms in high density. The advantage of this system is that it can be applied to any strain capable of feeding in liquid and does not require the use of drugs that can inhibit downstream analysis. This method should greatly enhance the ability of research to focus on ageing beyond early time-points and facilitate both genetic and pharmaceutical investigations into the efficacy of treatments in the AP window. Demonstrating the power of this technique, we identified 31 novel AP genes in an RNAi screen of chromatin and transcription-related factors. The success rate of this screen suggests that hundreds of AP genes may still remain undiscovered in the *C. elegans* genome and could lead to a revolution in identifying targets for ageing and its associated diseases. Non-communicable diseases are the highest cause of mortality globally and ageing is one of the biggest risk factors for all types (WHO 2017). The trend in ageing research, prior to now, has identified longevity interventions that must begin early in life to achieve full efficacy, as seen with *daf-2* interventions (Dillin *et al.* 2002). Other treatments such as rapamycin increase longevity independent of age but increase other degenerative occurrences such as cataracts and testicular degeneration (Wilkinson *et al.* 2012). The identification of genes, whose deleterious behaviour is specific to the mid to late-age period will provide a new point of action that bypasses these issues. This will allow for the targeting of ageing processes in the period of life when it is actually relevant and not beforehand where competing fitness effects are likely. The identification herein, that the late-life inhibition of autophagy can increase not only lifespan but also healthspan is a case in point. Autophagy is a process targeted in both cancer and neurodegenerative disease (Rubinsztein *et al.* 2007). As autophagy is a beneficial process when functioning in early life, many of these treatments aim to upregulate the autophagic process. However, based on our results this may be a less than optimal treatment as patients get older, when autophagy becomes increasingly dysfunctional and toxic. Indeed at least for neurodegeneration the inhibition rather than upregulation of autophagy may be the optimal strategy. This shows how important the age-specific context of how a process behaves

is and highlights the need for better tools and further investigation into the period of life where ageing actually exerts its effects.

The identification of autophagy as a detrimental process late in life is surprising given its normally vital cellular role. Inhibition of *pha-4* or *bec-1* greatly reduces total autophagic activity and appears to prevent the formation of multi-laminar autophagosomes, suggesting that autophagy becomes dysfunctional and toxic in late-life. Indeed its inhibition resulted in a global increase in healthspan and did not increase the percentage of life spent in frailty as see for other treatments (Bansal *et al.* 2015). From an evolutionary perspective, autophagy is a good platform for ageing to work through. It is an essential process for early life development and growth when resources need to be rapidly recycled. Once the onset of reproductive maturity is reached though, the energy demands of the organism decline sharply. This allows for a gradual failure of autophagic processes that are not immediately detrimental to the organism. Indeed, inhibiting autophagy exactly at this point is, on balance, neither beneficial nor detrimental to the subsequent lifespan. This, combined with a reduced force of natural selection allows for the later appearance of a detrimental phenotype. We find that autophagy is definitely an AP process and is more likely to have been shaped by a non-programmed evolutionary mechanism rather than a programmed one. This should add further evidence to the debate between these two theories for the origin of ageing. Additionally, the inhibition of autophagy may illuminate another AP process, that of fat storage. The ability to store fat for later use is a strong pro-fitness quality and a requirement for almost all known longevity phenotypes (Hansen *et al.* 2013). Indeed a recent study from the Brunet lab showed that feeding mono-unsaturated fatty acids to worms in early life extends lifespan (Han *et al.* 2017). However, the interaction of autophagy inhibition with other longevity mechanisms points to a role for reduced fat build up, a process normally performed through SKN-1, in the longevity phenotype. This point of action is likely epistatic with to the benefits gained from alleviating the pressure on dysfunctional autophagosomes and probably acts through a reduction in mitochondrial stress and reduced lipid toxicity. If found to be true, not only will it identify another AP process for late-life longevity but it will also demonstrate the need to assess lifespan interventions at numerous time-points across life to fully determine the benefits of such treatments.

# 6 Methods

### Worm strains and culture conditions

The following strains were used in this study: WT (N2 Bristol), NL2099/*rrf-3(pk1426)* II, CB4037/*glp-1(e2141)* III, CF1814/*rrf-3(pk1426)* II; *daf-2(e1370)* III, WM158/*ergo-1(tm1860)* V, DA1116/*eat-2 (ad1116)* II, PD4251/ccIs4251 [*(pSAK2) myo-3p::GFP::LacZ::NLS + (pSAK4) myo-3p::mitochondrial GFP + dpy-20(+)*]. All strains were acquired from the *Caenorhabditis* Genetics Centre (CGC) at the University of Minnesota, except the following: QU34/izEx5[*pAy39.1, bec-1p::BEC-1::RFP*], which was kindly provided by Alicia Meléndez (Queens College, Flushing, NY) and *eat-2(ad1116)* II *; rrf-3(pk1426)* II, which was kindly provided by Malene Hansen (Sanford Burnham Prebys, La Jolla, CA). Strains created for this project were BAT922/*rrf-3(pk1426)* II; adIs2122 [*lgg-1p::GFP::lgg-1* + *rol-6(su1006)*], BAT1032/*daf-16(mgDf47)* I; *daf-2(e1370)* III; *rrf-3 (pk1426)* II. *rrf-3* worms and all BAT strains were outcrossed to WT three times. Standard procedures for *C. elegans* strain maintenance were used (Brenner 1974). All stock animals were maintained on nematode growth medium (NGM) plates and fed with EV expressing HT115 bacteria. Experimental animals were maintained in a liquid culture system as described in the following section.

### Large-scale liquid culture with FuDR in Erlenmeyer flasks

For standard methods and reagents normal procedures for *C. elegans* maintenance are used (Stiernagle 2006). *C. elegans* WT worms were grown on NGM agar plates until high density. Worms were bleached using 1% NaOH. Eggs were hatched in M9 buffer with 0.05% TWEEN20 for 12 hrs to prevent starvation. Hatched L1s were counted and placed into S-medium at a density of 50,000 per litre and fed with OP50 *E. coli* bacteria at an OD of 3 and ampicillin 1 mM. The S-medium containing worms and food was placed in 1L Erlenmeyer Flasks with vented lids for aeration and rotated at 120 rpm at 20 °C. Worms were maintained in liquid culture for a minimum of three generations before experiments and grown to a density of 1 worm/µl. For experiments worms were established at the required density and allowed grow to the early L4 stage. Biological replicates were performed in parallel to reduce environmental variability but worms for each replicate were obtained from different ancestral stocks. Before the appearance of first eggs 150 µm FuDR was added to block reproduction. FuDR was maintained in the culture until day 6 of life. Worms were removed from culture daily and subject to a 30% sucrose preparation to remove dead worms. Samples were extracted from the cleaned population and remaining worms transferred back to liquid culture. Cultures were maintained until insufficient sample numbers remained for analysis.

### Analysis of histone modification with western blotting

Worms were grown in high speed liquid culture as described and harvested from liquid culture at day 0, day 6, day 12, day 16, day 20 and day 24. For each time-point a sample of at least 5,000 worms was harvested, washed 3 times with M9 buffer and once with $ddH_2O$, and suspended in 2x standard Laemmli buffer via 10 minute boiling. The samples were subjected to standard SDS-PAGE and western blotting. Antibodies were obtained from Active Motive and included all Histone 3 and Histone 4 modification antibodies offered by the company. Antibodies were all monoclonal mouse and used at 1:1000 with the exception of native H3 which was used at 1:10,000. Antibodies were diluted in PBST (5% milk powder, 0.1% Tween20) which was also used as blocking agent. Signals for histone modification were detected and quantified using ImageLab software from BioRad. Total H3 signal for each time-point was used as a control and all other signals were compared to this.

### Quantitative analysis of histone modification with mass spectrometry

Histone modification analysis was performed according to standard protocols for MALD-TOF mass spectrometry (Villar-Garea *et al.* 2008). Briefly, worms were grown in high speed liquid culture as described and harvested from liquid culture at day 2, day 6, day 12, day 16, day 20 and day 22. Samples were immediately frozen in liquid nitrogen for long term storage at -80 °C. For sample preparation worms were subject to 10 freeze thaw cycles in liquid nitrogen to crack the cuticle followed by pressure through a chilled cell homogeniser (Isobiotech) with an 8 micron gap to extract nuclei. Lysate was centrifuged for 10 min at 500 x g and pellet was resuspended in 0.25 M HCL at 4 °C overnight with rotation. Lysate was cleared to remove insoluble debris by cehtrifugation 5 min at 20,000 x g. Samples were dialysed three times for 2hrs at 4 °C against 0.1 M acetic acid with 1 mM DTT. Samples were freeze dried and reconstituted in 20 mM Tris HCL. To prevent trypsin cleavage after lysine residues histones were acylated following SDS gel separation. Extracted gel fragments were washed in 10 mM ammonium bicarbonate followed by 200 µl of 50% acetonitrile/50 mM ammonium bicarbonate and shaken for 60 min at 37 °C. Gel pieces were treated with 100 µl acetonitrile and shaken for 5 min at 37 °C. 5 µl anhydride (propanoic or acetic anhydride-d6) and 10 µl of 0.1 M ammonium bicarbonate were added to each gel piece. 35 µl of 0.1 M ammonium bicarbonate was added and samples incubated for 60 min at 37 °C with pH adjusted to between 7 and 8. Following incubation supernatant was removed and samples washed three times with water. Samples were then digested with 0.2 µg/µl trypsin. Digested samples were then subject to MALD-TOF acquisition and the spectra subsequently analysed

### Determination of protein abundance over age with label free mass spectrometry

Identification of protein abundance using label-free LC-MS/MS was performed according to standard protocols (Levin & Bahn 2010). Briefly, worm samples were collected at day 6, day 12, day 16, day 20 and day 24 and were derived from the same experiment as the samples for Histone modification quantification by western blot. Samples were flash frozen and subject to 10 freeze thaw cycles. Worm contents were then released by passing the samples through a cell homogeniser with a 20 micron gap. Lysate was centrifuged to remove debris and concentrated via centrifugal evaporation. Concentrated lysate was separated by SDS-page and bands corresponding to proteins ranging from 10kDa to 150kDa were extracted. Samples were digested with Tripsin and analysed via data-dependent acquisition on a Q-Tof Premier (Walters Corp., Milford, MA) mass spectrometer.

### DAVID analysis for enriched terms

DAVID is a freely available tool for the analysis of genetic and proteomic data sets for overlapping functional relevance (Huang *et al.* 2009, 2009, Dennis *et al.* 2003). Both gene and protein lists of interest were interrogated using two classification tools, Gene functional classification (Huang *et al.* 2007a) and functional annotation clustering (Huang *et al.* 2007b, Dennis *et al.* 2003, Hosack *et al.* 2003). Overrepresented terms or clusters were identified where the FDR was <1% and the minimum number of counts for any cluster was 3. Selected categories for functional annotation chart analysis were UP_KEYWORDS, UP_SEQ_FEATURE, GOTERM_BP_DIRECT, GOTERM_CC_DIRECT, and GOTERM_MF_DIRECT.

### RNA sequencing

Worms were grown in liquid culture as described. Worms were harvested at day 2, day 6, day 10, day 16, day 20 and day 22. 1,000 worms were collected and flash frozen in 400 µl of Trizol. RNA extraction and cDNA synthesis was carried out following the manufacturer's instruction. 60 NGS libraries were prepared using Illumina's TruSeq stranded Total RNA LT Sample Prep Kit following Illumina's standard protocol (Part # 15031048 Rev. E). The ribozero steps of the protocol for rRNA depletion were carried out manually, while the following steps of library preparation were performed using the Bravo Automated Liquid Handling Platform (Agilent). Libraries were prepared from a starting amount of 500 ng of total RNA and amplified in 11 PCR cycles. Libraries were profiled in a High Sensitivity DNA chip on a 2100 Bioanalyzer (Agilent technologies) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit 2.0 Fluorometer (Life technologies). 15 libraries were pooled together in equimolar ratio (60 libraries into 4 pools), and each pool was sequenced on 2 HiSeq 2500 lanes of a High Output run, single-read for 51 cycles plus 7 cycles for the index read. The libraries were sequenced on a HiSeq2500 with

a read length of 51 bp single read. Base calling and demultiplexing were performed using bcl2fastq (version 1.8.4). Reads were mapped against C. elegans genome assembly WBcel235 (ENSEMBL release 84) using STAR (Dobin & Gingeras 2013) (version 2.5.1b, additional parameters: --outFilterMultimapNmax 10 --outFilterMismatchNmax 2 --alignIntronMin 21 --sjdbOverhang 50). Strand specific read counts per gene were generated using featureCounts (Love *et al.* 2014) (version 1.4.6-p2 subread package). Differentially expressed genes were determined using DESeq2 (Liao *et al.* 2014) (version 1.12.3) with an FDR cutoff of 1% and default filtering criteria. Samtools (Quinlan & Hall 2010) (version 1.3), BEDTools (Kent *et al.* 2010) (version 2.25) and UCSC tools (Li *et al.* 2009) were used to generate coverage tracks.

**Real-time quantitative PCR**

Worms were grown in liquid culture as described. For RNAi knockdown experiments treatment commenced at day 0 or day 9. Samples were harvested at time-points indicated. Total RNA was isolated from flash frozen samples of at least 200 age synchronised worms using Trizol extraction. cDNA synthesis was performed using First Strand cDNA Synthesis Kit (ThermoFisher) with 800 ng of RNA and oligo-dT primers. Quantitative PCR was performed in a ViiA™ 7 Real-Time PCR System (ThermoFisher) using SYBR® Green PCR Master Mix (Life technologies). Values were normalized to *cdc-42* as an internal control.

**Lifespan assays**

Worms were synchronized to the appropriate age with the use of liquid culture sedimentation. Bacterial inoculations were grown for 14 hours until fully dense and then NGM agar plates, treated with 100 µg/ml Ampicillin and 1mM IPTG, were seeded with respective dsRNA expressing bacteria at native density and grown overnight at RT. Day 0 was determined by the appearance of the first internal eggs. Worms were transferred to a stock plate and directly picked to their relative RNAi treatment plates. A total of 105 - 140 animals were placed on 3 to 4 replicate plates with 35 worms per 6 cm plate. Picking was performed with a soft pick utilising a blunted eyelash hair to avoid any damage to the worms. Worms were picked to new plates and scored every two days. In the event of treatments prior to day 6, worms were passaged every day until no offspring remained. For double RNAi treatment, bacteria were diluted in a 1:1 ratio before being plated at native density. All assays were performed at 20 °C. For the temperature sensitive *glp-1* mutant strain, worms were maintained at the permissive temperature of 25 °C until day 0 and then transferred to 20 °C. For lifespan assays indicated in the supplementary table as plate only, these worms were maintained and synchronised only on plates. Worms were scored as alive until there was no movement at all after repeated prodding with

an eyelash. Worms were censored for the following reasons: crawling off the plate, bagging or bursting, dropping on transfer, contamination, and burrowing. Whole plates were censored when contamination was not removed upon worm transfer. For RNAi experiments, RNAi clones were obtained from the Ahringer libary. All clones were passaged four times on LB plates containing 100 µg/ml Ampicillin and 2.5 µg/ml Tetracycline to remove any non HT115 bacteria. All final clones were confirmed by DNA sequencing.

### Redeveloped age synchronous liquid culture of *C. elegans*

NGM plus liquid media was prepared with 100 µg/ml Ampicillin and EV HT115 bacteria re-suspended to a final density of $3x10^9$ cells $ml^{-1}$. Cell culture flasks with breathable filter caps were filled to the following capacities: 75 $cm^2$ to a maximum of 50 ml, 175 $cm^2$ to a maximum of 100 ml and 300 $cm^2$ to a maximum of 200 ml, in order to provide optimal aeration. Capacities were determined by the desired worm load in each flask with a maximum density of 0.5 worms per µl per flask. Stock worms were washed from two 10 $cm^2$ NGM plates, sedimented and placed in a 100 ml cell culture flask. All procedures from this point were carried out under a laminar flow hood to avoid contamination. Worm cultures were placed at 20 °C in a shaking incubator at 40 rpm. Once dense with egg producing adults, they were synchronised with NaOCl treatment followed by an over-night egg hatch in M9 buffer. L1 worms were counted and placed back into culture at the appropriate density. Worms were maintained in this manner for a minimum of three and a maximum of eight generations at optimal conditions. For experimental samples, L1 worms were harvested from age synchronous adults at no later than day 1 of life. These offspring were allowed to grow until day 0 and were then subject to twice daily sedimentation to remove offspring (Fig 12, A-D). The sedimentation protocol was as follows. Adult worms were allowed to settle in 50 ml Falcon tubes at a 1:1 ratio to the density of the liquid culture. Sedimentation proceeded for 5-10 minutes depending on the age of the worms, adult worms sediment faster than larvae. The supernatant containing L1-L3 worms was removed by aspiration. Worms were re-suspended in NGM plus containing EV bacteria at a density of $1x10^9$ cell $ml^{-1}$. This was repeated six times. Worms were then carefully layered onto a 15 ml column of 36% Percoll with NGM in a 50 ml Falcon (Fabian & Johnson 1994). Worms were allowed to settle through the Percoll for 10 minutes and the top two thirds of the Percoll containing: dead worms, bagging worms, and egg clumps was aspirated. Worms were washed a further three times with NGM plus before being transferred into a fresh food culture. Percoll sedimentation was performed twice daily for the first five days and then once daily thereafter. A final sedimentation of in 40% Percoll was performed on days where samples were extracted.

### RNAi screen

A comprehensive RNAi library comprising 785 dsRNA expressing HT115 *E.coli* bacteria specific to *C. elegans* gene regulatory factors was prepared from the Ahringer and Vidal libraries (Supplemental Table 6). All clones were cleaned by 4x passage and single colony selection on LB plates with 100 µg/ml ampicillin and 10 µg/ml tetracycline. All plasmids were DNA sequenced using the M13 forward primer to confirm the correct target identity. This library was grown overnight in 2x YT media in a 96 deep well format and seeded onto 24 well NGM agar plates prepared with 100 µg/ml Ampicillin and 1 mM β-D-isothiogalactopyranoside (IPTG) at 2x native density. Replicates were grown in one row per plate, controls were maintained on separate plates. Controls comprised of 4 plates of both EV and *gfp* RNAi HT115 bacteria. *gfp* RNAi was used as a non-targeting control. *rrf-3* mutant worms were synchronised via liquid culture until day 9 and then cleaned and sorted with the COPAS Biosorter with exactly 20 worms per well. Control plates were monitored until the vast majority of worms appeared dead and subsequently all treatment wells were scored. Scoring was performed by flushing each well with M9 buffer and immediately counting moving and dead worms. Only worms that could be identified as live or dead were scored, any missing worms were assumed to have crawled off the plate and as such were not counted. Contaminated wells or wells that contained offspring were not scored, combined these two conditions represented ~10% of all wells.

### Western blotting

Worms were grown as described and transferred to respective RNAi treatments at day 9. For each time-point or knockdown condition: 200 synchronized animals were harvested, washed 3 times with M9 buffer and once with $ddH_2O$, and suspended in 2x standard Laemmli buffer via 10 minute boiling. The samples were subjected to standard SDS-PAGE and western blotting. Antibodies used were monoclonal mouse anti-GFP (Roche,1:10,000) and mouse monoclonal [6G6] anti-RFP (Chromotek,1:1000). Antibodies were diluted in PBST (5% milk powder, 0.1% Tween20) which was also used as blocking agent. Each western blot is representative for similar results obtained in two independent biological replicates.

### Microscopy

Worms were grown as described and transferred to respective RNAi treatments at day 9. On the day of analysis, 100 worms from each knockdown condition were paralyzed using 0.5% NaAz and mounted on a 2% agar pad on a glass microscope slide. Fluorescent signals were acquired using a STED super-resolution microscope (Leica) at 100x magnifications. Images were taken of the hypodermis which was

identified by locating the plane between the muscle and cuticle. At least 60 total regions were imaged from 50 different worms per replicate with two biological replicates. All images used for comparative quantifications were taken on the same day, with the same settings and by the same user.

### Quantification of LGG-1::GFP foci

All GFP::LGG-1 foci were quantified using ImageJ according to standard procedures (Palmisano & Meléndez 2016, Alberti *et al.* 2010). Each image was processed in the same manner. Briefly, the original image is examined and the hypodermis outlined to avoid other regions such as muscle or seam cells in young worms (Supplemental Figure 3, A and B). Images are converted into a bit map following automatic threshold adjustment using the max entropy calculation to remove background (Supplemental Figure 3, C and D). The region corresponding to the outlined hypodermis is selected for analysis Particles are analyzed in this selected region with the following criteria: particles were selected for quantification based on a size range of 0.2 - 4 $\mu m^2$ and with a circularity of 0 – 1 (Supplemental Figure 3E). The outlines of the identified and counted particles are checked to avoid spurious counts (Supplemental Figure 3F). Images were not considered for quantification where the hypodermis was not clear or where foci were not visible following thresholding. In total, at least 60 total regions were quantified from 50 different worm samples per condition.

### Pharynx imaging and analysis

Worms were grown as described and transferred to respective RNAi treatments at day 9. On day 20, 50 aged-synchronized worms from each knockdown condition were paralyzed using 0.5% NaAz and mounted on a 2% agar pad on a glass microscope slide. The images of the pharynges were acquired on a SP5 microscope (Leica) using the Differential Intense Contrast (DIC) filter and a 63x/1.4 NA oil immersion objective. At least 30 whole worms per condition were imaged in each replicate with three technical replicates. Images were scored blind for pharynx degradation based on a three-point scale of damage related to changes in the structure of the corpus, isthmus or terminal bulb. Worms with no obvious damage to any of the three parts were scored as 1, worms with damage to only one or two parts were scored 2, and worms with damage to all three parts were scored 3. For comparative analysis all worms were imaged at the same time, with the same settings and by the same user. This experiment was carried out in two independent biological replicates.

### Muscle cell imaging and analysis

Worms were grown as described and transferred to respective RNAi treatments at day 9. On day 20, 30 aged-synchronized worms from each knockdown condition were incubated in fixation buffer (160 mM KCl, 100 mM Tris HCl pH 7.4, 40 mM NaCl, 20 mM $Na_2EGTA$, 1mM EDTA, 10 mM Spermidine HCl, 30 mM Pipes pH 7.4, 1% Triton-x-100, 50% Methanol) at room temperature for 1 hour rotating. Worms were washed twice with PBS and incubated in a 1:200 dilution of Phalloidin–Atto 565 (Sigma) in PBS-0.5% Triton-x-100 for 4 hours at room temperature rotating. Worms were then mounted on a 2% agar pad on a glass microscope slide. The 561 nm signals were acquired using a STED CW super-resolution microscope (Leica) and a 63x/1.4 NA oil immersion objective. At least 40 images, comprising 2-4 cells, from 20 different worms were imaged per replicate with three technical replicates. Images were scored blind, based on a five-point scale of muscle fiber degradation. Cells showing no obvious degradation were scored as 1, cells with kinks or striations were scored as 2, cells with small lesions combined with striations or other damage were scored 3, cells with muscle fiber breaks, gross striations and lesions were scored as 4, and cells where the muscle fibers were no longer intact were scored as 5. For comparative analysis, all worms were imaged at the same time, with the same settings and by the same user. This experiment was carried out in two independent biological replicates.

### Pharynx pumping assay

Worms were grown in liquid culture and transferred to respective RNAi treatments at day 9. On day 20, 50 aged-synchronized worms from each knockdown condition were transferred individually to an agar plate seeded with a bacterial lawn. The number of contractions in the terminal bulb of pharynx was scored during a 30 second period immediately upon transfer using a stereomicroscope. This experiment was carried out in two independent biological replicates.

### Movement scoring/thrashing assay

Worms were grown as described and transferred to respective RNAi treatments at day 9. On day 20, 50 aged-synchronized worms from each knockdown condition were transferred individually in a 20 µL drop M9 buffer on a petri dish. After a 30 second recovery period, the numbers of body bends were scored during a 30 seconds period using a stereomicroscope. A body bend was defined as a change in the reciprocating motion of bending at the mid-body. This experiment was carried out in two independent biological replicates.

### Mitochondria staining

Worms were grown as described and transferred to respective RNAi treatments at day 9. On day 20, 70 aged-synchronized worms were picked into M9 containing food and stained with 20 nM Mitosensor® or Mitotraker® (Thermo Fischer) for 3 hours at room temperature rotating. Worms were paralyzed by 0.5% NaAz and mounted on a 2% agar pad on a glass microscope slide. 488 nm, 561 nm or 633 nm signals were acquired using a STED CW super-resolution microscope (Leica) and a 63x/1.4 NA Oil immersion objective. At least 50 whole worms were imaged per condition.

### Electron microscopy

Worms were grown as described and where relevant transferred to respective RNAi treatments at day 9. Samples of 12 worms were taken per time-point or knockdown condition. Worm samples were frozen under high pressure (2100 bars) using the high pressure freezing machine (Engineering Office M. Wohlwend GmbH, Switzerland). The worms were enclosed and protected in a small volume between two specimen carriers and locked inside the chamber. Liquid nitrogen was used as a cooling medium. Following freezing, samples were put into the substitution medium containing 2% osmium tetroxide and 0.1% uranyl acetate inside the freeze substitution machine (EM, AFS, Leica Microsystems) for a total of 29h. The samples were slowly warmed up to 0°C and transferred to 100% acetone at room temperature. The infiltration was proceeded in EPON (acetone : EPON 2:1 for 30min, 1:1 for 30min, 1:2 for 30min, pure EPON for 1h and pure EPON overnight). The worms were embedded at the desired orientation and the polymerization took place at 60°C for 48h. Ultrathin sections were collected using a Leica ultramicrotome and were post stained with 4% uranyl acetate. The FEI Tecnai F20 transmission electron microscope was operated at 200kV. Electron micrographs were taken on a 2k charge-coupled device (CCD) camera (Gatan Ultrascan 1000).

### Apoptosis and necrosis staining

Worms were grown as described and transferred to respective RNAi treatments at day 9. On day 20, worms from each condition were were picked into 1 ml M9 and stained with either Acridine range 50 µl of a 1 mg/ml stock or Propidium Iodide 75 µl of a 1 mg/ml stock for 2 hrs. Worms were washed in M9 and paralyzed by 0.5% NaAz and mounted on a 2% agar pad on a glass microscope slide. 510 nm, 560 nm (AO) or 633 (PI) nm signals were acquired using a STED CW super-resolution microscope (Leica) and a 63x/1.4 NA Oil immersion objective. At least 20 whole worms were imaged per condition.

### Proteasome peptidase activity assay

*In vitro* proteasomal peptidase assay was performed as previously described (Kisselev & Goldberg 2005). Briefly, synchronised worms were grown in liquid culture and transferred to RNAi feeding plates at day 9. Day 16 old animals were homogenized in proteasome activity assay buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM ATP and 1 mM dithiothreitol), using an ultrasonicator (Bioruptor, Diagenode). The lysate was centrifuged at 10,000 x g for 15 minutes at 4 °C. 25 µg of total protein lysate was transferred to a 96-well black microplate (Greiner) and incubated with fluorogenic substrate. As a control condition, the lysate was treated with the proteasome inhibitor MG-132 (25µM in DMSO) and the other conditions were supplemented with the matching volume of DMSO as a vehicle control. Chymotrypsin-activity was assayed with hydrolysis of the fluorogenic peptide substrates Suc-LLVY-AMC (Enzo Life Sciences). Fluorescence (380 nm excitation, 460 nm emission) was recorded every 5 minutes for 1 hour at 25 °C with a microplate fluorometer (Infinite M1000, Tecan). Assays were performed in three biological replicates, each averaging two technical replicates.

**Bortezomib treatment**

To pharmacologically inhibit the proteasome, animals were treated with Bortezomib (EMD Millipore Calbiochem). Briefly, worms were grown as described in liquid culture until day 14.5 and subsequently incubated with 2 mM Bortezomib or DMSO vehicle control over night (~12 hours). The drug treatment was performed in M9 liquid medium supplemented with EV HT115 bacteria at a density of $3x10^9$ cell $ml^{-1}$, while shaking at 20 °C. The worms were harvested at day 15, following a 40% Percoll wash. This wash was repeated in the event that the cleaned worms contained less than 95% living worms.

**Statistics**

All statistical analysis was performed with Graph Prism 6. p-values for lifespan curves were calculated using the Log-Rank (Mantel Cox) test (Mantel 1966). Mean treated lifespan (MTL) was calculated as the mean remaining lifespan of the worms from the day of first treatment. p-values for quantification of GFP::LGG-1 foci and mitochondrial area were calculated using the non-parametric Mann Whitney U test. p-values for: muscle health, and pharynx integrity were calculated using a Chi-square test with two tails and 95% confidence interval. p-values for: qPCRs, body bend counts, pharynx pumping, and chymotrypsin assay were calculated using a two tailed t-test. GO-Term statistics were calculated by the DAVID software directly. Significance was scored as follows for all experiments: **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

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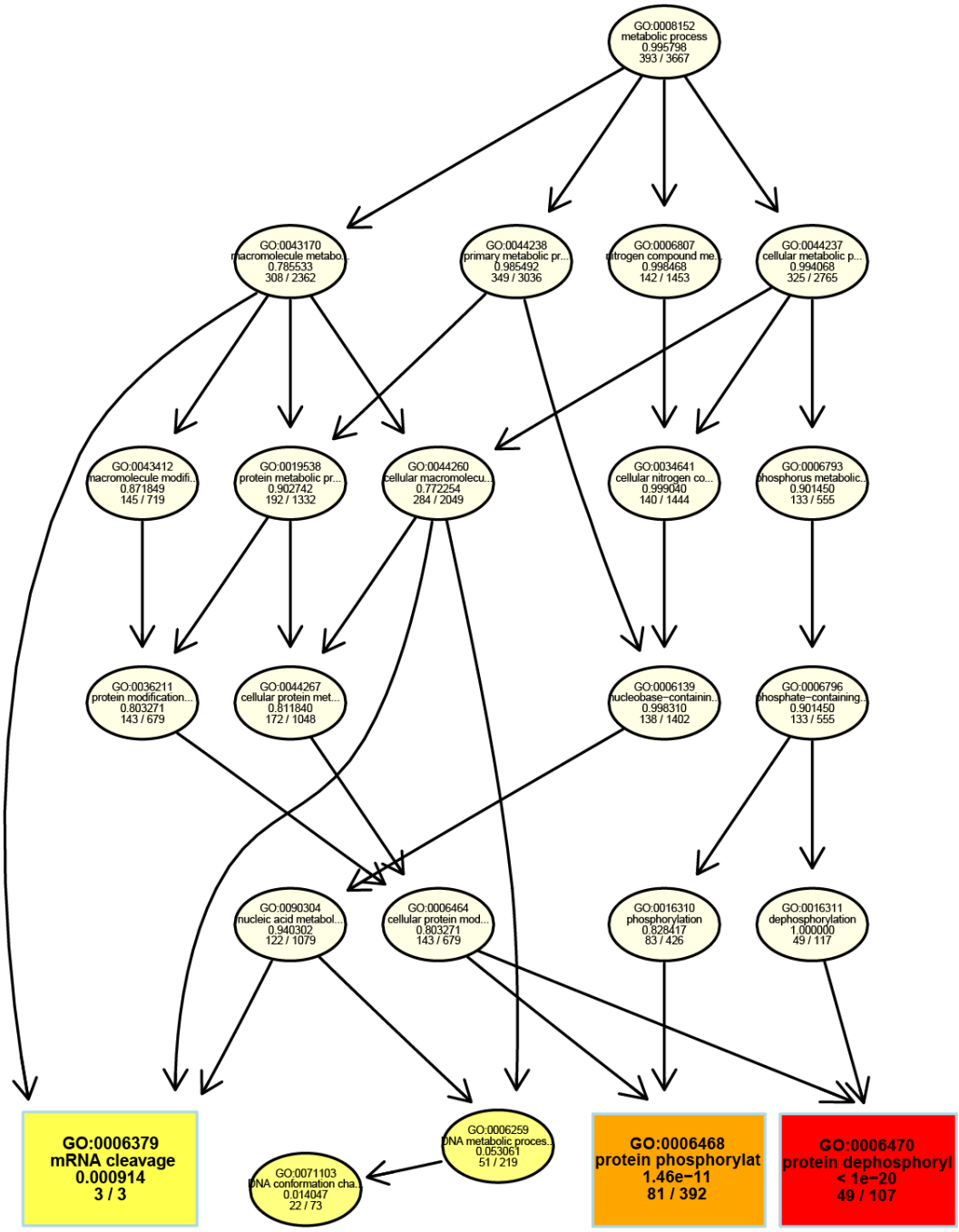
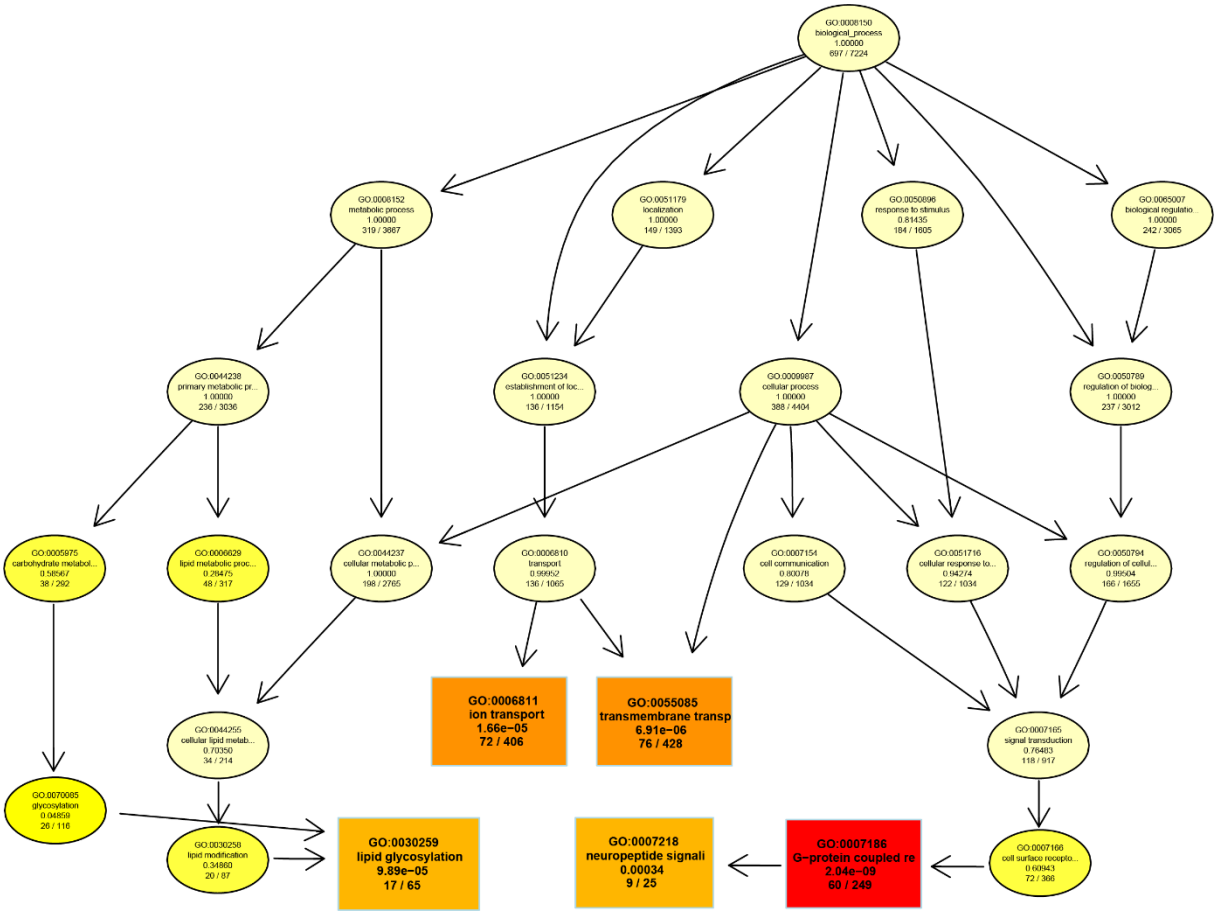
# 8 Supplemental Figures & Tables

## 8.1 Supplemental Figure 1



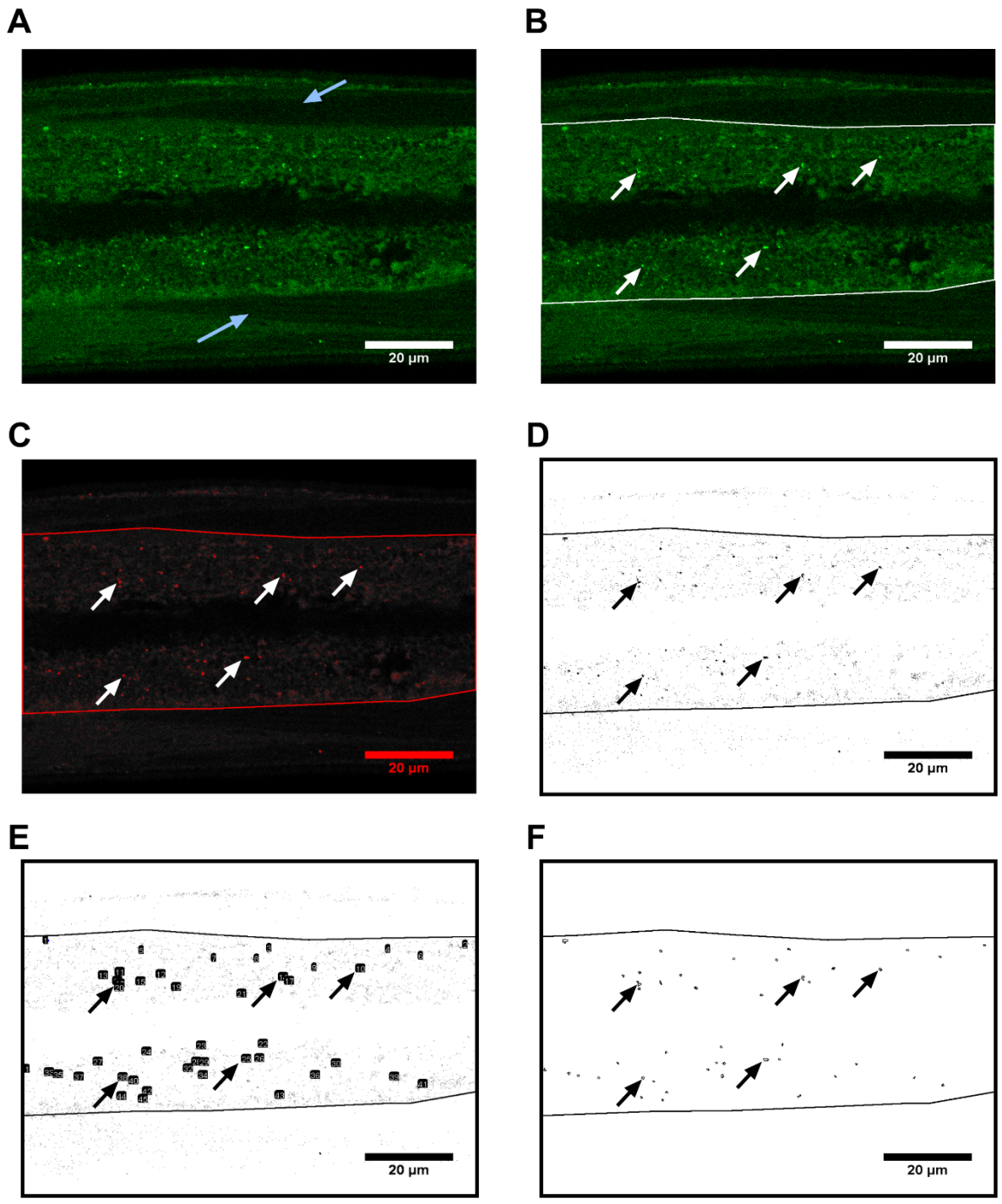

**Supplemental Figure 1.** Section of GO-Term analysis tree for the 727 highly variable genes identified by RNAseq. Image shows the GO hierarchy, the number of genes belonging to each term (out of all possible genes containing that term) and the FDR. Image is a section of the entire GO tree focusing on the highly enriched terms mRNA cleavage, protein phosphorylation, and protein dephosphorylation. Image relates to Figure 7, Table 3, and Supplemental Table 3.

## 8.2 Supplemental Figure 2




**Supplemental Figure 2.** Section of GO-Term analysis tree for the 286 highly variable genes identified by RNAseq as exhibiting a decreasing or trough expression pattern over life. Image shows the GO hierarchy, the number of genes belonging to each term (out of all possible genes containing that term) and the FDR. Image is a section of the entire GO tree focusing on the highly enriched terms ion transport, transmembrane transport, lipid glycosylation, neuropeptide signalling, and G-protein coupled receptors. Image relates to Figure 7, Table 3, and Supplemental Table 3.

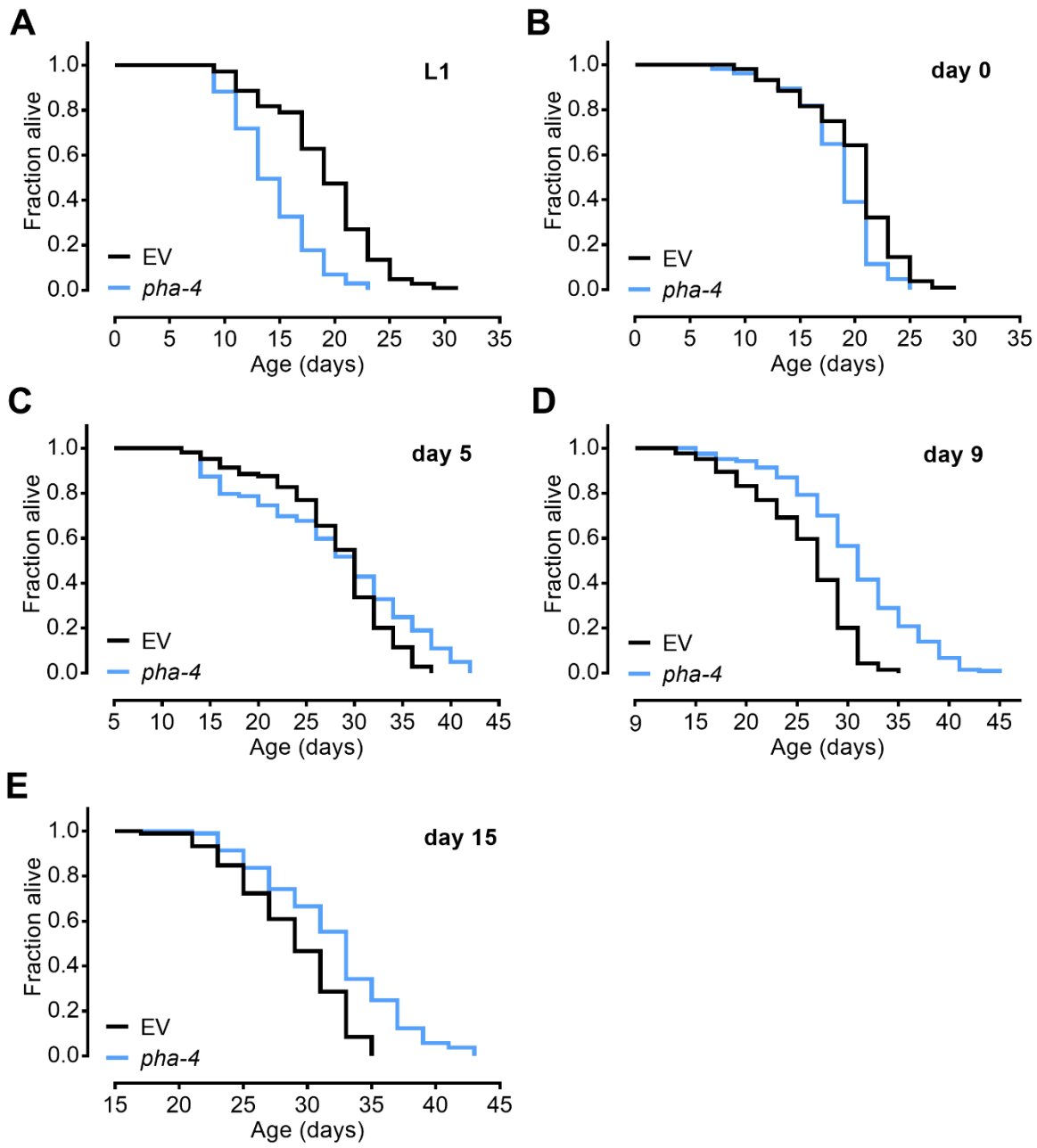
## 8.4 Supplemental Figure 3




**Supplemental Figure 3.** GFP::LGG-1 foci counting methodology. (A) Tissues not belonging to the hypodermis such as muscles (white arrows) are identified within the image. (B) The hypodermis is outlined and drawn to the image. Autophagic foci are indicated (white arrows). (C) Images are converted to 8 bit and subjected to automatic thresholding using the MaxEntropy tool. Identified particles are highlighted in red. (D) Thresholding is applied to create a binary bit map of the worm. (E) The region corresponding to the hypodermis is selected and particles are counted using the analyse

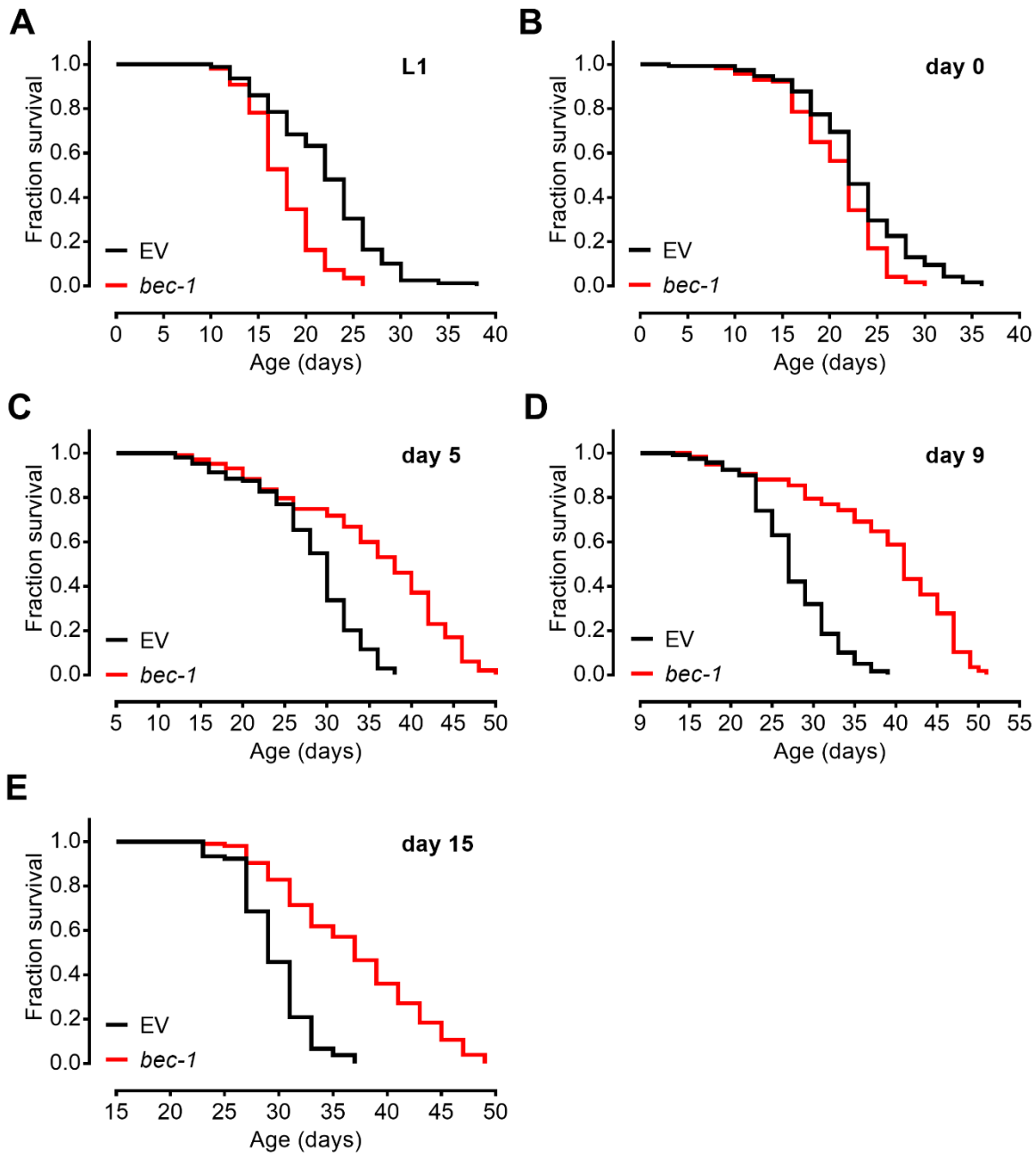
particles function. Counted foci are numbered. (F) Outlined autophagesomes are checked to ensure valid particles are counted. — Original Image was taken from 15 day old worms expressing GFP::LGG-1 fed with EV HT115 bacteria. Images were taken at 100x magnification. Images have been cropped to remove regions of no signal outside the worm, the complete worm remains displayed. For the purposes of illustration the brightness and contrast of the initial image was adjusted and all subsequent steps were performed with this adjusted image.

## 8.5 Supplemental Figure 4




**Supplemental Figure 4.** *pha-4* exhibits antagonistic effects on longevity depending on the timing of RNAi treatment. RNAi inactivation of *pha-4* in *rrf-3* worms results in: a reduction in MTL when initiated at L1 (A) and day 0 (B), no effect on MTL when initiated at day 5 (C), and a positive effect on MTL when initiated at day 9 (D) and day 15 (E). Peak MTL extension was observed at day 9 (26% $p<0.0001$). Days represent days post first egg lay. Initiation of RNAi is indicated by the first time-point on each lifespan curve. Statistics are presented in Supplemental Table 7.

## 8.6 Supplemental Figure 5




**Supplemental Figure 5.** *bec-1* exhibits antagonistic effects on longevity depending on the timing of RNAi treatment. RNAi inactivation of *bec-1* in *rrf-3* worms resulted in: a reduction in MTL when initiated at L1 (A) and day 0 (B), and a positive effect on MTL when initiated at days 5, 9, and 15 (C-E). Peak MTL extension was observed at day 9 (60% $p<0.0001$). Days represent days post first egg lay. Initiation of RNAi is indicated by the first time-point on each lifespan curve. Statistics are presented in Supplemental Table 7.

## 8.7 Supplemental Table 1

| **Falling Proteins** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ACT-4 | ASP-1 | DCT-16 | F01G4.6 | GSNL-1 | KYNU-1 | PAB-1 | PYK-1 | TBA-1 |
| AHCY-1 | B0238.11 | DDO-3 | F33D11.10 | GSP-2 | LBP-9 | PAM-1 | RACK-1 | TBB-1 |
| ALH-8 | C07D8.6 | DEB-1 | F36A2.7 | HCH-1 | LEC-6 | PAS-5 | RAN-1 | TOP-2 |
| ANT-1.1 | C31C9.2 | EAT-6 | F46H5.3 | HEL-1 | LEM-2 | PAT-6 | RET-1 | TSN-1 |
| ARF-3 | CHT-1 | ECH-6 | GDH-1 | ICL-1 | LET-75 | PCK-2 | RPS-5 | VDAC-1 |
| ASB-2 | CPL-1 | EEF-2 | GPD-3 | IDH-1 | MLC-4 | PHB-1 | SODH-1 | Y53G8AR.6 |
| ASG-2 | CPN-3 | EPS-8 | GPX-2 | INF-1 | MYO-3 | PYGL-1 | T13F3.6 | |
| **Rising Proteins** | | | | | | | | |
| ATP-5 | CHT-3 | F59B1.2 | H01G02.1 | RPN-12 | TBH-1 | ULE-1 | Y62H9A.3 | |
| B0513.4 | CLE-1 | FAR-1 | HSP-16.2 | RPS-24 | TEP-1 | ULE-3 | ZK856.7 | |
| C42D4.1 | EEF-1G | FAR-2 | MAI-2 | SIP-1 | TTR-15 | VHA-10 | | |
| C44B7.5 | ENDU-1 | FAR-6 | PERM-2 | SOD-1 | TTR-16 | W05H9.1 | | |
| C46C2.5 | F55B11.2 | FLN-1 | PERM-4 | T12D8.5 | TTR-32 | Y37D8A.19 | | |
| **Peaking Proteins** | | | | | | | | |
| ATP-4 | CEY-4 | E04F6.9 | HIL-3 | LIN-33 | PQN-53 | RPS-22 | TTR-25 | ULE-4 |
| ATTF-2 | CLIC-1 | EIF-2β | HIS-1 | LMN-1 | RPL-18 | SNR-3 | TTR-26 | W01F3.2 |
| C08F11.12 | CRI-1 | ERM-1 | HSP-16.1 | MIG-6 | RPL-25.2 | SPP-10 | TTR-4 | Y57G11B.5 |
| C39D10.7 | CYN-5 | F48E3.4 | HSP-16.48 | NAP-1 | RPL-30 | SWSN-3 | TTR-46 | Y62H9A.5 |
| C53B7.2 | D1086.1 | F49E2.5 | HTZ-1 | NID-1 | RPL-34 | T04A8.6 | TTR-5 | ZK105.1 |
| CEC-5 | D1086.11 | F53F4.11 | ICD-1 | NOL-2 | RPN-1 | TTR-18 | TTR-51 | ZK822.2 |
| CEY-2 | DID-2 | F53H4.2 | LAM-2 | PCCA-1 | RPN-2 | TTR-2 | TTR-59 | ZK970.7 |
| CEY-3 | E04F6.8 | FAR-3 | LEC-5 | PLP-1 | RPS-18 | TTR-24 | TTR-6 | |
| **Trough Proteins** | | | | | | | | |
| ACAA-2 | CAP-1 | CSQ-1 | GLP-4 | KAT-1 | MUP-2 | PYC-1 | TKT-1 | VHA-13 |
| ACT-5 | CAP-2 | CTC-2 | GOT-2.2 | KIN-3 | MYO-2 | PYP-1 | TNI-3 | VHA-16 |
| ANT-1.2 | CCT-1 | DLD-1 | GPD-4 | LAM-1 | NPA-1 | RAB-1 | TNI-4 | VHA-8 |
| ARF-1.2 | CDC-48.2 | DNJ-13 | GSR-1 | LEA-1 | NUO-5 | RLA-2 | TPI-1 | VIT-2 |
| B0303.3 | CHDP-1 | ECH-1.2 | GTA-1 | LEC-4 | PAS-4 | SARS-1 | TTR-45 | Y69A2AR.18 |
| C14F11.4 | CMD-1 | EEF-2 | HIS-24 | LYS-1 | PAS-7 | SCA-1 | UCR-2.1 | |
| C15H9.9 | COX-4 | F13E6.1 | HSP-110 | MDT-28 | PBS-3 | SUCG-1 | ULE-2 | |

**Supplemental Table 1.** List of proteins identified by mass spectrometry as significantly changing over life (FDR 1%). Proteins were identified as belonging to a pattern when trends were consistent across two biological replicates. Falling proteins display their highest abundance early in life and decline thereafter. Rising proteins have low abundance in early life that increases over time. Peaking proteins exhibit a maximal abundance between the first and last measured samples. Trough Proteins display a fall in abundance followed by a rise again at a later point. Table is related to Figure 6.

## 8.8 Supplemental Table 2

| Group type | Category | GO_term | Count | % of total | FDR |
|---|---|---|---|---|---|
| Falling Proteins | Biological process | Embryo development ending in birth or egg hatching | 38 | 62.30 | 8.32E-07 |
| | Enriched terms | Cytoplasm | 15 | 24.59 | 1.02E-05 |
| | Biological process | Nematode larval development | 28 | 45.90 | 9.43E-05 |
| | Cellular component | Cytoplasm | 22 | 36.07 | 1.56E-04 |
| | Enriched terms | Nucleotide-binding | 13 | 21.31 | 0.004899 |
| | Enriched terms | Hydrolase | 12 | 19.67 | 0.015558 |
| | Enriched terms | Oxidoreductase | 8 | 13.11 | 0.036813 |
| | Biological process | Determination of adult lifespan | 18 | 29.51 | 0.002315 |
| | Biological process | Reproduction | 25 | 40.98 | 0.040456 |
| | Enriched terms | Cytoskeleton | 7 | 11.48 | 0.043187 |
| Rising Proteins | Enriched terms | Signalling | 26 | 70.27 | 1.70E-07 |
| | Cellular component | Extracellular region | 9 | 24.32 | 8.50E-05 |
| | Enriched terms | Secreted | 7 | 18.92 | 0.001627 |
| | Biological process | Determination of adult lifespan | 6 | 16.20 | 0.002443 |
| Peaking Proteins | Protein domain | Transthyretin-like | 10 | 14.29 | 5.30E-09 |
| | Biological process | Nematode larval development | 24 | 34.29 | 1.17E-04 |
| | Enriched terms | Signalling | 33 | 47.14 | 6.99E-04 |
| | Cellular component | Extracellular space | 10 | 14.29 | 9.05E-04 |
| | Cellular component | Intracellular ribonucleoprotein complex | 8 | 11.43 | 0.002075 |
| | Enriched terms | Ribonucleoprotein | 7 | 10.00 | 0.037476 |
| | Biological process | Determination of adult lifespan | 14 | 20.00 | 0.04623 |
| Trough Proteins | Metabolism | Carbon metabolism | 12 | 15.79 | 1.12E-05 |
| | Biological process | Reproduction | 33 | 43.42 | 2.42E-05 |
| | Enriched terms | Cytoplasm | 16 | 21.05 | 2.81E-05 |
| | Cellular component | Mitochondrion | 14 | 18.42 | 2.01E-04 |
| | Metabolism | Biosynthesis of antibiotics | 13 | 17.11 | 4.01E-04 |
| | Protein domain | Thiolase, conserved site | 4 | 5.26 | 0.001148 |
| | Biological process | Nematode larval development | 28 | 36.84 | 0.002599 |
| | Biological process | Fatty acid beta-oxidation | 5 | 6.58 | 0.005693 |
| | Metabolism | Metabolic pathways | 22 | 28.95 | 0.005667 |
| | Metabolism | Valine, leucine and isoleucine degradation | 7 | 9.21 | 0.005744 |
| | Enriched terms | Transit peptide | 7 | 9.21 | 0.008089 |
| | Enriched terms | Nucleotide-binding | 14 | 18.42 | 0.010232 |
| | Metabolism | Acetyl-CoA acetyltransferase | 4 | 5.26 | 0.007978 |
| | Molecular function | Catalytic activity | 12 | 15.79 | 0.020101 |
| | Enriched terms | Actin-binding | 5 | 6.58 | 0.038923 |
| Combined | Biological process | Embryo development | 112 | 46.09 | 2.21E-18 |
| | Biological process | Reproduction | 90 | 37.03 | 3.46E-16 |
| | Biological process | Nematode larval development | 83 | 34.15 | 7.42E-15 |
| | Biological process | Determination of adult lifespan | 53 | 21.81 | 4.55E-12 |
| | Metabolism | Carbon metabolism | 20 | 8.23 | 7.19E-09 |
| | Metabolism | Biosynthesis of antibiotics | 21 | 8.64 | 1.98E-05 |
| | Biological process | Apoptotic process | 26 | 10.69 | 2.61E-04 |
| | Biological process | Translation | 19 | 7.81 | 2.66E-04 |
| | Metabolism | Valine, leucine and isoleucine degradation | 10 | 4.11 | 8.48E-04 |
| | Metabolism | Metabolic pathways | 39 | 16.04 | 0.002496 |
| | Molecular function | Actin binding | 10 | 4.11 | 0.005263 |
| | Biological process | Fatty acid beta-oxidation | 6 | 2.46 | 0.023018 |
| | Molecular function | H+ transmembrane transporter activity | 6 | 2.46 | 0.033977 |

**Supplemental Table 2.** DAVID analysis of proteins whose abundance changes over life. Enriched terms were identified where FDR <1% and minimum count for inclusion was 3. Analysis was selected to identify changes in enriched terms, keywords, and go terms for biological processes, molecular function and cellular components.

## 8.9 Supplemental Table 3

| Category | Term | Count | % of total | Fold Enrichment | FDR |
|---|---|---|---|---|---|
| **Goterm_cc_direct** | Pseudopodium | 28 | 4.01 | 26.09 | 6.21E-31 |
| **Goterm_cc_direct** | Cytoskeleton | 43 | 6.16 | 6.88 | 1.85E-20 |
| **Goterm_bp_direct** | Protein dephosphorylation | 37 | 5.30 | 8.15 | 1.32E-19 |
| **Goterm_mf_direct** | Protein tyrosine phosphatase activity | 29 | 4.15 | 10.09 | 1.76E-17 |
| **Up_keywords** | Cytoskeleton | 43 | 6.16 | 5.88 | 1.94E-17 |
| **Goterm_bp_direct** | Peptidyl-tyrosine dephosphorylation | 28 | 4.01 | 9.78 | 2.99E-16 |
| **Goterm_bp_direct** | Regulation of cell shape | 24 | 3.44 | 9.49 | 4.11E-13 |
| **Goterm_mf_direct** | Phosphoprotein phosphatase activity | 22 | 3.15 | 8.24 | 1.41E-10 |
| **Up_keywords** | Acetylation | 25 | 3.58 | 6.71 | 2.44E-10 |
| **Goterm_bp_direct** | Peptidyl-serine phosphorylation | 24 | 3.44 | 7.10 | 2.94E-10 |
| **Goterm_bp_direct** | Innate immune response | 36 | 5.16 | 4.40 | 3.03E-10 |
| **Up_keywords** | Signal | 220 | 31.52 | 1.57 | 3.10E-10 |
| **Up_keywords** | Cell projection | 26 | 3.72 | 5.67 | 4.18E-09 |
| **Goterm_mf_direct** | Structural constituent of cuticle | 24 | 3.44 | 5.62 | 3.67E-08 |
| **Goterm_mf_direct** | Protein kinase activity | 37 | 5.30 | 3.57 | 4.53E-08 |
| **Goterm_cc_direct** | Collagen trimer | 25 | 3.58 | 5.22 | 5.90E-08 |
| **Up_keywords** | Protein phosphatase | 18 | 2.58 | 6.61 | 1.33E-06 |
| **Goterm_bp_direct** | Protein phosphorylation | 36 | 5.18 | 3.22 | 2.00E-06 |
| **Up_keywords** | Collagen | 25 | 3.58 | 4.17 | 8.07E-06 |
| **Goterm_mf_direct** | Protein serine/threonine kinase activity | 27 | 3.87 | 3.65 | 2.29E-05 |
| **Up_keywords** | Cytoplasm | 50 | 7.16 | 2.12 | 0.001273 |
| **Goterm_mf_direct** | Non-membrane spanning protein tyrosine kinase activity | 10 | 1.43 | 8.03 | 0.003664 |

**Supplemental Table 3.** GO-term analysis of all 727 highly variable genes identified by RNAseq over life. Category lists the query origin for each identified term. BP is biological process, MF is molecular function, and CC is cellular component. Keywords are terms linked to genes and proteins and derived from UniProtKB annotations. The over-represented terms for each term are displayed with the number of genes belonging to each group, the % of the total sampled population, the enrichment score and the FDR. Terms were only considered significant where the FRD was <1%.

## 8.10 Supplemental Table 4

| Gene Class | Gene name | Pattern | Most significant change between |
|---|---|---|---|
| NURD | din-1 | Rising | d2-d22 |
| | dpl-1 | Oscilating | d6-d22 |
| | epc-1 | Trough | d6-d22 |
| | let-418 | Falling | d2-d20 |
| | lin-35 | Trough | d6-d16 |
| | sin-3 | Trough | d6-d22 |
| | spr-3 | Falling | d2-d20 |
| HMT | set-5 | Rising | d2-d22 |
| | set-11 | Peaking | d2-d16 |
| | set-14 | Trough | d6-d22 |
| | set-15 | Oscilating | d6-d22 |
| | set-19 | Falling | d2-d20 |
| | set-23 | Trough | d6-d20 |
| | set-24 | Trough | d6-d20 |
| | set-26 | Rising | d6-d22 |
| | set-30 | Peaking | d6-d22 |
| | lin-59 | Rising | d2-d22 |
| Dosage compensation | dpy-21 | Rising | d6-d22 |
| | dpy-27 | Rising | d2-d22 |
| | dpy-21 | Rising | d2-d22 |
| | dpy-27 | Rising | d2-d22 |
| | dpy-28 | Rising | d6-d22 |
| | sdc-1 | Falling | d2-d22 |
| | sdc-3 | Falling | d2-d20 |
| HDM | jmjd-2 | Rising | d6-d22 |
| | jmjd-3.2 | Falling | d2-d20 |
| | jmjd-1.2 | Peaking | d2-d20 |
| SWI /SNF | ssl-1 | Trough | d6-d22 |
| | swsn-3 | Trough | d6-d16 |
| | swsn-6 | Trough | d6-d20 |
| HAT | taf-1 | Trough | d6-d22 |
| | hat-1 | Falling | d2-d16 |
| HDAC | hda-2 | Peaking | d6-d22 |
| | hda-6 | Peaking | d6-d20 |
| | hdac-11 | Falling | d2-d22 |
| COMPASS | wdr-52. | Falling | d2-d20 |
| NURF | nurf-1 | Rising | d6-d22 |

**Supplemental Table 4.** Table listing all genes with epigenetic functions identified as differentially expressed in RNAseq data. Genes listed were identified from 1048 total genes belonging to 14 different gene families. Genes were included in the list when differentially expressed between at least 2 time-points. Gene names are listed along with their expression pattern and time period of greatest expression change. Table relates to Figure 10 and Table 4.

## 8.11 Supplemental Table 5

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | N | Control Mean treated lifespan +/- SD | N | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| WT (N2) Day 9 | *hda-6* | 10.817 +/- 0.072 | 103/105 | 11.633 +/- 1.730 | 105/105 | -7.01% | 0.6666 | |
| | *jmjd-1.2* | 12.363 +/- 0.672 | 104/105 | 11.633 +/- 1.730 | 105/105 | 6.28% | 0.2592 | 11A |
| | *ttr-24* | 10.555 +/- 0.288 | 103/105 | 11.633 +/- 1.730 | 105/105 | -9.27% | 0.5652 | 11A |
| | *hsp-16.2* | 10.474 +/- 0.678 | 104/105 | 11.633 +/- 1.730 | 105/105 | -9.96% | 0.5185 | 11A |
| | *rfp-1* | 12.007 +/- 0.616 | 105/105 | 11.633 +/- 1.730 | 105/105 | 3.21% | 0.0909 | 11A |
| | *rpn-1* | 6.940 +/- 0.121 | 105/105 | 11.633 +/- 1.730 | 105/105 | -40.34% | 0.0001 | |
| | *rpn-10* | 7.628 +/- 0.866 | 70/70* | 11.633 +/- 1.730 | 105/105 | -34.43% | 0.0009 | |
| | *rpn-2* | 7.867 +/- 0.538 | 69/70* | 11.633 +/- 1.730 | 105/105 | -32.37% | 0.0002 | |
| | *rpn-12* | 9.158 +/- 0.982 | 104/105 | 11.633 +/- 1.730 | 105/105 | -21.28% | 0.0009 | |
| | *taf-1* | 11.586 +/- 0.764 | 104/105 | 11.633 +/- 1.730 | 105/105 | -0.40% | 0.3333 | |
| | *set-11* | 15.212 +/- 1.157 | 104/105 | 14.766 +/- 0.611 | 105/105 | 3.02% | 0.1481 | |
| | *din-1* | 14.601 +/- 0.916 | 104/105 | 14.766 +/- 0.611 | 105/105 | -1.12% | 0.4500 | |
| | *ttr-12* | 14.630 +/- 0.055 | 104/105 | 14.766 +/- 0.611 | 105/105 | -0.92% | 0.9130 | |
| | *hda-2* | 13.740 +/- 0.236 | 105/105 | 14.766 +/- 0.611 | 105/105 | -6.95% | 0.9411 | |
| | *hat-1* | 14.956 +/- 0.647 | 104/105 | 14.766 +/- 0.611 | 105/105 | 1.29% | 0.1764 | 11B |
| | *jmjd-1.2* | 14.082 +/- 0.914 | 105/105 | 14.766 +/- 0.611 | 105/105 | -4.63% | 0.9047 | 11B |
| | *utx-1* | 14.107 +/- 0.105 | 103/105 | 14.766 +/- 0.611 | 105/105 | -4.46% | 0.6909 | 11B |
| | *spat-3* | 14.492 +/- 0.461 | 104/105 | 14.766 +/- 0.611 | 105/105 | -1.86% | 0.5384 | |
| | *swsn-6* | 14.643 +/- 0.385 | 103/105 | 14.766 +/- 0.611 | 105/105 | -0.83% | 0.3684 | 11B |
| | *set-24* | 14.275 +/- 0.325 | 104/105 | 14.766 +/- 0.611 | 105/105 | -3.33% | 0.2608 | |

**Supplemental Table 5.** Lifespan effects of genes identified as potential AP from MS and RNAseq data. Day 9 RNAi against these genes had no effect on lifespan in WT worms (Fig. 11, A & B) with the exception of gene members of the 20s proteasome complex. RNAi knockdown of *rpn-1, rpn-2* and *rpn-12* significantly shortened lifespan from day 9. — Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. N: number of dead worms/number of total worms. *: indicates where whole plates were scored out due to contamination. Worms from these plates were not used for statistical analysis.

## 8.12 Supplemental Table 6

### Plate 1

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Ena2A01 F47G6.1 Dyb-1 correct | EV | Ena3B10 T28F2.4 JmjC correct | Ena1C09 R119.6 Taf-4 correct | Ena3C03 D1037.1 correct | Ena1D06 F56A6.1 Sago-2 correct | Ena3D03 T12F5.4 Lin-59 correct | Ena4D12 C18E3.7 Ppw-1 also targets sago-2 (correct) high seq. homology – targets sago-2 as well | Ena4E03 C43E11.1 acin-1 by chance (supposed to be cogc-5) | Ena4E05 C43E11.3 Met-1 correct | Ena4E12 C43E11.10 Cdc-6 correct | Ena2F02 W10C8.2 Pop-1 correct |
| **B** | Ena4F04 C43E11.1 Acin-1 correct | Ena2G09 C45E1.4 correct | EV | Ena1H09 K12C11.4 Dapk-1 correct | Ena6A02 F54C1.3 Mes-3 correct | NT GFP | Supp V-14J8 Y59A8A.2 phf-14 correct | Cloned C06A5.3 correct | Ena6D02 F56A3.2 slx-1 correct | Ena6D09 all his1 (hil-2, hil-5, hil-6, his-24) by chance (supposed to be B0261.1) | Ena6E09 C01G8.9 Let-526 correct | Ena6E10 C01G8.8 Let-526 correct (C01G8.9) |
| **C** | Ena6G02 F55A12.8 Nath-10 correct | Ena7G08 C32F10.2 Lin-35 correct | Ena7G10 C32F10.5 Hmg-3 correct | Ena11A02 F22D6.6 Ekl-1 correct | Ena11A11 K10D3.3 Taf-11.2 correct | Ena12B03 D2030.9 Wdr-23 correct | AL I-3D18 F26A3.3 Ego-1 correct | Ena9C04 K04F10.6 Rde-3=mut-2 correct | Ena12C03 T23G11.2 Gna-2 correct | Ena11C08 C01H6.7 Tag-298 = swsn-9 correct | Ena11C10 C01H6.9 correct | AL:I-3G19 T23H2.3 correct |
| **D** | AL:I-3G24 K02F2.3 Teg-4/phi-6/tag-203 correct | Ena11D05 R06C7.7 Lin-61 correct | AL III-4J4 R107.2 correct | Ena11D12 F21C3.4 Rde-2 correct | Ena12D12 D2005.5 Drh-3 correct | Ena12E08 F30F8.8 Taf-5 correct | Gino:1C2 H06O01.2 Chd-1 correct | Ena12G12 C54G4.1 Rskn-2 correct | Ena11H07 C26C6.1 Pbrm-1 correct | Ena11H11 C26C6.5 Dcp-66 correct | Ena13A10 C09H6.1 Spr-4 correct | Ena15A12 W06D4.4 Prmt-7 correct |
| **E** | NT GFP | Ena15B02 W06D4.6 Rad-54 correct | Ena16B06 K07A1.11 Rba-1 correct seq.cl.mix | Ena16B07 K07A1.12 Lin-53 correct | Ena13D04 T01G9.6 Kin-10 correct | Ena13D11 C34B7.1 correct + targets mys-4 as well | Ena14D12 DY3.2 Lmn-1 correct | Ena13E03 F16D3.2 Rsd-6 correct | Ena15E06 C12C8.3 Lin-41 correct | Ena13F02 F02E9.4 Sin-3 correct | Ena15F08 C17E4.6 correct | Ena15F10 C17E4.6 correct |
| **F** | Ena13G07 D1081.8 correct | Ena14H02 F32H2.1 Gei-11 correct | AL III-6K7 Y56A3A.16 correct | Ena15H05 F30A10.10 Usp-48 correct | Ena15H06 C41G7.1 Smn-1 correct | Ena17B02 T23D8.8 Cfi-1 correct | Ena20B12 F15D3.1 Dys-1 correct | Ena17C05 B0379.3 Mut-16 correct | Ena18D08 T22A3.4 Set-18 correct | Supp I-10A5 F28D9.2 Sri-5 correct | Supp III-8G7 Y119D3B.11 orc-3 correct | Ena18G02 B0205.1 correct |
| **G** | Ena18G04 B0205.3 Rpn-10 correct | AL I-5O8 F55A3.3 Phi-16 correct | Ena17H07 F25D7.3 Blmp-1 correct | Ena20H11 R09B3.4 Ubc-12 correct | Ena20H12 R09B3.5 Mag-1 correct | AL X-1G12 C46H3.1 correct | Ena22A10 T15D6.8 correct | Ena 23A08 W02A11.4 Uba-2 correct | AL X-4D20 C11E4.6 correct | Ena22B01 T15D6.11 correct | Ena23B01 B0019.2 correct | Ena23B03 Y18D10A.1 attf-6 correct |
| **H** | Ena24B04 T04D3.5 correct | AL I-6G16 K11D2.1 correct | Ena21D11 R05D7.2 correct | Ena24D01 Y40B1B.6 Spr-5 correct | Ena22E02 W02D9.3 hmg-20 correct | AL I-7C7 W04A8.7 Taf-1 correct | Ena25C08 F47G4.6 Hmg-6 correct | Ena27C09 Y39G10AR.18 correct | AL X-5P12 F45E6.3 tbc-13 correct | Ena26E08 Y54E5B.4 Ubc-16 correct | Ena27E05 Y39G10AR.8 correct | Ena27E10 Y39G10AR.7 Y39G10AR.8 Y39010AR.30 correct |

## Plate 2

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Ena26F10 ZK909.2 Kin-1 correct | Supp I-9E5 Y47G6A.6 Pcaf-1 & Y47G6A.19 correct | AL I-3F21 R06C7.1 wago-1 correct | Ena26G11 F33H2.7 Set-10 correct | Ena25H03 Y105E8A.17 Ekl-4 correct | Ena29E12 Y71G12B.15 Ubc-3 correct | AL II-1A5 C01B12.2 correct | Ena31A09 C01B12.8 correct | Ena31A10 F23F1.1 Nfyc-1 correct | Ena34A09 W09B6.2 Taf-6.1 correct | Ena32C12 W04H10.3 Nhl-3 correct | Ena33C03 T07D3.7 Alg-2 correct |
| **B** | AL I-4G17 F52B5.5 cep-1 correct | Ena36A05 F52C6.12 correct | Supp II-10H20 ZK1240.1 correct | Ena37A06 ZK1240.2 correct | Ena37A07 ZK1240.3 correct | EV | Ena37H07 F34D6.4 Set-11 correct | AL II-2P24 T10D4.6 correct | Ena42A06 C16A11.3 correct | AL II-3B14 C16A11.4 correct | Ena42B11 H20J04.2 athp-2 correct | Ena41E08 Y14H12B.2 correct |
| **C** | Ena41F04 K07D4.3 Rpn-11 correct | Ena42G06 T05A7.4 Hmg-11 correct | NT GFP | Ena44A05 F58A6.8 Msp-45 correct | AL I-5C1 T23D8.7 correct | Ena44B09 C34F11.4 Msp-50 correct | Ena44B11 C34F11.6 Msp-49 correct | Supp I-10A8 D2030.6 prg-1 correct | NT GFP | Supp II-11J11 ZK1248.7 wago-5 correct | Ena46C06 H12I13.1 correct | Ena46C08 F21H12.1 rbbp-5 correct |
| **D** | Ena44D05 F09E5.1 Pkc-3 correct | Ena43D08 R05F9.3 Msp-32 correct | Ena46D05 C29H12.5 cec-9 correct | Ena43E01 R05F9.8 Msp-33 R05F9.5 correct | Ena43E06 R05F9.13 Msp-13 correct | Ena43E09 ZK546.3 ZK546.17 correct | Ena43E11 ZK546.6 Msp-152 correct | AL II-5E23 F18C5.2 wrn-1 correct | Ena46G10 C32D5.5 Set-4 correct | Ena45G12 ZK1248.17 correct | Ena45F05 K05F1.7 Msp-63 correct | Ena43H11 ZC8.3 set-30 by chance (supposed to be msp-74) |
| **E** | AL III-1J2 C14B1.6 nrde-1 correct | Ena49B04 F32A5.1 Ada-2 correct | Ena49B10 F32A5.7 Lsm-4 + ada-2 correct | AL III-5E16 ZK757.3 alg-4 correct | AL II-5I8 ZK1127.3 correct | Ena48E08 ZK1127.7 Cin-4 correct | Cloned Y18H1A.10 my stock I correct | AL:II-5I18 ZK1127.7 Cin-4 correct | AL: II-5J06 T09A5.8 cec-3 correct | Supp III-8B19 C16C10.3 hrde-1 correct | Ena49E10 D1022.1 Ubc-6 correct | Ena47G04 C18H9.7 Rpy-1 correct |
| **F** | Ena50G04 C08B11.2 Hda-2 correct | Ena50G05 C08B11.3 swsn-7 correct | Ena50G08 C08B11.6 Arp-6 correct | AL IV-3J8 F45E4.10 nrde-4 correct | AL IV-3O11 C01G5.2 prg-2 correct | Ena51C10 F35C11.4 correct | Ena51D12 C26D10.1 Ran-3 correct | Ena53D07 T23G7.1 Dpl-1 correct | Ena52E03 T01B7.5 correct | Supp II-10A24 K08F8.6 Let-19 correct | AL II-6M24 T07D4.3 Rha-1 correct | Ena53G04 D2013.9 Ttll-12 correct |
| **G** | Ena52H10 T21B10.5 Set-17 correct | Ena57B01 R06F6.4 Set-14 correct | Ena57B02 R06F6.5 Npp-19 correct | AL II-7I16 R166.1 Mab-10 correct | Ena55F07 ZK945.5 correct | Ena55G09 F33H1.4 correct | AL II-7N4 F07A11.4 correct | AL II-7N8 F07A11.6 Din-1 correct | Ena58G08 ZK20.5 Rpn-12 correct | Ena58G10 C47D12.1 Trr-1 correct | Ena60A10 C50E10.4 Sop-2 correct | Ena59B02 F43G6.11 Hda-5 correct |
| **H** | AL IV-6A22 T22B3.2 alg-3 correct | Ena60C02 C50E10.4 Sop-2 correct | Ena59C03 K12D12.1 Top-2 correct | EV | Ena59D08 W03C9.4 Lin-29 correct | AL V-1F11 R09A1.1 ergo-1 correct | Ena62E06 ZK131.3 His-9 + his-13,-9,-10,-25,-26 correct | Ena62E10 ZK131.7 His-13 + his-14 correct | Ena59F12 Y57A10A.1 correct | Ena61F03 W09H1.2 His-73 correct | Ena62F05 F08G2.3 His-42 correct | Ena61F07 F15D4.1 Btf-1 correct |

## Plate 3

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Ena59G01 Y57A10A.2 correct | Ena59G03 Y57A10A.3 Y57A10A.4 correct | Ena59G05 Y57A10A.5 Y57A10A.4 correct | Ena59G07 Y57A10A.5 Y57A10A.7 Y57A10A.4 correct | Ena59G09 Y57A10A.5 Y57A10A.6 Y57A10A.7 correct | Ena59G11 Y57A10A.7 correct | Ena59H01 Y57A10A.8 correct | Ena62G03 Y51H1A.5 Hda-10 correct | Ena63C06 Y48B6A.11 Jmjd-2 correc t | Ena65C04 Y110A2AR.2 ubc-15 by chance (supposed to target another gene) | AL V-1G23 T22H9.3 wago-10 correct | Ena63D07 Y48B6A.14 Hmg-1.1 correct |
| **B** | Ena64E06 Y53F4B.3 correct | Ena63F05 F26H11.2 Nurf-1 correct | Ena66B01 F54C4.2 Spt-4 correct | Ena66B02 F54C4.3 attf-3 correct | AL III-1C15 C29F9.5 correct | Ena67C11 F23H11.1 Bra-2 correct | Ena68C09 W04B5.1 correct | Ena66D04 F40G9.3 Ubc-20 correct | Gino 2D1/3H3 C14B1.4 Wdr-5.1 correct | Ena69E12 C44F1.2 gmeb-3 correct | Ena68F08 H05C05.2 correct | Ena69F09 R13G10.2 Amx-1 correct |
| **C** | Ena69G07 C36A4.8 Brc-1 correct | Ena70A11 T02C12.3 tftc-5 correct | Ena73A07 C34E10.5 Prmt-5 correct | Ena73A10 C34E10.8 correct | Ena70B01 E03A3.3 His-69 correct | Ena70C07 C16C10.4 correct | Ena70C10 C16C10.7 Rnf-5 correct | Ena72C04 F26F4.7 Nhl-2 correct | Ena72C11 C26E6.3 ntl-9 correct | Ena72D05 C26E6.9 Set-2 correct | Supp III-8E15 C26E6.12 correct | AL V-4H7 Y38A10A.6 smut-1 correct |
| **D** | Ena71G11 C38D4.3 Mel-28 correct | Ena70H03 R07E5.3 Snfc-5 correct | Ena70H09 R07E5.10 Pdcd-2 correct | AL III-2P1 K10D2.1 correct | Ena73H05 C05D2.5 Xnd-1 correct | Ena72H09 ZC155.2 correct | NT GFP | Ena77A04 ZK418.4 Lin-37 correct | Ena76C08 F37A4.8 isw-1 correct | Ena75D09 F47D12.4 Hmg-1.2 correct | Gino 2E6 C56G2.1 correct | Ena75E03 C56G2.1 correct |
| **E** | Ena74E10 C28H8.9 Dpff-1 correct | EV | Supp:III-8E14 C16A3.1 correct | Cloned R151.8 correct | Ena77E07 T20H4.4 Adr-2 correct | Ena75F01 C16A3.3 Let-716 correct | Ena74F05 F25B5.4 Ubq-1 correct | Ena76F01 T07E3.3 correct | Ena75H02 T26A5.5 jhdm-1 correct | Ena75H04 T26A5.7 Set-1 correct | Ena75H05 T26A5.8 correct | Ena77H05 T20B12.8 Hmg-4 correct |
| **F** | Ena76H12 R01H2.6 Ubc-18 correct | Ena79A05 F44B9.6 Lin-36 correct | Ena81A04 F54F2.9 correct | Ena80A10 ZK1236.2 Cec-1 correct | Ena79B04 K12H4.8 Dcr-1 correct | AL:III-4C10 C14B9.4 Plk-1 correct | Ena81B04 ZK637.7 Lin-9 correct | AL:III-4E8 C14B9.4 Plk-1 correct | Ena78C06 ZK783.4 Flt-1 correct | Ena79C06 C14B9.6 Gei-8 correct | Ena78D04 ZK112.2 ncl-1 correct | EV |
| **G** | AL III-4N12 F54G8.4 Nhl-1 correct | Cloned box3 CE7X_3.2 | NT GFP | Ena80H05 K02D10.3 correct | Ena81H06 T23G5.6 correct | Ena80H09 F54F2.2 Zfp-1 correct | Ena81H07 T02C1.1 correct | Ena80H10 F54F2.2 Zfp-1 correct | Gino 2F9 C29E4.5 Tag-250 correct | Ena83A05 R10E11.3 Usp-46 correct | AL III-5B2 K01G5.2 Hpl-2 correct | Ena84A08 T20G5.11 Rde-4 correct |
| **H** | Ena82B09 F54C8.2 Cpar-1 correct | Ena83B12 ZK632.13 Lin-52 correct | Ena85C01 C44B9.4 Athp-1 correct | Ena84D02 M03C11.3 correct | Ena84D03 M03C11.4 Hat-1/tag-235 correct | Supp:III-8C7 M03C11.8 correct | AL X-1A17 R04A9.2 nrde-3 correct | Ena82F05 F58A4.3 Hcp-3 correct | Ena82F12 F58A4.10 Ubc-7 correct | Ena82G11 C07A9.7 Set-3 correct | Ena83H04 ZK1128.5 Swsn-3 = ham-3 correct | Ena89A01 T28A8.6 correct |

## Plate 4

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Ena86B02 Y47D3B.9 Bed-2 correct | Ena87B10 Y49E10.6 His-72 correct | Ena87B11 Y49E10.7 & Y49E10.4 correct | Ena87B12 Y49E10.8 Y49E10.26 Y49E10.30 correct | Gino 2H4 T12D8.1 Set-16 correct | AL III-6D24 T12D8.7 Taf-9 correct | Supp III-8K22 Y55B1BR.3 cec-8 correct | Gino 2H2 Y43F4B.3 Set-25 correct | EV | AL III-6I14 Y111B2A.11 Epc-1 correct | Ena88E07 F53A2.5 Dro-1 correct | Ena87G12 Y37D8A.9 Mrg-1 correct |
| **B** | Gino 1E9 Y87G2A.11 correct | Ena86H03 Y75B8A.4 correct | Gino 3E3 F17C11.10 correct | Ena88H10 T28A8.3 correct | Ena88H11 T28A8.4 correct | EV | Ena89C06 ZK520.2 Sid-2 correct | Ena91E03 Y71H2AM.17 Swsn-3 correct | Gino 4B2 K07H8.3 correct | Ena90G04 Y53G8AR.6 & Y53G8AR.8 correct | Ena90G05 Y53G8AR.6 correct | Ena90H06 Y53G8AR.2 phf-15 correct |
| **C** | AL IV-1A4 C18H7.9 correct | NT GFP | Ena92B02 B0545.1 Tpa-1 correct | Ena92C10 T21D12.3 Pqbp-1.1 correct | Ena92E07 T07A9.1 Pqbp-1.2 correct | Supp:IV-9D12 C50A2.2 cec-2 correct | Ena92E10 T07A9.5 Eri-1 & T07A9.12 correct | Gino 4C9 K07C5.8 cash-1 correct | Ena95E05 M57.1 & B0212.3 correct | Ena95E09 B0212.3 & M57.1 correct | AL IV-1O4 F53H1.4 correct | Gino 4E3 R11E3.4 Set-15 correct |
| **D** | Gino 1F3 F39B2.2 uev-1 correct | Ena108B5 ZK1251.6 Msp-76 correct | AL IV-2C22 R08C7.3 Htz-1 correct | Gino 1H2 F40E12.2 correct | Ena99B06 ZK354.4 Msp-113 correct | Ena99B07 ZK354.5 Msp-51 correct | Ena99B12 ZK354.11 Msp-59 correct | NT GFP | Ena96C09 C35B1.1 Ubc-1 correct | Ena99C12 F41H10.6 Hda-6 correct | Ena97E12 F29B9.2 Jmjd-1.2 correct | Gino 1H7 ZK430.7 correct |
| **E** | Ena97F1 F29B9.4 psr-1 correct | Ena97F3 F29B9.6 Ubc-9 correct | Gino 1H9 F33G12.2 correct | Ena99F4 T12E12.2 cec-6 correct | AL IV-2O5 F15E6.1 Set-9 correct | Ena100A11 C06E7.1 Sams-3 correct | AL IV-3B8 F55G1.2 His-59 correct | Ena101B5 F38A5.13 Dnj-11 correct | Ena101B7 F15B10.2 Drh-1 correct | Ena103C12 F32E10.2 cec-4 correct | Ena100D3 B0350.2 Unc-44 correct | Ena100D4 B0350.2 Unc-44 correct |
| **F** | AL IV-3H6 F32E10.5 correct | Gino 4E12 F32E10.6 cec-5 correct | Ena101E3 C17H12.13 anat-1 correct | Ena103E3 F45E4.9 Hmg-5 correct | Ena101F3 T05A12.4 correct | Ena101F10 Recent T05A12.4 correct | Ena101G10 ZK381.4 Pgl-1 correct | Ena103G4 C46A5.9 Hcf-1 correct | Ena103G9 C33H5.6 Swd-2.1 correct | AL IV-4A16 F17E9.10 His-32 correct | Ena105B7 D2096.8 Nap-1 correct | Ena104C1 F20D12.1 Csr-1 correct |
| **G** | Ena106C5 C27B7.4 Rad-26 correct | Ena105E5 C09G4.4 & C09G4.5 Mes-6 correct | Ena105E6 C09G4.5 Mes-6 correct | Ena105E8 F42A9.2 Lin-49 correct | Ena107E2 F56D5.4 correct | AL IV-4J18 F59B8.2 Idh-1 correct | Ena110A7 R11A8.4 Sir-2.1 correct | Ena109B11 C04G2.4 Msp-36 correct | Gino 2B1 F28C6.3 cpf-1 correct | Ena108C8 T13F2.2 correct | Ena108D4 T13F2.10 Msp-79 correct | Gino 2B2 FsopC5.9 correct |
| **H** | Gino 2B5 D2013.2 wdfy-2 correct | Ena111D10 F36H1.2 Tag-144 correct | Ena108E2 K07F5.1 Msp-81 correct | Ena108E3 K07F5.2 Msp-10 +usp-81 correct | Ena109E3 K08F4.8 Msp-38 correct | Ena108E4 K07F5.3 Msp-56 correct | AL IV-5J14 M7.1 Let-70 correct | Ena108F8 F32B6.6 Msp-77 correct | Ena108G11 C47E12.4 Pyp-1 correct | Ena111G3 F01G4.1 Psa-4 = swsn-4 correct | Ena112B8 B0035.4 Pfd-4 correct | Ena112C9 F54E12.1 His-55 correct |

## Plate 5

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Ena112 C10 F54E12. 2 correct | Ena113D6 C29E6.2 Trpa-1 correct | Ena114D 4 F28D1.1 Wdr-46 correct | Ena112E 2 F22B3.2 His-63 correct | AL IV - 6J23 C25G4.4 Tag-347 correct | Ena112F 5 M04B2.1 Mep-1 correct | Ena112F 7 M04B2.3 Gfl-1 correct | Ena113F 12 K08E4.1 Spt-5 correct | Ena113 G6 F11A10. 1 Lex-1 correct | AL IV- 7A1 C39E9.1 2 correct | Ena117 A8 C08F11. 7 correct | AL IV- 7E9 JC8.6 Lin-54 correct |
| **B** | Gino 2B9 T01E8.4 mec-15 correct | Ena117E1 B0513.6 correct | Ena117E 10 Y37A1B. 1 Lst-3 correct | Ena116F 11 Y38H8A. 3 correct | EnaEna11 6G1 Y38H8A.5 correct | Ena117G 1 F52B11. 1 Cfp-1 correct | Ena116G 7 Y45F10A .5 Nlp-17 correct | AL IV- 7P18 Y51H4A. 12 Set-26 correct | AL:IV- 8A12 F26D10. 3 Hsp-1 correct (but bad seq) | Ena122 A6 Y41D4B .12 Set-23 Targets Y41D4B .14 & Y41D4B .19 correct | Ena122 B8 C26E6.9 set-2 by chance (suppos ed to target ced-2 & set-23) | Ena120D 2 Y116A8C .13 correct |
| **C** | Gino 2B12 ZK20.3 rad-23 correct | Ena121F1 Y73B3B.1 Y104H12 BR.1 correct | AL:IV- 8O17 F26D10. 3 Hsp-1 correct | Ena128 D10 Y46H3C. 4 correct | Gino 2C3 F43G6.6 jmjd-1.1 correct | Gino 3C4 T10B5.4 correct | Gino 3C5 F59A7.8 correct | Ena131E 5 F37B4.1 0 correct | Ena136 A12 F54D11. 2 correct | Ena134 B7 T27C4.4 Lin-40 correct | Ena136 D6 C18G1.5 Hil-4 correct | Ena138B 11 C24G6.6 correct |
| **D** | Ena137 C2 C04F5.1 Sid-1 correct | Ena138D5 CD4.7 Zhit-1 correct | Ena138E 6 C14C11. 6 Mut-14 correct | Ena137F 9 B0238.1 0 correct | NT GFP | Ena144D 11 D1014.8 Spr-1 correct | Ena144D 12 D1014.9 Spr-1 correct | Ena141E 5 VC5.4 Mys-1 correct | Ena144 E12 K07C11. 2 Air-1 correct | Ena141 G8 F09G2. 9 attf-2 correct | Gino 3D4 T23B12. 1 Phf-30 correct | Ena147B 4 F07C3.4 Glo-4 correct |
| **E** | Ena145 E3 F45F2.1 3 HIS-6 correct | Ena148F3 R07B5.9 lsy-12 by chance (suppose d to target T11A5.1) | Ena148H 3 C27H6.2 Ruvb-1 correct | Ena148 H11 K08H10. 7 Rde-1 correct | Ena150A6 Y32F6A.1 Set-22 correct | Ena150A 12 F22E12.4 Egl-9 correct | Ena152A 12 K03B8.4 correct | Ena150B 12 C35A5.9 Hdac-11 correct | Supp V- 14G17 ZK856.9 Zhit-3 correct | Ena149 E9 C12D8. 10 Akt-1 correct | Ena151 E4 C06H2.3 Jmjd-5 correct | Gino 2C12 H06I04.4 ubl-1 correct |
| **F** | Ena152 F7 T27F2.1 Skp-1 correct | Ena149G 4 B0024.12 Gna-1 correct | Ena151H 11 F47G9.4 correct | EV | Ena153D3 F57F5.5 Pkc-1 correct | Ena156D 7 M04G12 .4 Somi-1 correct | Ena155E 3 F54F3.1 Nid-1 correct | Ena156E 7 Y2H9A.1 Mes-4 correct | Ena153 G9 ZK863.6 Dpy-30 correct | Ena159 C6 C53A5. 3 Hda-1 correct | Ena157 D10 F55B12. 3 Sel-10 correct | Gino 3E12 C47E8.8 Set-5 correct |
| **G** | Ena162 B1 F28F8.7 correct | Ena163G 4 T10C6.13 HIS-2 correct | AL V- 11G22 Y102A5C .18 Efl- 1 correct | Ena171 C1 C25F9.5 correct | NT GFP | Ena172G 4 Y113G7B .17 prmt-1 correct | Ena175A 6 Y97E10A R.4 Y97E10A R.3 Y97E10A R.7 Y97E10A R.8 correct | Ena175A 7 Y97E10A R.3 Y97E10A R.8 correct | EV | AL V- 1H20 Y46H3C .4 + others correct | Ena176 A12 R04A9.7 correct | AL X- 1G3 F13C5.2 correct |
| **H** | AL X- 1K7 T04G9.1 correct | Ena178F1 2 ZC53.6 correct | Ena177G 3 ZK402.3 more targets correct | Gino 2F9 C29E4.5 tag-250 correct | Ena177G6 ZK402.5 correct | AL:X- 2C18 C12D12. 5 sox-2 correct | Ena182D 3 F48D6.1 Taf-11.1 correct | Ena182E 2 C52B9.8 correct | AL X- 2J11 C24A8.3 Pqn-15 correct | Ena183 F4 ZC8.3 Set-30 correct | Ena183F 5 ZC8.3 Set-30 correct | Ena180G 5 K09C4.3 Hsp-2 correct |

## Plate 6

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | AL X-2O6 F47F2.1 correct | Ena182 H9 C07A12.5 Spr-3 correct | Supp X-8A23 T03G11.1 Pqn-62 correct | EV | Ena187A10 C54D1.5 Lam-2 correct | Ena187C4 K03A1.1 His-40 correct | Ena184E11 T22B7.1 Egl-13 correct | Ena187E10 C10A4.8 Mnm-2 correct | Ena184F7 W01C8.2 Recent T22B7.1 Egl-13 correct | Gino 3G9 W01C8.3 Set-19 correct | Ena184F9 W01C8.4 Set-20 correct | Ena187F3 K08A8.2 Sox-2 correct |
| **B** | Supp X-8L14 F14B8.5 correct | Ena188A12 K09F5.5 Set-12 correct | Ena190A7 C28G1.1 Ubc-23 correct | AL X-4C5 C47C12.3 Ref-2 correct | Ena190B1 C06E2.3 Ubc-21 correct | EV | Ena188E4 F45E1.6 His-71 correct | Ena189E5 D2021.1 Utx-1 correct | Ena189F1 F18E9.5 Jmjd-3.1 correct | Gino 3A1 C33H5.7 swd-2.2 correct | Ena188G6 F22F1.1 Hil-3 correct | Ena188G8 F22F1.3 correct |
| **C** | Ena190G12 E01H11.1 Pkc-2 correct | Ena195A3 C49F5.5 correct | Ena192D6 F46F6.2 pkn-1 correct | Ena195E3 W06D11.4 correct | Ena195E6 F17A2.3 phf-32 correct | Ena194F5 C34E11.1 Rsd-3 correct | Ena194F8 C43E11.1 Taf-7.1 correct | Ena193G3 F38B2.1 Ifa-1 correct | Ena192G9 F13E6.3 phf-31 correct | Ena194G2 F54F7.7 correct | NT GFP | Ena194H9 T08D10.2 Lsd-1 correct |
| **D** | Ena194H12 C49F5.2 Set-6 correct | Ena197A1 K09A11.1 correct | Ena197A5 K09A11.5 phf-33 correct | Ena198A2 ZK1086.3 correct | Ena199A3 F23D12.5 Jmjd-3.2 correct | Ena199A6 M163.3 His-24 correct | Ena197B5 F46G10.3 Sir-2.3 correct | Ena197B9 F46G10.7 Sir-2.2 correct | Ena198B8 F48F7.1 Alg-1 correct | Gino 3A8 T13F2.3 pis-1 correct | Ena196C11 T22H6.6 Gei-3 correct | Ena197C11 C29F7.6 Jmjd-3.3 correct |
| **E** | Gino 3A11 R13.4 miz-1 correct | Ena196D1 T14G8.1 Chd-3 correct | Gino 4A5 F02D10.7 Set-8 correct | Gino 4F8 D1053.2 correct | Ena198E5 F28H6.4 correct | Ena197F8 F54B11.6 Bra-1 correct | Ena196H10 K08H2.6 Hpl-1 correct | Gino 1B12 K06A5.8 correct | Supp I-9F2 ZK973.2 cec-10 correct | Ena201A12 F53H4.6 F53H4.2 correct | Ena202A6 F22H10.5 correct | Gino 4B1 C10E2.3 Hda-4 correct |
| **F** | NT GFP | AL X-4L16 C17G1.4 nra-3 correct | AL X-7K21 K09A9.4 Usp-33 correct | EnaS1A3 B0207.4 Air-2 correct | EnaS1A11 Y71F9B.7 Plk-2 correct | EnaS1C3 Y47G6A.6 Pcaf-1 correct | EnaS1C6 Y71G12B.1 Chaf-2 correct | EnaS1C12 Y119C1B.8 Tag-332 = bet-1 correct | EnaS2B3 T06D10.2 Chaf-1 correct | EnaS3A1 F26A3.8 correct | EnaS3A7 C41G7.4 Set-32 correct | cloned Y43F11A.5 Set-24 correct |
| **G** | EnaS3C9 Y71A12B.9 Usp-3 correct | EnaS3H3 Y74C9A.4 correct | EnaS4B3 B0205.9 correct | EnaS5C9 Y92H12A.2 correct | EnaS6B1 ZK1320.12 Taf-8 correct | Supp:II-10O11 Y52E8A.2 correct | Supp:II-10A10 Y48E1B.13 Csp-1 correct | EnaS7B3 C13B4.2 Usp-14 correct | EnaS7E2 Y17G7B.2 Ash-2 correct | EnaS8B9 Y51H1A.4 Ing-3 correct | EnaS9A8 F10B5.7 Rrf-3 correct | EnaS9E1 K12H6.11 Set-13 correct |
| **H** | cloned T07C4.11 Jmjd-4 correct | EnaS14B12 Y47D3A.16 correct | EnaS14D1 Y71H2AM.8 Set-27 correct | EnaS15C3 BE0003N10.3 correct | EnaS15E6 Y111B2A.16 Taf-7.2 correct | EnaS15H2 Y67D2.7 correct | EnaS18A1 F40F12.7 correct | EnaS18H5 K03H1.10 correct | EnaS20B6 VT23B5.1 ncRNA correct | EnaS20C3 Y24D9A.2 Set-21 correct | Supp:IV-9A24 Y37E11B.4 Taf-2 correct | Supp:IV-9G04 Y55F3AM.14 correct |

## Plate 7

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | EnaS23D3 C27B7.1 Spr-2 correct | EnaS24A6 Y94H6A.6 Ubc-8 correct | EnaS28A6 F26F12.7 Let-418/evl-11, chd-4 correct | EnaS29A10 C01B7.6 Rpm-1/rpm-3/ sam-1/ sad-3/syd-3 correct | Supp:V-14E24 Y113G7B.23 Psa-1/swsn-1 correct | EnaS30F4 K04A8.6 Dre-1 correct | Supp:V-15E20 D2023.4 correct | EnaS37C6 T07C12.14 Suds-3 correct | EnaS38D4 F59A7.4 Hil-6 & B0414.3 his-1 correct | EnaS40C6 F55G7.2 correct | Supp:X-8G5 M163.2 Ztf-14 correct | Supp:X-8A4 F28H6.1 Akt-2 correct |
| **B** | EnaS42B9 Y40A1A.1 correct | Supp:X-8F5 F45B8.4 Pag-3 correct | 10002-F11 B0205.7 Kin-3 - not in Supp correct | 10012-B5 ZK909.2 Kin-1 - not in Supp correct | 10012-G1 ZK909.2 Kin-1 - not in Supp correct | 10013-E11 Y51H1A.4 Ing-3 correct | AL II-9E3 W03H9.1 correct | 10018-F9 B0207.4 Air-2 correct | 10018-H5 Y48E1B.13 Csp-1 correct | 10020-C5 C13B4.2 Usp-14 correct | 10020-C11 Y48E1B.13 Csp-1 correct | 10021-G6 F09E5.1 Pkc-3 correct |
| **C** | 10022-F1 E01H11.1 Pkc-2 correct | 10022-G5 R06A4.7 Mes-2 correct | AL IV-3G8 C17H12.1 dyci-1 correct | 10036-G4 Y54G2A.31 Ubc-13 correct | 10037-B1 Y87G2A.9 Ubc-14 correct | 10051-F4 F40F12.7 correct | 10114-C8 C15F1.8 correct | 10154-B12 F45B8.4 Pag-3 correct | Supp III-8F21 Y54H5A.1 correct | 11002-D3 Y37E11B.4 Taf-2 correct | 11010-D11 Y47D3A.26 Smc-3 correct | 11010-E3 Y47D3A.16 correct |
| **D** | AL IV-3C1 C06E7.3 sams-4 correct | NT GFP | Gino 2G4 R10E11.1 Cbp-1 correct | Cloned F57C7.1 bet-2 | 11014-D11 F47G4.6 Hmg-6 correct | 11014-G3 F02E9.2 lin-28 correct | 11019-C1 Y54E10BR.8 Ztf-23 correct | 11019-G7 C41G7.4 Set-32 correct | 11020-H1 F33H2.7 Set-10 correct | 11021-C3 Y74C9A.4 rcor-1 correct | 11021-F5 Y47G6A.19 correct | 11021-H6 B0205.9 correct |
| **E** | 11022-A2 Y71F9B.7 Plk-2 correct | 11022-A11 Y47G6A.6 Pcaf-1 correct | Cloned C04F12.1 (vsr-1) | Cloned Y110A7A.13 chp-1 | 11023-C6 Y71F9B.10 Sop-3 correct | 11023-D10 Y119C1B.8 Tag-332 = bet-1 correct | Cloned Y37D8A.11 cec-7 | 11023-H11 K12C11.4 Dapk-1 correct | EV | 11025-B9 K05F1.2 Msp-142 correct | Cloned R06C1.1 Hda-3 | 11031-H3 Y9D1A.1 correct |
| **F** | Cloned C25A1.10 Dao-5 | 11036-D6 Y17G7B.2 Ash-2 correct | EV | Cloned C43E11.11 Cogc-5 | Cloned MS box4 C18E3.7 ppw-1 | 11040-F5 B0035.10, his-45 = his-55, F54E12.1 correct targets many diff. histones | Cloned B0261.1 | from Thomas B0261.1 | Cloned MS box4 Y73B3B.2 set-28 | 11054-H2 Y71A12B.9 Usp-3 inSupp correct | 11054-H5 Y111B2A.22 Ssl-1 correct | 11055-F5 D2023.4 correct |
| **G** | Cloned MS Y110A7A.18 Ppw-2 | 11068-F5 F59A7.4 Hil-6 correct | NT GFP | 11071-F10 Y113G7B.23 Psa-1/swsn-1 correct | 11071-H9 F38B7.5 Duo-1 correct | 11077-B9 F55G7.2 correct | 11081-E2 W10G6.2 Sgk-1 correct | 11201-B2 Y48E1B.13 Csp-1 correct | 11303-F8 Y57A10A.3 correct | 11304-E2 Y57A10A.6 correct | JAP-MS box2 B0041.7 Xnp-1/slr-8 correct | JAP-MS box2 C25F9.5 correct |
| **H** | JAP-MS box2 Y111B2A.22 Ssl-1 correct | JAP-MS box2 F26F12.7 let-418/evl-11, chd-4 correct | JAP-MS box2 F19B2.5 correct | JAP-MS box2 Y43F8B.14 correct | NT GFP | Cloned MS box2 Y71F9AL.18 pme-1 correct | Cloned MS box2 T21B4.4 ador-1 correct | Cloned MS box2 T23B5.1 prmt-3 correct | Cloned MS box2 C15H11.5 set-31/tag-338 correct | Cloned MS box2 R07B5.9 lsy-12/mys-3 correct | Cloned MS box2 ZK380.5 correct | Cloned MS box2 ZK616.4 swsn-6/psa-13 correct |

## Plate 8

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Cloned MS box3 Y92H12B R.6 set-29 | Cloned MS box3 Y37E3.15 npp-13 | Cloned MS box3 Y39G10AR .18 | Cloned MS box3 H12I13 .1 | Cloned MS box3 Y110A2A R.2 ubc-15 | Cloned MS box3 Y41D4B. 10 dsl-3 | Cloned MS box3 Y45F10C.1 | Cloned MS box3 F07B7. 12 targets rpm-1 as well | Cloned MS box3 Y108F1. 3 set-33 targets set-21 as well | Cloned box3 Y48G8A L.1 herc-1/hpo-23 | Cloned box3 C18E3. 2 swsn-2.2 | Cloned box3T04D 1.4 Chd-7/tag-192 |
| **B** | Cloned box3 Y46H3C. 4 targets K08E5.1 and top-2 as well | Cloned box3 Y73B3B. 1 | EV | Cloned box3 C04A2. 3 egl-27 | Cloned box3 F15D4.1 btf-1 | Cloned box3 T22B7.1 egl-13 | Cloned box3 B0564.11 rde-11 | Cloned box3 C34B7. 4 mys-4 | Cloned MS M01E5. 6 Sepa-1 | Cloned MS ZK1098. 8 Mut-7 | Cloned MS Y73E7 A.9 adpr-1 | Cloned MS F53H10.2 saeg-1 |
| **C** | Cloned F21G4.4 phf-34 | Cloned Y54E10A. 11 | Cloned W02D3.9 unc-37 | Cloned F59E1 2.9 | Cloned T01H8.1 Rskn-1 | Cloned Y49F6A. 1 wago-11 | Cloned ZK863.3 elpc-3 | Cloned F55A12 .1 wago-2 | Cloned T06A10 .4 lsy-13 | Cloned ZK337.2 | Cloned F56D1 2.5 Vig-1 | Cloned F59H5.1 |
| **D** | Cloned C44B7.1 Psmd-9 | Cloned F52G2.2 Rsd-2 | Cloned Y47G6A.4 Rde-10 | Cloned C11G6 .1 Taf-3 | Cloned Y47D3A. 6 Tra-1 | Cloned T24G10. 2 | Cloned Y54F10BM .14 phf-5 not in AL | Cloned Y56A3A .4 Taf-12 | C09E10 .2 dgk-1 not in AL | Cloned Y75B8A. 6 | Cloned ZK593. 4 Rbr-2 | Cloned K08F4.2 |
| **E** | Cloned F54H5.4 Klf-3 | Cloned Y116A8C .22 athp-3 | Cloned C39F7.2 Trim-9 | ZC302. 2 Wdr-5.3 | Cloned F33E11.6 phf-10 not in AL | Cloned F40E10. 2 Sox-3 | Cloned K04G11.4 Wdr-5.2 | Cloned T07F8.4 100% hom with F10E7.1 1 | Cloned F10E7.1 1 100% hom with T07F8.4 | AL I-9L05 F02E9.2 lin-28 | Ena1A 12 F53G1 2.5 Mex-3 correct | Ena1H07 K12C11.2 F29d11.2 Capg-1 correct |
| **F** | Ena7A04 T05E8.2 Hil-8 correct | Ena14B0 6 F43G9.1 2 correct | Ena36H12 B0281.3 correct | AL II-3P2 F10G7. 2 Tsn-1 correct | Ena45B0 8 C17G10. 4 Cdc-14 correct | Ena59D 03 Y17G7A. 1 Hmg-12 correct | Ena74A12 B0336.7 correct | Supp:III -8J20 C56G2. 15 correct | Ena78F 01 ZK652. 6 correct | Ena79H 12 R05D3.1 1 Met-2 correct | Gino 1E1 F45H1 1.2 ned-8 correct | Ena88H12 T28A8.5 correct |
| **G** | Ena95A1 2 F42A6.5 correct | Ena97C0 6 R08C7.1 0 Wapl-1 correct | Ena139F7 K12B6.1 Sago-1 correct | Gino 2D6 T10F2. 4 prp-19 correct | Ena172G 1 Y113G7B .14 correct | Ena173B 11 B0250.9 Dhcr-7 correct | Ena185A1 F49E10.5 Ctbp-1 correct | Ena190 D6 F47E1.3 correct | Ena192 H3 T01C1. 3 Mbr-1 correct | AL X-7C6 F53H4.5 correct | EV | 11013-D6 C01G8.9 Let-526 correct |
| **H** | Cloned K03D10. 3 Mys-2 | Cloned H15N14. 1 Adr-1 | 11071-D10 K04A8.6 Dre-1 correct | JAP-MS box2 M04C3 .1 correct | NT GFP | | | | | | | NT GFP |

Note: all our cloned RNAi clones sequenced and correct

**Plate 9 (Additional genes of interest)**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Daf-16 | HSf-1 | Unc-15 | Mxl-3 | Cdl-1 | Age-1 | Nrde-2 | Y51B9A.5 | Dpy-27 | NT GFP | F56F3.4 | Vps-22 |
| **B** | Swsn-5 | C48D4.7 | Unc-86 | Dep-28 | EV | Mec-3 | Lin-3 | Let-60 | Nhr-7 | Mig-32 | Nhr-68 | End-1 |
| **C** | NT GFP | Unc-39 | Pha-4 | Spat-3 | Sdc-2 | Hnd-1 | Mes-1 | EV | Hrld-1 | Cul-4 | Ubh-4 | Y51B9A.5 |
| **D** | Brd-1 | Cyy-1 | C02F5.7 | Sqv-3 | Cdk-1 | Elb-1 | Daf-2 | Prmt-6 | H34C03.2 | Ddb-1 | Eri-5 | Rbx-1 |
| **E** | F25B3.6 | Math-33 | Zfp-3 | C14C10.5 | Cul-2 | Sel-11 | Tlk-1 | Sdc-1 | Y1A5A.1 | Sdc-3 | | |
| **F** | | | | | | | | | | | | |
| **G** | | | | | | | | | | | | |
| **H** | | | | | | | | | | | | |

**Supplemental Table 6.** List of all genes used in RNAi screening approach. Genes listed in plates 1-8 were provided by Ena Kolundzic and Martina Hajduskova from the Tursun lab and the annotations are theirs. Plate 9 lists the 54 additional genes that were included by us as part of the library. Highlighted genes for plates 1-8 indicate target identity was checked by sequencing. For non-highlighted genes, target sequences were cloned individually to ensure sequence match. All genes in plate 9 were sequenced. Vector controls were added randomly to each plate to serve as inter-plate variation controls. Empty vector (EV) HT115 and RNAi targeting GFP (a non-targeting [NT] control) were used to determine lifespan of control worms. Tables relate to Figure 13.

## 8.13 Supplemental Table 7

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | N | Control Mean treated lifespan +/- SD | N | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| *rrf-3* Day 9 | *ego-1* | 18.951 +/- 0.384 | 103/105 | 16.900 +/- 0.519 | 102/105 | 12.14% | 0.0054 | |
| | | 20.271 +/- 0.141 | 104/105 | 16.058 +/- 0.082 | 103/105 | 26.24% | < 0.0001 | |
| | | 18.538 +/- 0.515 | 104/105 | 15.661 +/- 0.543 | 104/105 | 18.37% | < 0.0001 | 14 |
| | *eri-1* | 19.005 +/- 0.229 | 102/105 | 16.900 +/- 0.519 | 102/105 | 12.46% | 0.0021 | |
| | | 20.486 +/- 0.181 | 102/105 | 16.058 +/- 0.082 | 103/105 | 27.58% | < 0.0001 | |
| | | 17.467 +/- 0.693 | 105/105 | 15.661 +/- 0.543 | 104/105 | 11.53% | 0.0068 | 14 |
| | *dot-1.1* | 18.556 +/- 1.163 | 97/105 | 16.900 +/- 0.519 | 102/105 | 9.80% | 0.0079 | |
| | | 19.219 +/- 0.117 | 105/105 | 16.058 +/- 0.082 | 103/105 | 19.68% | < 0.0001 | |
| | | 18.286 +/- 1.258 | 105/105 | 15.661 +/- 0.543 | 104/105 | 16.76% | 0.0001 | 14 |
| | *cyk-3* | 19.467 +/- 0.472 | 101/105 | 16.900 +/- 0.519 | 102/105 | 15.19% | 0.0002 | |
| | | 18.514 +/- 0.093 | 105/105 | 16.058 +/- 0.082 | 103/105 | 15.29% | 0.0027 | |
| | | 18.059 +/- 0.416 | 104/105 | 15.661 +/- 0.543 | 104/105 | 15.31% | 0.002 | 14 |
| | *pha-4* | 20.867 +/- 0.152 | 104/105 | 15.661 +/- 0.543 | 104/105 | 33.24% | < 0.0001 | 14 |
| | | 21.029 +/- 0.855 | 105/105 | 17.867 +/- 0.644 | 105/105 | 17.70% | < 0.0001 | |
| *rrf-3* L1 | *pha-4* | 16.083 +/- 0.479 | 98/105 | 20.898 +/- 0.751 | 99/105 | -23.04% | < 0.0001 | |
| | | 17.908 +/- 0.499 | 96/105 | 22.445 +/- 0.751 | 69/105 | -20.21% | < 0.0001 | |
| | | 16.371 +/- 0.083 | 102/105 | 21.099 +/- 0.262 | 104/105 | -22.41% | < 0.0001 | S4A |
| | *bec-1* | 16.730 +/- 0.552 | 103/105 | 22.619 +/- 0.242 | 98/105 | -26.04% | < 0.0001 | |
| | | 16.400 +/- 0.392 | 101/105 | 21.370 +/- 0.407 | 89/105 | -23.26% | < 0.0001 | |
| | | 17.673 +/- 0.407 | 55/90* | 22.097 +/- 1.167 | 79/90 | -20.02% | < 0.0001 | S5A |
| *rrf-3* Day 0 | *pha-4* | 19.924 +/- 0.397 | 105/105 | 21.320 +/- 0.496 | 104/105 | -6.55% | < 0.0001 | |
| | | 18.463 +/- 0.316 | 104/105 | 24.398 +/- 0.355 | 87/105 | -24.33% | < 0.0001 | |
| | | 19.251 +/- 0.166 | 104/105 | 21.657 +/- 0.573 | 102/105 | -11.11% | < 0.0001 | |
| | | 17.581 +/- 0.725 | 105/105 | 18.648 +/- 0.477 | 103/105 | -5.72% | 0.0009 | S4B |
| | *bec-1* | 21.010 +/- 0.150 | 105/105 | 22.033 +/- 0.407 | 102/105 | -4.64% | 0.0603 | |
| | | 21.041 +/- 0.527 | 137/140 | 22.351 +/- 1.001 | 135/140 | -5.86% | < 0.0001 | |
| | | 22.690 +/- 0.739 | 117/120 | 22.884 +/- 0.899 | 115/120 | -9.59% | < 0.0001 | S5B |
| | | 22.050 +/- 0.688 | 103/105 | 21.911 +/- 0.662 | 103/105 | 0.63% | 0.6242 | |
| *rrf-3* Day 5 | *pha-4* | 19.420 +/- 1.038 | 102/105 | 18.529 +/- 0.693 | 102/105 | 4.81% | 0.2058 | |
| | | 21.926 +/- 0.206 | 93/105 | 18.295 +/- 0.473 | 95/105 | 19.85% | < 0.0001 | |
| | | 22.977 +/- 0.390 | 101/105 | 23.162 +/- 1.543 | 104/105 | -0.80% | 0.0318 | S4C |
| | *bec-1* | 22.815 +/- 0.748 | 62/105* | 19.880 +/- 0.611 | 104/105 | 14.76% | 0.0004 | |
| | | 30.232 +/- 1.497 | 101/105 | 23.162 +/- 1.543 | 104/105 | 30.52% | < 0.0001 | S5C |
| | | 25.862 +/- 0.341 | 99/105 | 22.576 +/- 0.457 | 98/105 | 14.60% | < 0.0001 | |
| *rrf-3* Day 9 | *pha-4* | 22.579 +/- 0.611 | 103/105 | 17.881 +/- 0.432 | 104/105 | 26.27% | < 0.0001 | S4D |
| | | 22.433 +/- 0.422 | 149/150 | 17.950 +/- 0.688 | 148/150 | 24.97% | < 0.0001 | 17A |
| | | 22.820 +/- 1.261 | 119/120 | 18.622 +/- 0.915 | 90/120 | 22.54% | < 0.0001 | |
| | *bec-1* | 28.194 +/- 0.849 | 103/105 | 17.881 +/- 0.432 | 104/105 | 57.68% | < 0.0001 | |
| | | 29.275 +/- 1.363 | 116/150* | 17.950 +/- 0.688 | 148/150 | 63.09% | < 0.0001 | 17A |
| | | 28.573 +/- 0.304 | 88/120* | 18.622 +/- 0.915 | 90/120 | 53.44% | < 0.0001 | |
| | | 29.540 +/- 1.148 | 116/120 | 18.414 +/- 0.460 | 119/120 | 60.42% | < 0.0001 | S5D |
| *rrf-3* Day 15 | *pha-4* | 19.182 +/- 1.053 | 65/70* | 14.958 +/- 0.876 | 98/105 | 28.24% | < 0.0001 | |
| | | 18.390 +/- 0.512 | 104/105 | 15.705 +/- 0.512 | 105/105 | 17.10% | < 0.0001 | |
| | | 17.029 +/- 0.855 | 105/105 | 13.867 +/- 0.644 | 105/105 | 22.80% | < 0.0001 | S4E |
| | *bec-1* | 22.021 +/- 0.178 | 104/105 | 14.629 +/- 0.610 | 105/105 | 50.53% | < 0.0001 | S5E |
| | | 19.505 +/- 0.980 | 62/105* | 14.990 +/- 0.819 | 104/105 | 30.12% | < 0.0001 | |
| | | 15.017 +/- 0.695 | 120/120 | 20.462 +/- 0.607 | 118/120 | 36.26% | < 0.0001 | |
| *rrf-3* Day 9 Plate only | *pha-4* | 19.936 +/- 0.755 | 104/105 | 15.467 +/- 0.648 | 105/105 | 28.89% | < 0.0001 | 17B |
| | | 19.524 +/- 0.554 | 99/105 | 16.655 +/- 0.557 | 98/105 | 17.23% | < 0.0001 | |
| | | 19.344 +/- 0.700 | 70/105* | 16.346 +/- 0.093 | 104/105 | 18.34% | 0.0003 | |
| | *bec-1* | 22.515 +/- 0.835 | 105/105 | 16.655 +/- 0.557 | 98/105 | 35.18% | < 0.0001 | 17B |
| | | 24.293 +/- 0.842 | 104/105 | 15.467 +/- 0.648 | 105/105 | 57.06% | < 0.0001 | |
| | | 21.968 +/- 1.227 | 102/105 | 16.346 +/- 0.093 | 104/105 | 34.40% | < 0.0001 | |

**Supplemental Table 7.** Day 9 RNAi inactivations of candidate AP genes extend MTL (Fig. 14). Day 9 RNAi against *pha-4* and *bec-1* extends MTL (Fig. 17A). Inactivation of *bec-1* and *pha-4* at different time

points has converse effects on MTL (Fig. 19 & Supplemental Figures 4 & 5). Day 9 inactivation of *bec-1* and *pha-4* only maintained on NGM agar plates extends MTL (Fig. 17B). — Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. N: number of dead worms/number of total worms. *: indicates where whole plates were scored out due to contamination. Worms from these plates were not used for statistical analysis.

## 8.14 Supplemental Table 8

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | N | Control Mean treated lifespan +/- SD | N | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| WT(N2) L1 | *pha-4* | 12.606 +/- 0.389 | 73/105 | 19.960 +/- 0.206 | 99/105 | -36.84% | < 0.0001 | |
| | | 13.838 +/- 0.460 | 100/105 | 20.524 +/- 0.235 | 91/105 | -32.58% | < 0.0001 | 17E |
| | | 12.222 +/- 0.736 | 77/105 | 20.293 +/- 1.385 | 94/105 | -39.77% | < 0.0001 | |
| | *bec-1* | 13.220 +/- 0.066 | 95/105 | 19.960 +/- 0.206 | 99/105 | -33.77% | < 0.0001 | |
| | | 14.845 +/- 0.794 | 96/105 | 20.524 +/- 0.235 | 91/105 | -27.67% | < 0.0001 | 17E |
| | | 15.805 +/- 0.480 | 97/105 | 20.986 +/- 0.246 | 102/105 | -24.69% | < 0.0001 | |
| WT(N2) Day 0 | *pha-4* | 18.131 +/- 0.192 | 103/105 | 21.191 +/- 0.506 | 68/70* | -14.44% | < 0.0001 | 17E |
| | | 19.926 +/- 0.231 | 103/105 | 25.582 +/- 0.249 | 102/105 | -22.11% | < 0.0001 | |
| | | 15.687 +/- 0.571 | 103/105 | 19.014 +/- 1.054 | 192/210 | -17.50% | < 0.0001 | |
| | | 14.782 +/- 0.357 | 173/175 | 20.548 +/- 0.562 | 202/210 | -26.17% | < 0.0001 | |
| | | 15.366 +/- 0.263 | 102/105 | 20.427 +/- 0.486 | 103/105 | -24.78% | < 0.0001 | |
| | *bec-1* | 19.063 +/- 0.607 | 104/105 | 21.191 +/- 0.506 | 68/70* | -10.04% | 0.0041 | 17E |
| | | 21.675 +/- 0.375 | 103/105 | 25.582 +/- 0.249 | 102/105 | -15.27% | < 0.0001 | |
| | | 20.625 +/- 0.770 | 105/105 | 23.562 +/- 0.750 | 104/105 | -12.46% | 0.0007 | |
| WT(N2) Day 9 | *pha-4* | 12.180 +/- 0.313 | 96/105 | 12.096 +/- 0.293 | 101/105 | 0.69% | 0.8095 | |
| | | 11.744 +/- 0.453 | 101/105 | 12.774 +/- 0.156 | 100/105 | -8.06% | 0.0814 | |
| | | 14.223 +/- 0.740 | 102/105 | 13.924 +/- 0.397 | 105/105 | 2.15% | 0.5546 | |
| | | 12.838 +/- 0.444 | 105/105 | 12.114 +/- 0.491 | 105/105 | 5.98% | 0.3994 | 17D |
| | *bec-1* | 14.868 +/- 0.910 | 135/140 | 11.080 +/- 0.606 | 136/140 | 34.19% | < 0.0001 | |
| | | 14.431 +/- 0.608 | 137/140 | 10.143 +/- 0.700 | 140/140 | 42.28% | < 0.0001 | |
| | | 14.154 +/- 0.996 | 137/140 | 11.151 +/- 0.798 | 137/140 | 26.93% | < 0.0001 | |
| | | 16.184 +/- 0.719 | 104/105 | 12.114 +/- 0.491 | 105/105 | 33.60% | < 0.0001 | 17D |
| *ergo-1* Day 9 | *pha-4* | 23.574 +/- 0.397 | 102/105 | 18.714 +/- 1.818 | 105/105 | 25.97% | < 0.0001 | |
| | | 23.215 +/- 0.824 | 90/105 | 18.866 +/- 1.692 | 104/104 | 23.05% | < 0.0001 | 17C |
| | | 21.605 +/- 0.859 | 70/70* | 18.096 +/- 0.072 | 104/105 | 19.39% | < 0.0001 | |
| | *bec-1* | 26.873 +/- 1.001 | 102/105 | 18.714 +/- 1.818 | 105/105 | 43.60% | < 0.0001 | |
| | | 26.606 +/- 1.054 | 104/104 | 18.866 +/- 1.692 | 104/104 | 41.03% | < 0.0001 | 17C |
| | | 24.820 +/- 1.741 | 105/105 | 18.096 +/- 0.072 | 104/105 | 37.16% | < 0.0001 | |

**Supplemental Table 8.** Inhibition of *pha-4* and *bec-1* in WT worms at L1 or day 0 reduces lifespan (Fig. 17E). Inhibition of autophagy formation at day 9 through *bec-1* but not *pha-4* extends lifespan in WT worms (Fig. 17D). Inhibition of both *pha-4* and *bec-1* extend MTL in the RNAi hypersensitive strain *ergo-1(tm1860)* from day 9 (Fig. 17C). Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. N: number of dead worms/number of total worms. *: indicates where whole plates were scored out due to contamination. Worms from these plates were not used for statistical analysis.

## 8.15 Supplemental Table 9

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | n | Control Mean treated lifespan +/- SD | n | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| *rrf-3* Day 9 | *bec-1* | 28.041 +/- 1.604 | 101/105 | 19.990 +/- 0.529 | 105/105 | 40.28% | < 0.0001 | 18C |
| | | 26.014 +/- 1.504 | 104/105 | 19.088 +/- 0.445 | 136/140 | 36.28% | < 0.0001 | |
| | | 27.280 +/- 0.309 | 102/105 | 16.908 +/- 0.146 | 136/140 | 61.34% | < 0.0001 | |
| | EV + *bec-1* | 27.920 +/- 0.968 | 102/105 | 19.990 +/- 0.529 | 105/105 | 39.67% | < 0.0001 | 18C |
| | | 27.486 +/- 1.382 | 140/140 | 19.088 +/- 0.445 | 136/140 | 44% | < 0.0001 | |
| | | 25.743 +/- 1.420 | 138/140 | 16.908 +/- 0.146 | 136/140 | 52.25% | < 0.0001 | |
| | *bec-1* | 28.041 +/- 1.604 | 101/105 | 27.920 +/- 0.968 | 102/105 | 0.43% | 0.9256 | 18C |
| | | 26.014 +/- 1.504 | 104/105 | 27.486 +/- 1.382 | 140/140 | -5.36% | 0.3981 | |
| | | 27.280 +/- 0.309 | 102/105 | 25.743 +/- 1.420 | 138/140 | 5.97% | 0.1109 | |
| *rrf-3* Day 9 | EV + *bec-1* | 30.095 +/- 1.590 | 105/105 | 20.438 +/- 0.397 | 105/105 | 47.25% | < 0.0001 | |
| | | 25.880 +/- 0.837 | 90/105 | 18.902 +/- 0.241 | 101/105 | 36.92% | < 0.0001 | 18A |
| | | 24.386 +/- 1.245 | 103/105 | 16.900 +/- 0.694 | 101/105 | 44.30% | < 0.0001 | |
| | | 27.700 +/- 2.107 | 140/140 | 18.661 +/- 0.926 | 104/105 | 48.40% | < 0.0001 | |
| | *bec-1 + pha-4* | 27.855 +/- 1.299 | 102/105 | 20.438 +/- 0.397 | 105/105 | 36.29% | < 0.0001 | |
| | | 22.347 +/- 0.284 | 97/105 | 18.902 +/- 0.241 | 101/105 | 18.23% | < 0.0001 | 18A |
| | | 21.846 +/- 0.939 | 99/105 | 16.900 +/- 0.694 | 101/105 | 29.27% | < 0.0001 | |
| | | 27.131 +/- 1.271 | 103/105 | 18.661 +/- 0.926 | 104/105 | 45.35% | < 0.0001 | |
| | *bec-1 + pha-4* | 27.855 +/- 1.299 | 102/105 | 30.095 +/- 1.590 | 105/105 | -7.44% | 0.0168 | |
| | | 22.347 +/- 0.284 | 97/105 | 25.880 +/- 0.837 | 90/105 | -13.65% | 0.0005 | 18A |
| | | 21.846 +/- 0.939 | 99/105 | 24.386 +/- 1.245 | 103/105 | -10.42% | 0.0132 | |
| | | 27.131 +/- 1.271 | 103/105 | 27.700 +/- 2.107 | 140/140 | -2.05% | 0.4822 | |
| WT(N2) Day 9 | EV + *bec-1* | 14.934 +/- 0.638 | 99/105 | 12.212 +/- 0.444 | 100/105 | 22.29% | < 0.0001 | |
| | | 17.047 +/- 0.148 | 99/105 | 13.316 +/- 0.807 | 104/105 | 28.02% | < 0.0001 | 18B |
| | | 18.943 +/- 0.821 | 99/105 | 16.048 +/- 0.381 | 59/70 | 18.04% | 0.0013 | |
| | | 16.400 +/- 0.173 | 100/105 | 12.631 +/- 0.163 | 102/105 | 29.84% | < 0.0001 | |
| | | 15.507 +/- 0.307 | 91/105 | 14.350 +/- 0.387 | 100/105 | 8.06% | 0.0506 | |
| | *bec-1 + pha-4* | 14.039 +/- 0.317 | 96/105 | 12.212 +/- 0.444 | 100/105 | 14.96% | 0.0011 | |
| | | 15.107 +/- 0.323 | 98/105 | 13.316 +/- 0.807 | 104/105 | 13.45% | 0.0039 | 18B |
| | | 17.730 +/- 0.566 | 98/105 | 16.048 +/- 0.381 | 59/70 | 10.48% | 0.022 | |
| | | 13.524 +/- 0.448 | 105/105 | 12.631 +/- 0.163 | 102/105 | 7.07% | 0.2909 | |
| | | 14.322 +/- 0.478 | 98/105 | 14.350 +/- 0.387 | 100/105 | -0.19% | 0.9227 | |
| | *bec-1 + pha-4* | 14.039 +/- 0.317 | 96/105 | 14.934 +/- 0.638 | 99/105 | -5.99% | 0.1817 | |
| | | 15.107 +/- 0.323 | 98/105 | 17.047 +/- 0.148 | 99/105 | -11.38% | 0.0123 | 18B |
| | | 17.730 +/- 0.566 | 98/105 | 18.943 +/- 0.821 | 99/105 | -6.40% | 0.1048 | |
| | | 13.524 +/- 0.448 | 105/105 | 16.400 +/- 0.173 | 100/105 | -17.54% | < 0.0001 | |
| | | 14.322 +/- 0.478 | 98/105 | 15.507 +/- 0.307 | 91/105 | -7.64% | 0.0504 | |

**Supplemental Table 9.** Epistasis interactions of *pha-4* and *bec-1*. *bec-1* inactivation produces a similar MTL extension when fed to the worms undiluted or as a 50:50 mixture with EV (Fig. 18C). Day 9 inactivation of *bec-1* in combination with *pha-4* reduces MTL compared to *bec-1* alone in both *rrf-3(pk1426)* and WT(N2) worms. (Fig. 18, A & B). — Dark grey shaded values indicate where RNAi treatment was compared to EV + *bec-1* to test for significant effects compared to the *bec*-1 lifespan. Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. n: number of dead worms/number of total worms.

## 8.16 Supplemental Table 10

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | N | Control Mean treated lifespan +/- SD | N | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| *rrf-3* Day 9 | EV + *bec-1* | 26.692 +/- 0.222 | 69/105* | 17.897 +/- 0.468 | 104/105 | 49.14% | < 0.0001 | |
| | | 25.307 +/- 0.373 | 100/105 | 18.373 +/- 0.306 | 102/105 | 37.74% | < 0.0001 | |
| | | 27.975 +/- 2.248 | 101/105 | 19.280 +/- 1.806 | 100/105 | 45.10% | < 0.0001 | 22D |
| | EV + *ced-4* | 18.711 +/- 0.192 | 104/105 | 17.897 +/- 0.468 | 104/105 | 4.55% | 0.1352 | |
| | | 19.189 +/- 0.246 | 104/105 | 18.373 +/- 0.306 | 102/105 | 4.44% | 0.2722 | |
| | | 19.563 +/- 0.416 | 104/105 | 19.800 +/- 1.806 | 100/105 | 1.47% | 0.7879 | 22D |
| | *bec-1* + *ced-4* | 25.721 +/- 0.496 | 91/105 | 17.897 +/- 0.468 | 104/105 | 43.72% | < 0.0001 | |
| | | 24.865 +/- 1.979 | 104/105 | 18.373 +/- 0.306 | 102/105 | 35.33% | < 0.0001 | |
| | | 25.629 +/- 3.004 | 104/105 | 19.800 +/- 1.806 | 100/105 | 32.93% | < 0.0001 | 22D |
| | *bec-1* + *ced-4* | 25.721 +/- 0.496 | 91/105 | 26.692 +/- 0.222 | 69/105* | -3.64% | 0.8847 | |
| | | 24.865 +/- 1.979 | 104/105 | 25.307 +/- 0.373 | 102/105 | -1.75% | 0.8169 | |
| | | 25.629 +/- 3.004 | 104/105 | 27.975 +/- 2.248 | 101/105 | -8.39% | 0.528 | 22D |
| *rrf-3* Day 9 | *bec-1* | 26.417 +/- 0.425 | 100/105 | 18.962 +/- 1.339 | 103/105 | 39.32% | < 0.0001 | 22A |
| | | 26.765 +/- 1.212 | 102/105 | 17.619 +/- 0.408 | 105/105 | 51.91% | < 0.0001 | 25C |
| | | 31.153 +/- 1.662 | 96/105 | 19.171 +/- 0.807 | 140/140 | 62.50% | < 0.0001 | |
| | *vps-34* | 26.171 +/- 0.566 | 105/105 | 18.661 +/- 0.926 | 104/105 | 40.24% | < 0.0001 | |
| | | 25.491 +/- 2.037 | 117/120 | 19.121 +/- 1.026 | 119/120 | 33.31% | < 0.0001 | |
| | | 25.549 +/- 1.545 | 100/105 | 18.962 +/- 1.339 | 103/105 | 34.74% | < 0.0001 | 22A |
| | *epg-8* | 24.857 +/- 1.200 | 70/70 | 19.257 +/- 0.181 | 140/140 | 29.08% | < 0.0001 | |
| | | 24.796 +/- 3.179 | 89/105 | 18.661 +/- 0.926 | 104/105 | 32.88% | < 0.0001 | |
| | | 25.517 +/- 0.602 | 116/120 | 19.121 +/- 1.026 | 119/120 | 33.45% | < 0.0001 | |
| | | 25.647 +/- 1.778 | 102/105 | 18.962 +/- 1.339 | 103/105 | 35.25% | < 0.0001 | 22A |
| *rrf-3* Day 0 | *vps-34* | 17.229 +/- 0.199 | 68/105* | 19.728 +/- 1.208 | 101/105 | -12.67% | 0.0039 | |
| | | 17.960 +/- 0.182 | 103/105 | 20.245 +/- 0.488 | 104/105 | -11.29% | 0.004 | |
| | | 16.949 +/- 0.348 | 102/105 | 18.560 +/- 0.466 | 103/105 | -8.68% | 0.0153 | |
| | | 16.727 +/- 0.121 | 66/105* | 21.405 +/- 1.085 | 90/105 | -21.85% | < 0.0001 | 22B |
| | *epg-8* | 18.343 +/- 0.352 | 105/105 | 19.728 +/- 1.208 | 101/105 | -7.02% | 0.0284 | |
| | | 18.867 +/- 0.152 | 104/105 | 20.245 +/- 0.488 | 104/105 | -6.81% | 0.0541 | |
| | | 17.484 +/- 0.267 | 104/105 | 18.560 +/- 0.466 | 103/105 | -5.80% | 0.0892 | |
| | | 17.916 +/- 0.376 | 90/105 | 21.405 +/- 1.085 | 90/105 | -16.30% | 0.0002 | 22B |
| *rrf-3* Day 9 | *pink-1* | 17.557 +/- 0.153 | 104/105 | 16.908 +/- 0.146 | 136/140 | 3.84% | 0.2099 | 25B |
| | | 17.512 +/- 0.377 | 103/105 | 17.828 +/- 0.224 | 105/105 | -1.77% | 0.3667 | |
| | | 17.887 +/- 0.488 | 104/105 | 17.619 +/- 0.408 | 105/105 | 1.52% | 0.5451 | |
| | *dct-1* | 19.210 +/- 1.650 | 105/105 | 19.171 +/- 0.807 | 140/140 | 0.20% | 0.5989 | |
| | | 18.286 +/- 0.168 | 105/105 | 17.771 +/- 0.284 | 105/105 | 2.90% | 0.5986 | 25C |
| | | 17.710 +/- 0.529 | 104/105 | 18.380 +/- 0.654 | 103/105 | -3.65% | 0.1400 | |
| | *drp-1* | 16.057 +/- 0.631 | 105/105 | 17.619 +/- 0.408 | 105/105 | -8.87% | 0.1388 | |
| | | 17.760 +/- 0.319 | 105/105 | 18.072 +/- 0.262 | 105/105 | -1.73% | 0.1182 | |
| | | 18.571 +/- 0.247 | 105/105 | 17.771 +/- 0.284 | 105/105 | 4.50% | 0.5232 | 25C |
| | EV + *bec-1* | 25.743 +/- 1.420 | 138/140 | 16.980 +/- 0.146 | 136/140 | 51.61% | < 0.0001 | 25D |
| | EV + *dct-1* | 17.557 +/- 0.153 | 104/105 | 16.980 +/- 0.146 | 136/140 | 3.40% | 0.2099 | |
| | *bec-1* + *dct-1* | 27.280 +/- 0.309 | 102/105 | 16.980 +/- 0.146 | 136/140 | 60.66% | < 0.0001 | |
| | *bec-1* + *dct-1* | 27.280 +/- 0.309 | 102/105 | 25.743 +/- 1.420 | 138/140 | 5.97% | 0.1109 | |

**Supplemental Table 10.** Examining routes of possible *bec-1* mediated lifespan extension. Inactivation of *bec-1* in combination with the apoptotic gene *ced-4* does not reduce the MTL of *bec-1* inactivation alone (Fig. 22D). Members of the BEC-1 nucleation complex extend MTL when inhibited at day 9 (Fig. 22A). These genes reduce lifespan when inhibited at day 0 (Fig. 22B). Inactivation of genes involved in mitophagy, *pink-1, drp-1* and *dct-1,* have no effect on MTL (Fig. 25, B & C). Inactivation of *bec-1* combined with *dct-1* does not reduced the MTL of *bec-1* alone (Fig. 25D). — Dark grey shaded values indicate where RNAi treatment was compared to EV + *bec-1* to test for significant effects compared to

the *bec*-1 lifespan. Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. N: number of dead worms/number of total worms. *: indicates where whole plates were scored out due to contamination. Worms from these plates were not used for statistical analysis.

## 8.17 Supplemental Table 11

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | n | Control Mean treated lifespan +/- SD | n | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| WT(N2) Day 9 | EV + *bec-1* | 16.542 +/- 0.151 | 97/105 | 12.171 +/- 0.261 | 103/105 | 35.91% | < 0.0001 | 27G |
| | | 16.811 +/- 0.340 | 67/105 | 13.146 +/- 0.403 | 66/105* | 27.88% | < 0.0001 | |
| | | 17.005 +/- 1.079 | 101/105 | 11.857 +/- 0.646 | 105/105 | 43.42% | < 0.0001 | |
| | | 18.431 +/- 0.660 | 68/105* | 13.899 +/- 0.561 | 104/105 | 32.61% | < 0.0001 | |
| | | 16.569 +/- 0.400 | 102/105 | 13.328 +/- 0.576 | 103/105 | 24.32% | < 0.0001 | |
| | EV + let-363 | 14.019 +/- 0.151 | 104/105 | 12.171 +/- 0.261 | 103/105 | 15.18% | 0.0021 | 27G |
| | | 12.621 +/- 0.067 | 100/105 | 13.146 +/- 0.403 | 66/105* | -3.99% | 0.7797 | |
| | | 13.610 +/- 1.387 | 105/105 | 11.857 +/- 0.646 | 105/105 | 14.78% | 0.0073 | |
| | | 16.231 +/- 0.003 | 104/105 | 13.899 +/- 0.561 | 104/105 | 16.78% | 0.0008 | |
| | | 14.898 +/- 0.768 | 103/105 | 13.328 +/- 0.576 | 103/105 | 11.78% | 0.0551 | |
| | *bec-1 + let-363* | 18.895 +/- 1.148 | 99/105 | 12.171 +/- 0.261 | 103/105 | 55.25% | < 0.0001 | 27G |
| | | 17.937 +/- 0.566 | 101/105 | 13.146 +/- 0.403 | 66/105* | 36.44% | < 0.0001 | |
| | | 17.810 +/- 0.613 | 103/105 | 11.857 +/- 0.646 | 105/105 | 50.21% | < 0.0001 | |
| | | 20.657 +/- 0.200 | 70/105* | 13.899 +/- 0.561 | 104/105 | 48.62% | < 0.0001 | |
| | | 18.891 +/- 0.215 | 99/105 | 13.328 +/- 0.576 | 103/105 | 41.74% | < 0.0001 | |
| | *bec-1 + let-363* | 18.895 +/- 1.148 | 99/105 | 16.542 +/- 0.151 | 97/105 | 14.22% | 0.0252 | 27G |
| | | 17.937 +/- 0.566 | 101/105 | 16.811 +/- 0.340 | 67/105 | 6.70% | 0.2796 | |
| | | 17.810 +/- 0.613 | 103/105 | 17.005 +/- 1.079 | 101/105 | 4.73% | 0.4082 | |
| | | 20.657 +/- 0.200 | 70/105* | 18.431 +/- 0.660 | 68/105* | 12.08% | 0.0118 | |
| | | 18.891 +/- 0.215 | 99/105 | 16.569 +/- 0.400 | 102/105 | 14.01% | 0.0163 | |
| *rrf-3* Day 9 | EV + *bec-1* | 22.552 +/- 1.266 | 104/105 | 18.086 +/- 0.057 | 70/105* | 24.69% | < 0.0001 | |
| | | 21.930 +/- 1.442 | 101/105 | 16.711 +/- 0.611 | 103/105 | 31.23% | < 0.0001 | |
| | | 22.913 +/- 1.457 | 103/105 | 17.230 +/- 0.038 | 104/105 | 32.98% | < 0.0001 | 27F |
| | | 29.145 +/- 0.67 | 104/105 | 19.257 +/- 0.181 | 140/140 | < 0.0001 | 51.35% | |
| | EV + let-363 | 17.362 +/- 0.499 | 105/105 | 18.086 +/- 0.057 | 70/105* | -4.00% | 0.099 | |
| | | 17.962 +/- 0.108 | 105/105 | 17.230 +/- 0.038 | 104/105 | 4.25% | 0.5834 | 27F |
| | | 16.581 +/- 0.344 | 105/105 | 16.711 +/- 0.611 | 103/105 | -0.78% | 0.7248 | |
| | | 19.509 +/- 0.790 | 102/105 | 19.257 +/- 0.181 | 140/140 | 0.794 | 1.31% | |
| | *bec-1 + let-363* | 22.456 +/- 0.730 | 101/105 | 18.086 +/- 0.057 | 70/105* | 24.16% | < 0.0001 | |
| | | 24.300 +/- 1.273 | 102/105 | 17.230 +/- 0.038 | 104/105 | 41.03% | < 0.0001 | 27F |
| | | 23.590 +/- 1.991 | 101/105 | 16.711 +/- 0.611 | 103/105 | 41.16% | < 0.0001 | |
| | | 28.867 +/- 1.391 | 101/105 | 19.257 +/- 0.181 | 140/140 | < 0.0001 | 49.90% | |
| | *bec-1 + let-363* | 23.590 +/- 1.991 | 101/105 | 21.930 +/- 1.442 | 101/105 | 7.57% | 0.2616 | |
| | | 22.456 +/- 0.730 | 101/105 | 22.552 +/- 1.266 | 104/105 | -0.43% | 0.7693 | |
| | | 24.300 +/- 1.273 | 102/105 | 22.913 +/- 1.457 | 103/105 | 6.05% | 0.0914 | 27F |
| | | 28.867 +/- 1.391 | 101/105 | 29.145 +/- 0.67 | 104/105 | 0.5372 | -0.95% | |
| *eat-2:rrf-3* Day 9 | *bec-1* | 16.973 +/- 1.080 | 101/105 | 16.432 +/- 0.671 | 104/105 | 3.30% | 0.1724 | |
| | | 19.260 +/- 1.637 | 103/105 | 19.771 +/- 0.260 | 105/105 | -2.58% | 0.3342 | |
| | | 22.720 +/- 0.529 | 101/105 | 18.836 +/- 0.533 | 102/105 | 20.62% | 0.0041 | 27E |
| | | 20.179 +/- 0.213 | 103/105 | 20.517 +/- 2.060 | 104/105 | -1.65% | 0.3838 | |
| | | 23.990 +/- 0.512 | 105/105 | 19.876 +/- 0.561 | 105/105 | 20.69% | 0.0011 | |
| *eat-2* Day 9 | *bec-1* | 19.771 +/- 1.210 | 100/105 | 20.857 +/- 0.284 | 105/105 | -5.21% | 0.0302 | |
| | | 20.990 +/- 0.512 | 105/105 | 19.876 +/- 0.561 | 105/105 | 5.60% | 0.1008 | |
| | | 19.300 +/- 0.664 | 103/105 | 19.971 +/- 0.326 | 105/105 | 3.36% | 0.3772 | 27E |
| *rrf-3* Day 9 | EV + *bec-1* | 26.299 +/- 1.039 | 103/105 | 19.695 +/- 0.353 | 103/105 | 33.53% | <0.0001 | |
| | | 27.390 +/- 0.096 | 69/70* | 19.912 +/- 0.206 | 68/70* | 37.56% | <0.0001 | |
| | | 26.334 +/- 1.075 | 103/105 | 19.001 +/- 0.860 | 102/105 | 38.59% | <0.0001 | 27D |
| | *EV + rsks-1* | 20.264 +/- 0.400 | 104/105 | 19.695 +/- 0.353 | 103/105 | 2.89% | 0.4187 | |
| | | 20.227 +/- 0.935 | 103/105 | 19.912 +/- 0.206 | 68/70* | 1.58% | 0.2002 | |
| | | 19.537 +/- 0.051 | 69/70* | 19.001 +/- 0.860 | 102/105 | 2.82% | 0.0606 | 27D |
| | *bec-1 + rsks-1* | 27.324 +/- 0.858 | 63/70* | 19.695 +/- 0.353 | 103/105 | 38.74% | <0.0001 | |
| | | 25.323 +/- 0.911 | 102/105 | 19.912 +/- 0.206 | 68/70* | 27.17% | <0.0001 | |
| | | 28.613 +/- 0.750 | 102/105 | 19.001 +/- 0.860 | 102/105 | 50.59% | <0.0001 | 27D |
| | *bec-1 + rsks-1* | 27.324 +/- 0.858 | 63/70* | 26.299 +/- 1.039 | 103/105 | 3.90% | 0.6481 | |
| | | 25.323 +/- 0.911 | 102/105 | 27.390 +/- 0.096 | 69/70* | -3.90% | 0.1197 | |
| | | 28.613 +/- 0.750 | 102/105 | 26.334 +/- 1.075 | 103/105 | 8.65% | 0.2087 | 27D |

**Supplemental Table 11**

The effect of *bec-1* inhibition on aspects of the TOR pathway. RNAi against *bec-1* in combination with *let-363* increases the lifespan extension of *let-363* alone in WT(N2) worms from day 9 (Fig. 27G). *let-363* inhibition failed to extend the MTL of *rrf-3(pk1426)* worms and had no effect on *bec-1* MTL (Fig. 27F). Inhibition of *bec-1* in *eat-2(ad1116)* mutants had no effect on MTL from day 9 (Fig. 27E). The effect of day 9 *bec-1* inhibition in *eat-2(ad1116);rrf-3(pk1426)* showed a significant lifespan increase for only 2/5 experiments. Results in this group are listed in order of the replicates in Figure 27C. Inhibition of both *bec-1* and *rsks-1* in *rrf-3(pk1426)* worms from day 9 produced no further extension of *bec-1* mediated MTL increase. Inhibition of *rsks-1* alone did not affect MTL (Fig. 27D). — Dark grey shaded values indicate where RNAi treatment was compared to EV + *bec-1* to test for significant effects compared to the *bec*-1 lifespan. Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. n: number of dead worms/number of total worms. *: indicates where whole plates were scored out due to contamination. Worms from these plates were not used for statistical analysis.

## 8.18 Supplemental Table 12

| Strain and Start Day | RNAi Treatment | Mean treated lifespan +/- SD | n | Control Mean treated lifespan +/- SD | n | Mean Change | p-Value | Figure |
|---|---|---|---|---|---|---|---|---|
| *glp-1* Day 9 | *bec-1* | 22.040 +/- 0.769 | 99/105 | 17.773 +/- 0.365 | 102/105 | 24.01% | < 0.0001 | |
| | | 24.182 +/- 1.068 | 91/105 | 17.914 +/- 0.569 | 103/105 | 34.99% | < 0.0001 | |
| | | 23.107 +/- 2.919 | 104/105 | 17.429 +/- 0.697 | 105/105 | 32.58% | < 0.0001 | |
| | | 21.295 +/- 0.108 | 105/105 | 15.314 +/- 0.445 | 105/105 | 39.06% | < 0.0001 | 28A |
| *daf-2;rrf-3* Day 9 | *bec-1* | 38.780 +/- 1.713 | 138/140 | 31.971 +/- 0.698 | 140/140 | 21.30% | < 0.0001 | 28B |
| | | 40.238 +/- 1.457 | 101/105 | 36.170 +/- 1.712 | 103/105 | 11.25% | < 0.0001 | |
| | | 46.522 +/- 0.459 | 97/105 | 39.530 +/- 1.249 | 100/105 | 17.69% | < 0.0001 | |
| *daf16;daf-2;rrf-3* Day 9 | *bec-1* | 12.743 +/- 0.280 | 105/105 | 9.960+/- 0.181 | 103/105 | 27.94% | < 0.0001 | |
| | | 12.401 +/- 0.445 | 104/105 | 10.190 +/- 0.071 | 105/105 | 21.70% | < 0.0001 | |
| | | 12.152 +/- 0.311 | 105/105 | 9.314 +/- 0.168 | 105/105 | 30.27% | < 0.0001 | 28C |
| | | 10.895 +/- 0.285 | 105/105 | 10.476 +/- 0.800 | 105/105 | 3.67% | 0.349 | |
| *rrf-3* Day 9 | EV + *bec-1* | 26.692 +/- 0.222 | 69/105* | 17.897 +/- 0.468 | 104/105 | 49.14% | < 0.0001 | |
| | | 22.913 +/- 0.799 | 104/105 | 17.638 +/- 0.593 | 105/105 | 29.91% | < 0.0001 | 28D |
| | | 25.743 +/- 1.420 | 138/140 | 16.908 +/- 0.146 | 136/140 | 52.25% | < 0.0001 | |
| | *EV + skn-1* | 14.933 +/- 0.391 | 105/105 | 17.897 +/- 0.468 | 104/105 | -16.56% | < 0.0001 | |
| | | 15.733 +/- 0.591 | 105/105 | 17.638 +/- 0.593 | 105/105 | -10.80% | < 0.0001 | 28D |
| | | 13.676 +/- 0.776 | 105/105 | 16.908 +/- 0.146 | 136/140 | -19.12% | < 0.0001 | |
| | *bec-1 + skn-1* | 26.249 +/- 0.273 | 104/105 | 17.897 +/- 0.468 | 104/105 | 46.67% | < 0.0001 | |
| | | 22.571 +/- 0.585 | 105/105 | 17.638 +/- 0.593 | 105/105 | 27.97% | < 0.0001 | 28D |
| | | 23.767 +/- 0.905 | 103/105 | 16.908 +/- 0.146 | 136/140 | 40.57% | < 0.0001 | |
| | *bec-1 + skn-1* | 22.571 +/- 0.585 | 105/105 | 22.913 +/- 0.799 | 104/105 | -1.49% | 0.206 | |
| | | 26.249 +/- 0.273 | 104/105 | 26.692 +/- 0.222 | 69/105* | -1.66% | 0.6362 | 28D |
| | | 23.767 +/- 0.905 | 103/105 | 25.743 +/- 1.420 | 138/140 | -7.68% | 0.2416 | |

**Supplemental Table 12.** Inhibition of *bec-1* extends late-life MTL independent of canonical longevity pathways. Day 9 *bec-1* inactivation extends the MTL of *glp-1(e2141)* worms (Fig. 28A). Day 9 *bec-1* inactivation extends the lifespan of *rrf-3(pk1426);daf-2(e1370)* worms (Fig. 28B) and *daf-16(mgDf47);daf-2(e1370);rrf-3(pk1426)* worms (Fig. 28C). Knockdown of *skn-1* from day 9 reduces MTL and inhibition of *bec-1* compensates for *skn-1* absence restoring longevity (Fig. 28D). — Dark grey shaded values indicate where RNAi treatment was compared to EV + *bec-1* to test for significant effects compared to the *bec*-1 lifespan. Mean lifespans were calculated from the day of RNAi treatment. p-values for lifespans were calculated using the Log-Rank (Mantel-Cox) statistical test from triplicate samples of 35 worms each. n: number of dead worms/number of total worms. *: indicates where whole plates were scored out due to contamination. Worms from these plates were not used for statistical analysis.