

Chromatin regulation of lifespan in *C. elegans*.

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ABSTRACT

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The biological basis of lifespan regulation is a subject of intense interest, and epigenetic control of gene expression is thought to play an important role in the ageing process. A library of RNAi clones against chromatin factors was used to screen for their effects on lifespan in *C. elegans*, utilising microscopic examination of lipofuscin accumulation as a primary biomarker of ageing. A secondary RNAi screen involving full lifespan monitoring confirmed knock-downs of four genes, *isw-1*, *cbp-1*, *mes-2* and *jmjd-3.2*, results in extension of lifespan. The use of mutant alleles of these genes also resulted in statistically significant lifespan extension. Intriguingly, three of those genes encode H3K27 modifiers: *cbp-1* is an acetyl transferase, whilst *mes-2* and *jmjd-3.2* are H3K27 methyltransferase and demethylase respectively.

Mutation in another gene of the *jmjd-3.2* family, *utx-1,* is also known to have a lifespan prolonging effects by increasing H3K27 methylation on the *daf-2* promoter (Jin et al, 2012). In addition to its lifespan effects, *utx-1* is also an essential developmental gene. Its role in development has, however, been confirmed to be independent of its demethylase activity (Vandamme et al. 2012) raising questions about the biological significance of UTX-1-mediated H3K27 demethylation. I used a demethylase-dead form of UTX-1 to demonstrate that enzymatic activity is absolutely required for UTX-1 function in lifespan regulation.

Intriguingly, it is not just *utx-1* loss of function that causes lifespan extension; overexpression of *utx-1* from a transgenic array is associated with even more dramatic lifespan extension. Moreover, *mes-2* and *jmjd-3.2* overexpression also increases the lifespan of transgenic animals. Interestingly, however, lifespan extension driven by overexpression of *jmjd-3.2* is not dependent on its demethylase function, suggesting a different mode of action to *utx-1*. Epistasis analysis suggests that the insulin signalling pathway is a crucial target of regulation for all the factors I identified, but ChIP analysis implies that lifespan extension driven by *utx-1* overexpression may involve a different pathway from H3K27me3 regulation at the *daf-2* locus.

Overall, this work pinpoints H3K27 modifications at key target genes as critical determinants of longevity, and furthermore identifies the crucial importance of the fine balance of factors controlling H3K27 methylation status.

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All these people helped to make this happen and I am extremely grateful to all of them.

LIST OF ABBREVIATIONS

ADP/ATP = adenosine di/tri- phosphate

CBP = CREB-Binding Protein

ChIP-qPCR = Chromatin Immunoprecipitation coupled to quantitative PCR

CGC = *Caenorhabditis* genetics Center

CpG = cytosine –phosphate -guanine

DAPI = 4',6-diamidino-2-phenylindole

DD = demethylase dead

DNA = deoxyribonucleic acid

DNMTs = DNA methyl transferases

DR = dietary restriction

FOXO = Forkhead Box O

FUDR = 5-fluoro-2'-deoxyuridine

GDP = gross domestic product

GFP = green fluorescent protein

H3 = histone 3

H3K27me1/me2/me3 = mono/di/tri- methylation at histone 3 lysine 27

HATs = histone acetyltransferases

HDACs = histone deacetylases

HDMs = histone demethylase

HGPS = Hutchinson Gilford progeria syndrome

HMTs = histone methyltransferase

HTA= High Temperature Arrest

IIS = insulin/insulin-like signallins

IPTG = isopropyl ß-D-1-thiogalactopyranoside

KATs = lysine acetyltransferases (includes HATs)

KDACs = lysine deacetylases (includes HDACs)

KMTs = lysine methyltransferases (includes HMTs)

KDMs = lysine demethylases (includes HDMs = histone demethylase group)

L1, L2, L3, L4 = *C. elegans* larval stages

miRNA = micro RNA

NAD = nicotinamide adenine dinucleotide

ncDNA = non-coding DNA

ncRNA = non-coding RNA

NGM = nematode growth media

PCR = polymerase chain reaction

RNA = ribonucleic acid

RNAi = RNA intereference

ROS = reactive oxygen species

siRNA = small interfering RNA

SASP = senescence-associated secretory phenotype

qRT-PCR = quantitative reverse transcriptase PCR

synMuv = synthetic multivulvae

TF = transcription factor

TOR = target of rapamycin

UV = ultra violet

WS = Werner syndrome

WT = wild-type

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CHAPTER 1: INTRODUCTION

1.1 WHY DO WE AGE?

We live in a fast-changing world; the astonishing progress in the medical field has caused a significant increase in the average length of human life. However positive this might seem at first, there are many consequences. A greater chance of living to an old age also means a greater chance of developing one of the diseases associated primarily with old age: Alzheimer's, type II diabetes, coronary heart disease, most types of cancer. Scientists are still to discover the reasons why ageing makes us so much more susceptible to diseases - especially chronic ones. The current challenge in biomedical research is not necessarily to extend lifespan, but to increase the "healthspan" of a human life: the time spent free of major disease and disability. To do this we need to understand the process of biological ageing itself. A key to understanding a process or a problem is to define it, and it is difficult to give a simple definition of ageing, although it has been broadly described by one of the important researchers in the field as "a progressive, generalized impairment of function, resulting in an increasing vulnerability to environmental challenge and a growing risk of disease and death" (Kirkwood 2005).

The world population is ageing. In more developed regions, by 1998 the older population (over 65) had already surpassed the population of children. In 2013, the 'old' fraction represented 23 per cent of the total population of the developed world. According to recent predictions, this will steadily increase, with a share of around 32 per cent by 2050. To further illustrate the point, it is worth noting that median age in developed countries increased from 28 to 40 years between 1950 and 2010. This shift towards an older population is happening because of two reasons. Firstly, average family size has been significantly decreasing over the last 60 years from 5 down to an average of 2.5 children/woman. Secondly, life expectancy has been increasing because of increased hygiene, vaccine use and better access to medicine (data

from: “World Population Ageing 2013”, United Nations, Department of Economic and Social Affairs, Population Division (2013)).

It is necessary to recognise the importance of ageing research, especially in terms of both personal and socioeconomic impact. Understanding the ageing process will allow the improvement of the health of older people and allow them to enjoy their lives in full. Furthermore, spending on health care is projected to see the largest rise of all elements of age-related spending, rising from an annual cost of 6.8% to 9.1% of GDP in UK between 2016/17 and 2061/62, a rise of 2.3% of GDP - equivalent to a rise of around £36bn in today’s money (data from: “The cost of our ageing society”, The International Longevity Centre – UK, Daniela Silcock and David Sinclair (2012)). Therefore, from a fiscal perspective, improving the health of an ageing population is sound policy. Targeting the ageing process itself might allow us to postpone or even eradicate several diseases at once, instead of focusing on each one separately, which is a more efficient approach.

Scientists have long struggled with describing ageing coherently and specifically enough to form a useful hypothesis. On one hand, ageing is something that we can all easily observe and experience; on the other ageing consists of many disparate symptoms some of which are often striking contradictions. Additionally, within any single species, the lifespan of individuals is highly variable. Even in isogenic populations like *C. elegans* the variation in maximal lifespan is usually around ten days – i.e. over 50% of the average lifespan of 15 days! In human monozygotic twins (containing identical genomes) one can still observe differences in the rate of ageing (Kirkwood 2005). These observations have led scientists to hypothesize that in contrast to developmental processes, ageing is not tightly controlled but is a rather passive, stochastic process. There is, however, an observed strong genetic component to ageing. The lifespan between even closely related species varies considerably: wild mice live for up to four years, while some squirrels (which are mammals in the same order of rodents) live over 20 years (Gorbunova, Bozzella, and Seluanov 2008). This suggests that lifespan is inherent to

species identity – i.e. is, to an extent, expression of its genetics. Supporting this idea are observations that *single* genes have been discovered that can drastically affect lifespan in many model organisms. Even at the cellular level similar contradictions seem to emerge: it was shown early on that primary cells have a limited division potential before undergoing replicative senescence (Hayflick 1965), suggesting that cells possess a programmed function that limits their division capability, presumably to inhibit overproliferation and cancer. Conversely, the time at which senescence initiates in a cell population is, again, highly variable. This may suggest that there is some genetic control over the ageing process but that it is strongly influenced by environmental factors.

Over the years, various explanations for why ageing occurs at all have been suggested. Before the advancement of proper scientific tools, a broadly accepted theory focused upon the idea that ageing and eventual death of individual animals benefits a species as a whole by removing the 'worn out' non-competitive individuals from the population. In this way ageing prevents overcrowding, secures the generation turnover and allows adaptation of the species to the changing environment. However, there are three major flaws in this argument, as explained by Kirkwood (2005): firstly, most of the mortality in a wild environment is due to external factors (predators, lack of resources and so forth), therefore there is no obvious need to get rid of aged individuals. Furthermore, organisms rarely live up to their "potential" maximal lifespan and therefore there is only a very rare opportunity for the genes 'for ageing' to evolve. Secondly, hypothesis is circular, as it is based on the idea that older individuals are meant to become reproductively 'exhausted' whilst failing to explain why this is the case, except as a consequence of ageing. Finally, this theory suggests that the benefits are mostly to the whole species or a group, which means that evolutionary "cheating" has to be taken into consideration – if genetic mutation for immortality (or longevity) was acquired, the individual would presumably benefit so highly from it that the mutation would spread quickly (assuming reproduction was still possible).

It is clear, therefore, that other explanations are necessary for the evolution of ageing. One might think that lengthened lifespan would be a straightforwardly positive phenotype and therefore theoretically by favoured by natural selection. However, the fact that organisms mostly reproduce at a young age means that unless mutations causing longevity have other positive effects on organismal fitness at or before reproductive age, the long-lived phenotype will not be selected for. The increased lifespan could only evolve due to mutation that enhances reproductive fitness of the organism and in this way the benefit is also a lengthened life.

Early work by Medawar (1952) suggested a novel approach to the topic that arose whilst studying Huntington's disease. Huntington's was known at the time to be a genetically recessive disease that should theoretically have been lost from the population as it is rare to find homozygous recessive individuals. And yet the disease continued to be present in the population. One feature that particularly interested Medawar was the worsening of the condition with age. People with Huntington's first display symptoms in their 30s, often after already having children, and therefore having passed one of the recessive mutant copies of the gene to the next generation. Medawar hypothesized that a similar rule might be applied to ageing itself – if the mutation acquired is not degenerative early in life and therefore does not affect the reproductive success or fitness of an organism, these mutations can accumulate over time. Late-acting alleles would not, therefore, be eliminated by natural selection. If an individual then escaped extrinsic mortality, it would experience the accumulation of late adverse acting alleles as the symptoms we call ageing (known as "mutation accumulation theory").

This work was further expanded by Williams (1957), who first suggested the "antagonistic pleiotropy" theory of ageing in which mutations can be beneficial early in life but be harmful later as, by this point selection pressure would have eased. However, interestingly, not many genes have been found to behave this way. The most common example used is p53, which

protects from cancer in youth but might subsequently drive tissue ageing as mutational defects arise over time. This was suggested by Tyner et al. (2002) who showed that increased p53 activity coincided with a much lower prevalence of cancer but an increased rate of ageing.

Finally, another major evolutionary theory of ageing was developed by Kirkwood (1977). Kirkwood suggested that the allocation of resources between a "disposable soma" and the germline is what drives ageing. He concluded that somatic maintenance *only* needs to be good enough to keep the organism in good condition as long as it has a chance of survival in the wild i.e. if a mouse usually dies in its first year of life, investment of energy (costly and a finite resource) into surviving longer would benefit only a small fraction of the population. It would be more beneficial to put any extra energy into propagation of the species (for example by germline protection). This theory also shows imperfections as is suggests that longevity should come at a cost to reproduction, in other words longer lived species would have fewer progeny. This, however, is known to be untrue both for a number of species, and even within a species of *C. elegans*, there are examples of long-lived mutants that have the same brood sizes, or even larger than wild type worms (Kenyon 2010b).

1.2 MOLECULAR THEORIES OF AGEING

The evolutionary theories of ageing, though satisfying in the extent to which they provide an explanation for the age structure of populations, do not however explain the molecular changes in the organism as it ages or the biological processes that become affected in the aged animal. Over recent decades, research has been conducted to unravel the factors that are crucial to control ageing and longevity. As continued research explores possible avenues to extend life, more molecular theories have started to appear claiming to be the "holy grail" of ageing research. Below, I will introduce the most established of these.

1.2.1 Telomere loss

The protective caps of ncDNA at the ends of chromosomes have been shown to shorten with each cell division in somatic cells, owing to the absence of the enzymatic component of telomerase in somatic cells (as opposed to germline or stem cells). In organisms that rely on telomerase to solve the end-replication problem, telomere attrition might be thought of as a protective measure preventing unregulated proliferation (cancer). Yet this also creates age-related dysfunction as the eventual price for this protection. This theory has been contested as it has also been shown that oxidative stress can significantly accelerate the loss of telomeres (Von Zglinicki 2002), suggesting that telomere shortening is not just a simple measure of chronological ageing although does correlate with biological ageing e.g. following stress. Additionally, simple reactivation of telomerase in somatic tissue does not constitute a cure for ageing, as it poses a significant cancer risk due to inactivation of the 'molecular clock' of the cell divisions (Granger, Wright, and Shay 2002) (Jaskelioff et al. 2011).

1.2.2 Mitochondrial theory

Mutations in mitochondrial genes have been confirmed to increase with age in several mouse tissues like muscle (Müller-Höcker 1989), gut (Taylor et al. 2003) and brain (Cottrell et al. 2000). Accumulation of these mutations during the cellular lifespan has been correlated with impaired ATP production and an eventual decline in cell and tissue bioenergetics. This can create problems in every cellular pathway required for healthy cells, as they depend on oxidative phosphorylation as a source of energy (in contrast to cancer cells, which due to defects in oxidative phosphorylation, use the glycolysis pathway for energy maintenance, reviewed in Gatenby and Gillies (2004)).

1.2.3 Altered proteins and waste accumulation

Impaired protein turnover has been shown to coincide with age. This impedance causes the accumulation of damaged proteins that in turn have been correlated with several age-related diseases such as cataracts, Alzheimer's and Parkinson's. It has been suggested that this waste

product accumulation is an effect of the functional decline in the activities of both proteasomes (responsible for protein degradation (Carrard et al. 2002)) and chaperones (active in protein folding (Soti and Csermely 2003)). Different diseases associated with aggregates could be results of aggeration of proteins in various cell types and cellular localisations however, they all eventually cause chronic ER stress leading to cytotoxicity (Colla et al. 2012; Kikuchi et al. 2006; Nunziante et al. 2011).

1.2.4 Somatic mutation theory

Age-related increases in somatic mutation and other forms of DNA damage (as a simple accumulation of mistakes over time) can result in genomic instability and often lowers a cell's capacity for DNA repair; this is due to a positive feedback in which loss of DNA repair contributes to increased mutation rate, which in turn can cause additional defects in DNA repair pathways and so on. These events can eventually lead to cancer progression. Clear examples of that are human DNA repair diseases like Werner syndrome or *Xeroderma Pigmentosum*; both syndromes are associated with significantly higher cancer risk. Additionally insight from studies utilising Ercc1$^{-/\Delta}$ mice which contain a mutation in DNA repair endonuclease suggest that endogenous DNA damage can contribute to accelerated ageing phenotype and can be used as a model for discovery of molecular mechanisms by which somatic DNA damage can drive ageing (Gregg et al. 2012). Further, longer-lived organisms seem to invest more energy and resources into protecting their genome by having more efficient repair or more types of maintenance. For example, higher levels of PARP-1 signalling, a key player in DNA repair, are associated with longer lifespan (Bürkle 2001).

1.2.5 Oxidative Stress

Progressive and irreversible accumulation of oxidative damage caused by reactive oxygen species *in vivo* is correlated with biological ageing (reviewed in Liochev (2013)). ROS can cause changes in transcription factor activation and gene expression, and might trigger

chronic inflammation which itself causes cellular and tissue damage (Kregel and Zhang 2007).

1.2.6 Senescence

Senescent cells are cells that have stopped dividing and experience an essentially irreversible growth arrest. They can be defined by a series of phenotypes: enlarged morphology, SA-beta-galactosidase activity, generation of a permanent DNA damage response, senescence-associated secretory phenotype (SASP) and $p16^{INK4a\alpha}$ or $p21^{CDKN1}$ locus expression (in vertebrates) (Campisi 2011). It has been shown that the number of senescent cells in some organisms rises with age (Wang et al. 2009; Jeyapalan et al. 2007) and that this accumulation is particularly evident in tissues that show the pathologies of age related diseases like Alzheimer's, pulmonary fibrosis or osteoarthritis (van Deursen 2014). However, the first true evidence towards senescence as a driver of ageing came from two studies in BubR1 progeroid mice (Baker et al. 2008; Baker et al. 2011) where the targeted removal of $p16^{INK4a}$ expressing cells from the organism resulted in the attenuation of progression of several age-related pathologies. Different studies have shown that SASP can also be a major force in disrupting local cellular environment and inducing chronic tissue inflammation (Freund et al. 2010).

1.2.7 Complexity of ageing phenotypes can be explained by multiple theories

Taken singly, none of the current theories discussed above is sufficient to account for all ageing phenotypes. It is likely that different mechanisms are important in different cell types or tissues in the organism, or even in various organisms. For example, dividing cells will be more likely to acquire somatic mutations and end up with shortened telomeres, and be more vulnerable to loss of replication – dependent DNA repair processes. Postmitotic cells (like neurons) might well be more vulnerable to the consequences of the accumulation of aberrant proteins and metabolic waste. With this in mind, it makes it even more striking that mutation of a single gene can have such disproportionate effects on lifespan. For example, in *C. elegans*, lifespan extension has been induced by changes in: oxidative stress (Hartwig et al.

2009), low temperature, chemosensory signals (Alcedo and Kenyon 2004; Apfeld and Kenyon 1999), thermosensory signals (Lee and Kenyon 2009), signals from the reproductive system (Hsin and Kenyon 1999), reduction in respiratory rate (Copeland et al. 2009) or reduction in translation rate (Syntichaki, Troulinaki, and Tavernarakis 2007).

This underlines just how variable the causes of ageing might be, and how manipulable the consequences. Early realization for this came from random mutagenesis screens in yeast, *C. elegans* and *D. melanogaster* as well as from research into human progeroid diseases like Hutchinson Gilford Progeria syndrome (Scaffidi and Misteli 2005) or Werner syndrome (Goldstein, Murano, and Reis 1990). Model organisms with particular single mutations can have life extended ten-fold, an extraordinarily large difference, or conversely, have lives significantly shortened.

1.3 *C. ELEGANS* AS A MODEL ORGANISM FOR USE IN AGEING STUDIES

A significant proportion of ageing research to date has utilised the nematode worm, and there are very good reasons for that - *C. elegans* is a well-established invertebrate model organism for many aspects of biological research. It was originally established by Sydney Brenner to study development and neurobiology (Brenner 1974), however the field has grown through the years and now worms are used to study apoptosis, cell signalling, sex determination, establishment of cell polarity, metabolism, gene regulation and many other biological mechanisms. Over the years, advancement in biomedical research and understanding of human disease has been helped by discoveries in *C. elegans*. For example in 1993 the first evidence of the mode of action of *sel-12* and *lin-12* genes was discovered in a worm (Sundaram and Greenwald 1993); these genes are homologues of human presenilin-1 (later associated with early onset form of Alzheimer's Disease). Subsequent work has also shown astonishing functional similarity between worm and human proteins, as expression of human presenilin-1 in *C. elegans* rescues the neuronal deficiencies in the worm *sel-12* mutant

(Levitan et al. 1996; Wittenburg et al. 2000). Another example of translational research in worms is the identification of negative regulators of the insulin pathway, and particularly the *daf-16* gene, a homologue of a mammalian FOXO transcription factor (Ogg et al. 1997). Five years after this discovery, mutations in FOXO were shown to rescue the diabetic phenotype of insulin-resistant mice (Nakae et al. 2002). These few examples are a tiny portion of the data that have been acquired using worms, and show how important basic biological research on a simple model organism like *C. elegans* is and how useful the insights it can provide in human disease research.

C. elegans is a truly powerful model organism due to several important advantages. Firstly, the genome is highly conserved, and around 70% of human genes have homologues in the *C. elegans* genome (Kaletta and Hengartner 2006). The *C. elegans* genome has been sequenced and fully annotated, and gene expression is relatively easy to manipulate by RNAi or mutations. Observable phenotypes are also very well described. Furthermore, from a more practical point of view, worms are very easy to culture as they are small, and large numbers can be grown simply and economically in the lab. They can also be fed various strains of bacteria, although non-pathogenic strains of *E. coli* are used as standard. Worms are mostly maintained as hermaphrodites that produce sperm early in development and then switch to producing oocytes during adulthood. This, as well as the fact that males can be generated by a heat shock (33°C for four hours (Corsi 2006)) is very useful for generation of new mutants by genetic crosses. The worm life cycle is presented in Figure 1.1, showing how after hatching from an egg, within three days (at 20°C), a worm goes through four larval stages (L1-L4) and eventually becomes an adult, being able to lay eggs itself. Interestingly there is an alternative developmental stage in *C. elegans* called the dauer state, which can be induced by lack of food, overcrowding or other environmental stress. This has been shown to be a stage of suspended animation in which the animals can survive for much longer than their usual

lifespan, and is another trait which can be manipulated as a powerful tool when using the worm as a model organism.

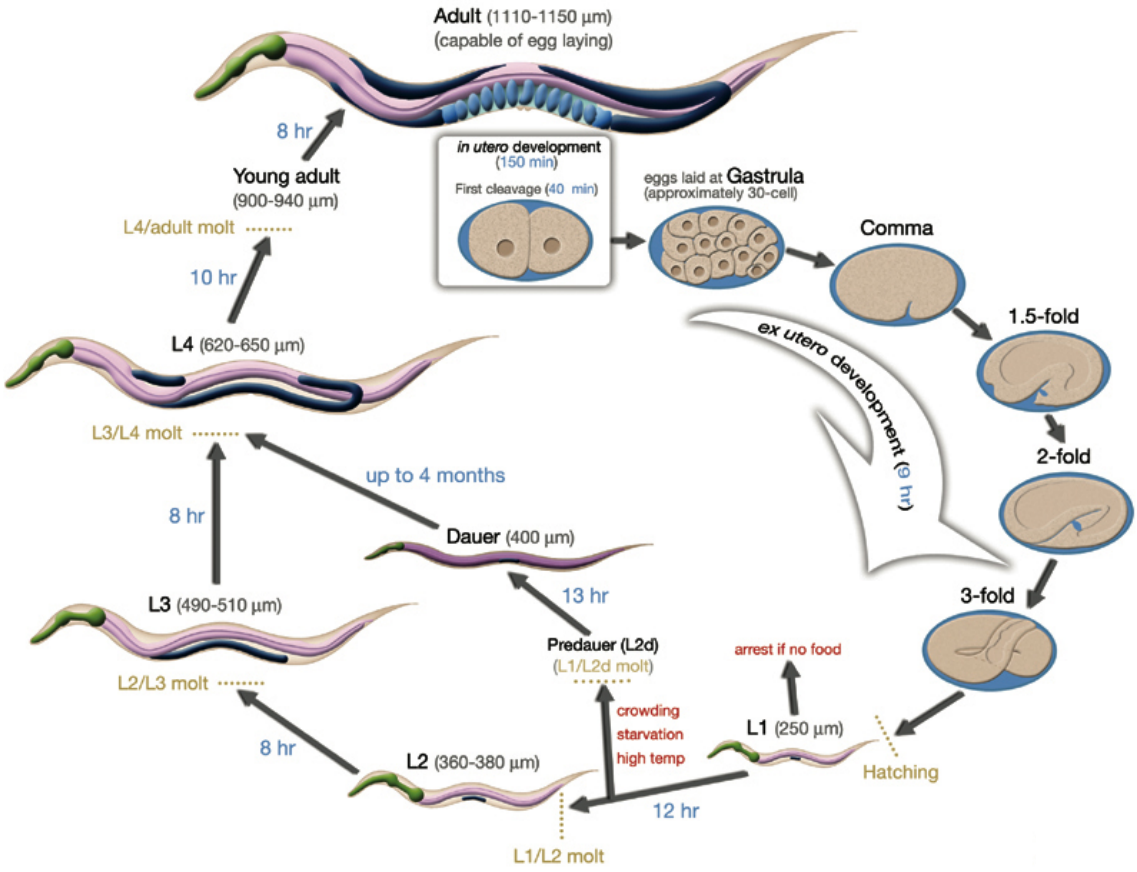


Figure 1.1 *C. elegans* life cycle – figure reused from Worm Atlas. Figure presents the timing of the life cycle of *C. elegans* at 22°C with respect to fertilisation at 0 min. *C. elegans* eggs are laid by adult hermaphrodite around 150 min post fertilisation and develop ex utero for approximately 9 hours until L1 stage larvae hatch from an egg. In favourable conditions (food abundance and accommodating temperature), L1 larvae develop through subsequent larval stages (L1-L4) into a young hermaphrodite. L1 larvae can also enter an alternative life cycle when faced with adverse conditions (starvation, overcrowding, high temperature), in which case, they develop into a dauer (state of suspended animation). Dauers can survive up to 4 months until a change in conditions and subsequent transformation into an adult hermaphrodite. Blue numbers show the amount of time spent in each stage. Approximate sizes of each larval stages are provided in black. Hatching and larval molts indicated in yellow.

The development of *C. elegans* has been extensively studied and every cell in the developing embryo can be followed through the larval stages and into adulthood. Together with the existence of the established isogenic populations, this allows scientists to identify even subtle developmental phenotypes. An additional useful trait, *C. elegans* are transparent which allows the use of advanced microscopy tools and especially *in vivo* fluorescent markers, to highlight distribution and changes in protein localisation in the living organism. Despite its small size, it is a multicellular organism with a division between somatic cells and germline, complicated organs, and tissue separation, and so the tissues can be readily correlated to similar layouts in humans (muscle, hypodermis, intestine, glands, nervous system).

C. elegans is particularly well suited to ageing studies: the organism shares fundamental biological processes with humans, and has a short wild-type lifespan of around three weeks that makes it viable to track and investigate lifespan changes. Statistical significance is easy to achieve experimentally as each organism produces ~300 offspring, allowing the generation of very large data sets in a short time frame. Other advantages are that, unlike mice and other vertebrates, work on the nematode is not subject to regulation by Home Office guidelines. Further, from the epigenetic point of view, histone modifications in the worm have similar functions to the histone marks in higher organisms. It must be noted that global DNA methylation does not exist in the nematode, and this simplifies the possibilities of potential layers of genetic regulation and thus might help to provide clearer results.

Ageing in worms has proved to have some surprisingly similar phenotypic correlation to human ageing, such as slowed movement, tissue disintegration and gene misregulation. Elderly *C. elegans*, like humans, also suffer from sarcopenia (an age related decline in muscle mass and sarcomere integrity). In their work, Herndon et al. (2002) has found no sign of neurodegeneration in the context of normal biological ageing, however they acknowledged that potential changes in neuronal biophysical properties (nerve conduction, synaptic transmission) have not been investigated (Herndon et al. 2002). Other work on

neurodegeneration in worms has been on-going since, and provided evidence of decreased neuronal function with age. For example decreased regeneration following laser axonomy (Wu et al. 2007) or decrease in learning ability (food-odorant association) with age (Kauffman et al. 2010). It has also been suggested that accumulation of macromolecules like yolk, cuticle proteins and lipids all play a role in worm ageing (Ackerman and Gems 2012); these proteins are produced during development and early adulthood as a necessary part of these processes, yet can have negative effects later in life (an example of antagonistic pleiotropy). It has also been suggested that due to the high variation in ageing progression seen not only between individuals in a population but also between cell types in one animal, an element of stochasticity is important in ageing. This is in agreement with the somatic mutation accumulation theory of ageing (Herndon et al. 2002).

Another reason for using *C. elegans* in ageing studies is that they are a very good model for premature ageing – for example mutation of the homologue of the human WRN gene replicates aspects of human Werner syndrome (WS) (Lee et al. 2004). Current work in this laboratory and others has shown that WS worms age faster and display an increased accumulation of ageing biomarkers (Lees, Cox, Woollard, University of Oxford – personal communication and Lee et al. (2004)).

Although worms are in many respects an excellent choice to study ageing, there are obviously some disadvantages too. Firstly, it is a very simple animal and therefore many of the more complex human disease models cannot be phenocopied. This leads to another potential problem - there is often no direct comparison between a particular type of human pathology and a phenotype in *C. elegans*. Human diseases can really only be studied in this model if we know the underlying molecular mechanisms involved and can therefore correlate these within the worm. Due to their well-developed cuticle, it can also be difficult to administer drugs to worms, as some molecules cannot penetrate the layers of lipids and diffuse through the hypodermis. One approach to this is to utilise cuticle permeability mutants like *bus-6*

(Gravato-Nobre et al. 2005). The other approach is to design bioavailable drugs that can be administered via the gut with the bacteria the worms eat – the active molecules would be designed to pass through the gut membranes and be directed into the target tissues.

Finally, the fact that a single hermaphrodite can have up to 300 progeny is a very useful feature if large populations of worms are needed. It can however, be a hurdle as well, especially in ageing studies where one has to follow single worms throughout their lifespan. Several solutions to the problem of overcrowded plates have been suggested. Firstly, the most laborious option is to pick the worm under investigation to a new plate every day over its egg-laying period. This can be very effective, but because of the excessive manipulation involved, the worms can be damaged or stressed – something which might affect the results of the experiment. This option is also not possible to use in high throughput screens due to the sheer amount of hours of manual picking involved. The second option is the use of feminised mutants that lack sperm (Hodgkin 1986). This causes the worms to lay unfertilised eggs and therefore the worm in question can be left on the same plate throughout the experiment as necessary. A potential disadvantage is that the presence of the background '*fem*' mutations might obscure or interfere with the data obtained. Finally it is possible to use chemicals like FUDR (5-fluoro-2'-deoxyuridine), which stop DNA replication and therefore when administered at the correct time (L4 stage), stop new embryos from developing. Due to the fact that *C. elegans* is largely a 'post-mitotic' organism i.e. non-germline cells mostly finish dividing before adulthood, it has been assumed that FUDR does not have a strong effect on *C. elegans* lifespan and is therefore an option often chosen for high throughput screening (Hamilton et al. 2005; Hansen et al. 2005).

In the next section I will discuss some of the molecular pathways in *C. elegans* and other model organisms which can be manipulated to cause dramatic differences in the lifespan of the organism.

1.4 DIETARY RESTRICTION

We know that there are many mutations that can extend lifespan and that many of these seem to affect either stress response or nutrient sensing pathways. In conditions where food is plentiful and there is low environmental stress these genes control growth and reproduction, In harsh conditions, however, their expression can change to cause a physiological shift to instead increase cell protection and maintenance, for example by increasing stress responses, and ultimately causing lifespan extension.

Early experiments studying the effects of DR on rats were performed during the great depression, as it was feared that chronic hunger might shorten lifespan. Unexpectedly, the result of that study suggested that the opposite was true (McCay et al. 1935). Initially it was thought that dietary restriction caused lifespan extension due to a reduced rate of cellular damage as a result of lower nutrient metabolism and less demand on waste disposal. Later, elegant experiments in *Drosophila* (Mair et al. 2003), showed that DR actually causes a decrease in mortality rate, countering the causes of ageing. DR has been shown to work via a nutrient sensing pathway including TOR (Kapahi et al. 2004), AMP kinase (Greer et al. 2007), sirtuins (Rogina and Helfand 2004) and insulin/insulin-like (IIS) growth factor signalling (Honjoh et al. 2009). The method of inducing the DR phenotype can notably change the exact pathway that mediates lifespan extension, suggesting a highly complicated and interlinked network (Greer and Brunet 2009). DR rodents not only live longer but also show an improvement in health; they are protected against cancer, cataracts, diabetes, motor decline, osteoporosis, nephropathy and other age-related diseases (reviewed in Swindell (2012)).

Interestingly, it has been recently suggested (Mattison et al. 2012) that DR does not have such a profound effect in primates as was suggested initially (Colman et al. 2009). In previous research it was proposed that both lifespan and healthspan improve in rhesus monkeys on the

DR diet- in this case semipurified, nutritionally fortified, low fat diet (Colman et al. 2009). A recent publication suggests that in primates, it is mostly the increased healthspan aspects (like protection from cardiovascular diseases) that occur robustly, whilst lifespan is not increased. Furthermore, in higher vertebrates a variety of factors like genetic background or dietary composition have been shown to profoundly affect the life-prolonging effect of DR (Mattison et al. 2012).

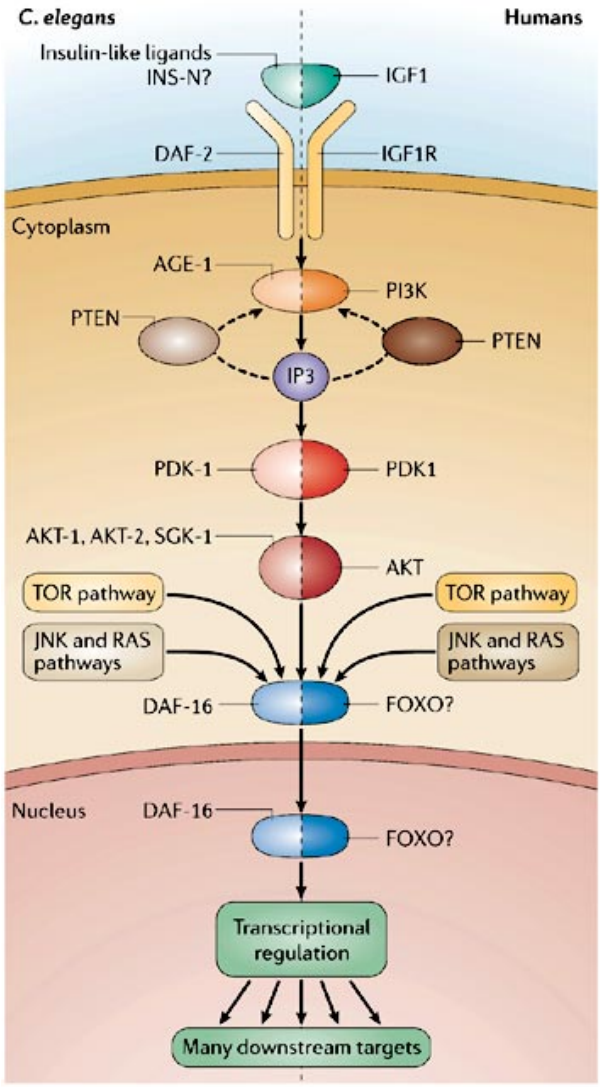


Figure 1.2 IIS pathway in *C. elegans* and humans – comparison. Figure from (Christensen, Johnson, and Vaupel 2006). Depicted is a canonical IIS pathway and homologous proteins are shown on two sides of the graph.

1.5 THE IIS PATHWAY IN *C. ELEGANS* IS A CONVERGENCE POINT FOR MANY LIFESPAN-REGULATING INPUTS.

In *C. elegans* the insulin/insulin-like pathway consists of DAF-2 (insulin/IGF-1 receptor) (Kimura et al. 1997); PDK-1, AKT-1 and AKT-2 (serine/threonine kinases) (Paradis et al. 1999; Paradis and Ruvkun 1998); AGE-1 and AAP-1 (PI 3-kinases) (Morris, Tissenbaum, and Ruvkun 1996; Wolkow et al. 2002); and DAF-16 (homologue of FOXO transcription factor) (Lin et al. 1997; Ogg et al. 1997) (Figure 1.2). Stimulation of this pathway by DAF-2-binding ligands results in the eventual phosphorylation of DAF-16 and its consequent sequestration from the nucleus (preventing the expression of *daf-16*-dependent genes important for lifespan

regulation like stress response genes and antimicrobial peptides) (Landis and Murphy 2010; Hertweck, Göbel, and Baumeister 2004; Kenyon 2010b). This pathway is conserved through evolution from yeast to humans (Barbieri et al. 2003); its perturbations have an effect on longevity in most model organisms and mutations in IGF-1 receptor, AKT and FOXO have been correlated with longevity in human studies (Suh et al. 2008; Kojima et al. 2004; Pawlikowska et al. 2009).

In *C. elegans*, there are several inputs that can activate or downregulate IIS signalling, such as germline loss (through KRI-1 and TCER1) (Kenyon 2010a), respiration (Kayser et al. 2004), dietary restriction (through sensing insulin levels) and diet composition (for example a high glucose diet was negatively correlated with activity of DAF-16 and longevity (Lee, Murphy, and Kenyon 2009)). Additionally, some of the major pathways regulating the response to nutrient signalling (TOR) and stress (JNK/Ras) have also been shown to affect the IIS pathway (Kenyon 2010b).

The convergence of so many signalling pathways through IIS suggests it is a major player in regulating longevity of model organisms. Because of this, perturbations of any genes that extend lifespan are often studied in the light of possible dependence on IIS signalling.

1.6 THE GERMLINE IS A MAJOR TRANSDUCER OF LONGEVITY SIGNALLING

The specific connection between the germline and longevity was first suggested in trade-off models relating fecundity to lifespan as described previously (section 1.1). While this might be true for some of the long-lived mutants that show decreased numbers of progeny, it is not necessarily true for all. For example, the long-lived fly *indy* mutant has even *more* progeny than wild-type flies (Rogina et al. 2000). Further, while removal of germline precursors does increase *C. elegans* lifespan by about 60%, complete removal of the entire reproductive system does not have any effect on the length of the worm's life (Kenyon 2005). Lifespan

extension via germline removal in *C. elegans* works through activating DAF-16/FOXO by steroid signalling and through the adaptor protein KRI-1 and transcription/elongation/splicing factor TCER-1. This lifespan increase can also be induced by TCER-1 overexpression in fertile animals, but in both cases it is dependent on DAF-16-induced lipase genes (Wang, O'Rourke, and Ruvkun 2008). Some effort has been made to replicate these experiments in rodents, one result showing that if ovaries from young mice are transplanted into an old mouse, the latter one lives for longer than sham transplanted controls (Mason et al. 2009).

The germline of an organism is often thought of as "immortal" or "non-ageing", as germ cells have the potential to divide indefinitely. This seems to be confirmed by one case in the simple freshwater animal genus Hydra, which has been described as a species that does not age at all (Bell 1984). *Hydra hydra* does not contain any post-mitotic cells: all can divide continuously and the animal can regenerate itself from virtually any body part, suggesting no distinction between soma and germline. Indeed, other organisms without a germ-soma distinction also tend not to age (Bell 1984). Taking this further, one might conclude that any unicellular organism would not age, given that there is no soma/germline distinction possible. However, this is clearly not the case. The budding yeast *S. cerevisiae* has been established as a model of ageing due to its asymmetric division (the new bud receives newly synthesised organelles, while the 'mother cell' eventually shows signs of ageing after a certain number of cell divisions, the number of bud scars at death can be used to asses age) (Lai et al. 2002; Kirkwood 2005). Even *E. coli*, that had been thought to divide symmetrically, does not exactly do so. One daughter cell per division has been shown to age due to inheritance of an 'old cell pole' (Stewart et al. 2005).

1.7 EPIGENETIC REGULATION OF GENE EXPRESSION AND AGEING

The discovery of epigenetic control of transcriptional regulation has added an additional layer of complexity to understanding the control of cellular processes. An epigenetic trait has been

described as a “stably heritable phenotype resulting from changes in the chromosome without alteration in the DNA sequence” (Berger et al. 2009). Epigenetic regulation can be broadly divided into three categories: DNA methylation, non-coding RNAs and histone modifications. All of these changes can affect transcription and not surprisingly all have been found to be involved in crucial areas of biology like development, cancer and ageing.

DNA methylation in eukaruotes refers to the addition of a methyl group to cytosine in CpG dinucleotides of the DNA sequence. The majority of DNA methylation has been connected to repetitive DNA sequences like transposons and pericentric and subtelomeric chromatin regions. There, it acts to prevent recombination or translocation in the genome. A widespread loss of DNA methylation has been regarded as one of the hallmarks of ageing tissue (Marioni et al. 2015). However, certain specific promoters (e.g. IGF-II, c-fos, e-cadherin) also get hypermethylated, causing gene misregulation and altering gene expression profiles. Hypermethylation of these promoters has also been found in tumourigenesis, therefore linking ageing to cancer progression. The global loss of methylation and changes in methylation profiles (e.g. hypermethylation of CpG islands) have been linked to misregulation of DNA methyl transferases (DNMTs) in aged cells (Casillas et al. 2003).

Non coding RNAs are mostly involved in heterochromatin formation and transcriptional and posttranscriptional gene silencing, and are composed of: siRNAs (21-25 nucleotide long, targeted to chromatin, participating in gene silencing and heterochromatin assembly), miRNAs (generated from single RNA molecules that form hairpin structures and found to induce post-trancriptional silencing by inhibiting translation) and piwi-associated RNAs (28-33 nucleotides long, functioning in preventing transposon jumping) (Gonzalo 2010). There is a very interesting co-regulation between histone modifiers and ncRNAs, as histone modifications can alter the expression of some of the miRNAs (Saito and Jones 2006) while these in turn can regulate the levels of expression of histone modifying enzymes (Fabbri et al. 2007; Friedman et al. 2009). There is also evidence for microRNAs being involved in the

regulation of lifespan. Firstly, experiments in *C. elegans* show upregulation of *lin-4* miRNA which controls levels of LIN-14 protein, an essential transcription factor that is involved in developmental timing and life span regulation via the IIS signalling pathway (Lee, Feinbaum, and Ambros 1993; Boehm and Slack 2005). Other miRNAs have also been found to have functions that might be involved in ageing. For example *Drosophila* miR-34 has been shown to play a role in modulating neurodegeneration (Liu et al. 2012), while miR-16 has been shown to regulate cell proliferation following UV-induced DNA damage in HeLa cells (Pothof et al. 2009). Finally, a diverse group of 24 miRNAs were shown to be involved in replicative and chemically-induced cell senescence in human fibroblasts (Faraonio et al. 2012).

Nucleosome-based packaging of DNA plays an important role in the regulation of gene expression. The DNA is wrapped around the core histone proteins, the structure of which allows their "tails" to be modified at particular residues. Figure 1.3, reproduced from Graff and Tsai (2013) shows the variety of histone modifications present in cells. Types of histone tail modifications include phosphorylation of serine and threonine, acetylation of lysines, ADP ribosylation of glutamic acids, methylation, sumoylation, crotonylation and ubiquitination of lysines, methylation of argenines and proline isomerisation. Such changes to the histone tail can affect the physical properties of histones making the chromatin more likely to acquire an open or closed conformation. Modified histones can also become platforms for the binding of specific factors, which in turn can permit or restrict the access of other regulatory proteins to DNA. The types of modifications most extensively studied in connection to ageing are acetylation and methylation.

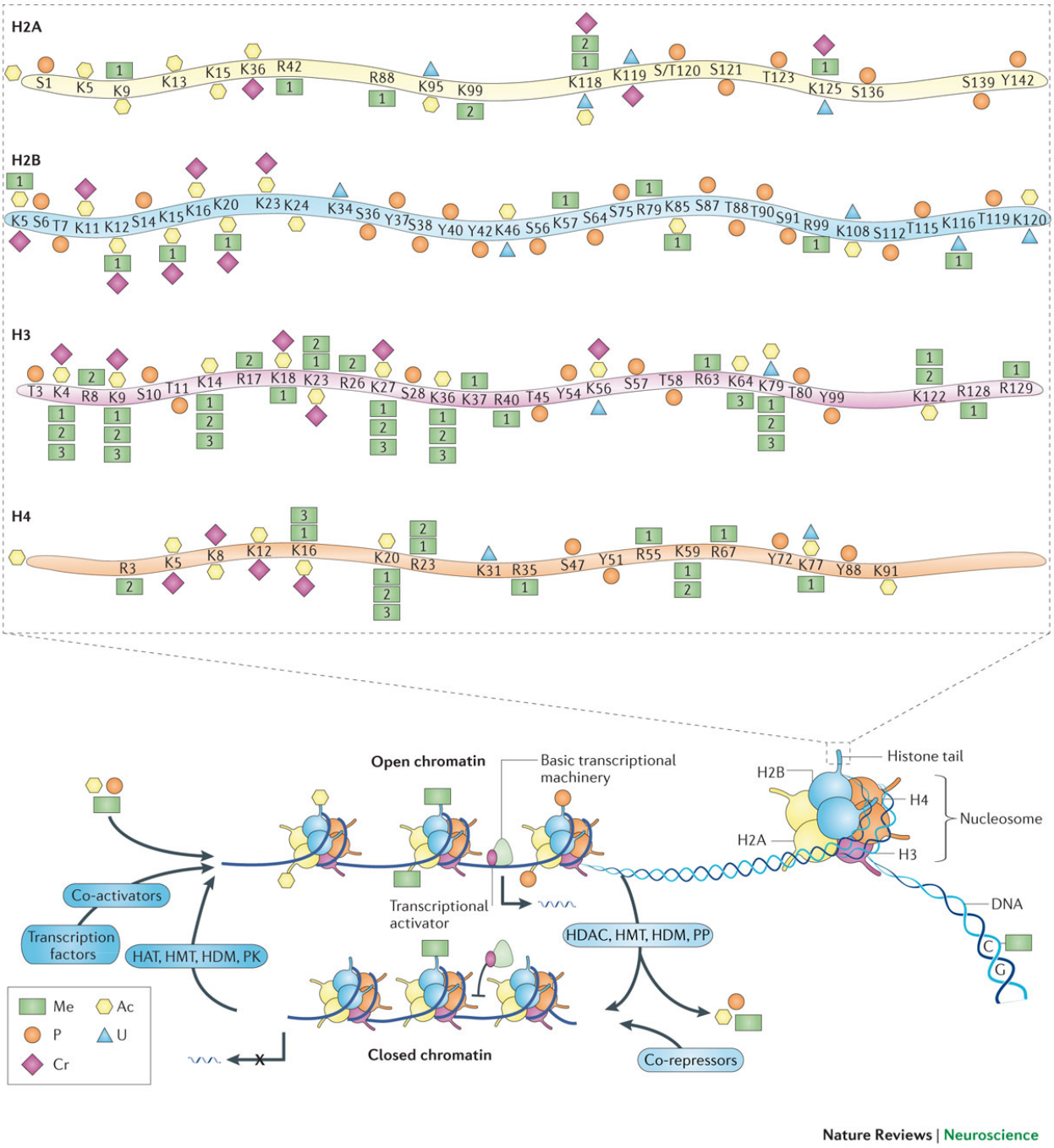


Figure 1.3 Histone tail modifications and structure of the open and closed chromatin in vertebrates. Figure from Graff and Tsai (2013) Top panel shows the known post-translational modifications on histone tails (Ac, acetylation; Me, methylation; P, phosphorylation, U, ubiquitination; Cr, crotonylation). Bottom panel, shows a simplified cartoon of open and closed chromatin states (euchromatin and heterochromatin) and their regulation by various histone-modyfying enzymes.

Acetylation of lysines on histone tails can by controlled by two groups of enzymes of opposing functions: HATs (histone acetyl transferases) and HDACs (histone deacetylases). Their effect on organisms has become an area of active research, especially after pioneering work in yeast by Kennedy et al. (1995) showed that expression of the sirtuin homologue complex (Sir2/Sir3/Sir4) correlates with yeast longevity. Furthermore, deletion of one of the components of the complex, Sir4, caused a decrease in lifespan, while a gain-of-function mutation induced a relocalisation of the whole complex and extended lifespan by 30%. Later studies using the overexpression of Sir2 orthologs (NAD^+-dependent histone deacetylases) in both flies and worms showed that they can lead to lifespan extension (Tissenbaum and Guarente 2001; Rogina and Helfand 2004), although a later publication has suggested that certain conclusions about the activity of sirtuins in ageing might be flawed (it is possible that background mutations in the animal strains confounded the data) (Burnett et al. 2011). Conversely, it has been argued that some of these concerns have been tackled before, as Bauer et al. (2009) used a different method of overexpressing Sir2, (a chemical trigger in *D. melanogaster*) which allowed for control of background mutations. Additionally, more recent research from Mouchiroud et al. (2013) has suggested an alternative pathway for sirtuin-dependent lifespan extension based on the fact that aged mice and worms show a decrease in NAD levels due to increase of activity of PARP (which consumes NAD+ as a co-factor necessary for function of the enzyme). The authors showed that genetic or pharmacological replenishment of NAD levels in *C. elegans* lead to a *sir-2.1*-dependent lifespan increase.

In mammalian systems, two of the sirtuin family homologues have been particularly studied, SIRT1 (responsible for deacetylating H4K16, H3K9 and p53) and SIRT6 (deacetylase activity towards histone H3K9Ac and H3K56Ac) (Oberdoerffer et al. 2008). It has been proposed that as levels of SIRT1 decrease with age, this could promote genomic instability and therefore certain ageing phenotypes. This seems to be confirmed by the fact that high SIRT1 levels are

known to be a hallmark of many tumours (which are genetically unstable), and that high expression of SIRT1 promotes survival in a mouse model of genomic instability (Oberdoerffer et al. 2008). Finally, deficiency of the other family member, SIRT6, leads to a degenerative ageing-like phenotype in mice, as shown by Mostoslavsky and collegues (2006). Discussion therefore continues about involvement of sirtuins in ageing (Longo and Kennedy 2006; Burnett et al. 2011), especially as more recent work in mammals confirms that brain-specific overexpression of SIRT1 can extend lifespan in mice (Satoh et al. 2013).

Histone methylation is probably the most wide-spread and studied epigenetic modification, but it is also one of the most complex as it can be formed both on arginine and lysine and each residue can be modified to a different extent (mono-, di- and tri- methylation). These modifications can have very different functions: for example H3K4me3 is a mark of active promoters, while H3K27me3 marks transcriptional repression, and H3K9me3 and H3K20me3 are marks associated with constitutive heterochromatin (like telomeric and pericentric regions) (reviewed in Graff and Tsai, 2013).

Therefore, not surprisingly, investigating these modifications and their effects on ageing phenotypes has produced varied outcomes; some results in the literature are in fact contradictory.

One of the more recent discoveries comes from work in *C. elegans* by Greer and colleagues, who identified a link between elongated lifespan and silencing of members of the COMPASS complex (ASH-2, WDR-5, SET-2) responsible for H3K4 trimethylation (Greer et al. 2011). Deficiencies in this complex not only increased the lifespan of the affected organism but also their descendants up to three generations later. Further, overexpression of RBR-2, an H3K4 demethylase, also increased lifespan. However all this held true only for organisms with a functioning germline and continuously laying fertilised eggs, as Greer and colleagues indicate that ASH-2-regulated genes only change in wild-type worms and not in the *glp-1* (germless)

mutants, and further, that RNAi against *ash-2* does not extend lifespan of the *fem-3(e2006ts)* mutant (which produces unfertilised eggs). This suggests that it is in fact functional egg production rather than simply the presence of the germline that is necessary for this lifespan-extending pathway. Therefore, the authors propose a germline-specific action for these histone modification enzymes in extending lifespan. The reasons why the deficiency of H3K4me3 seems to be beneficial for worm longevity has not yet been clarified (Greer et al. 2010; Greer et al. 2011).

In another, comprehensive study, Wang, Tsai, Yew, et al. (2010) used a senescence accelerated mouse model (SAMP8) and found increased H3K27me3 and H3K79me/me2 with concomitantly decreased levels of H4K20me and H3K36me3 in the mouse brain. However, a different publication indicated that H4K20me/me2 levels do not vary significantly in kidney tissue of normally aged rodents (Sarg et al. 2002).

Some epigenetic regulation has also been shown to be linked to IIS pathways, for example in *C. elegans* the loss of lysine demethylase UTX-1 (known to act on H3K27) increases trimethylation of H3K27me3 at the *daf-2* locus, resulting in *daf-2* transcriptional downregulation that in turn causes re-localisation of DAF-16 to the nucleus and activates anti-ageing genes downstream of the IIS pathway. H3K27me3 levels were additionally shown to decrease in somatic cells while *utx-1* mRNA levels increase with age (Maures et al. 2011).

This observed age-dependent global decrease in H3K27me3 levels in *C. elegans* does not agree with previously reported research (Wang, Tsai, Yew, et al. 2010), however it *does* match with studies of the human premature ageing disease Hutchinson Gilford Progeria Syndrome, caused by expression of a truncated version of A-type lamin (this mutated version being termed progerin). It has been reported that H3K27me3 levels decrease in these prematurely aged cells (De Sandre-Giovannoli et al. 2003; Eriksson et al. 2003). Interestingly, normal aged human fibroblasts also express progerin late in life, which links HGPS to

physiological ageing, and provides a viable accelerated model of ageing (Scaffidi and Misteli 2006). It has been shown that HGPS cells have many epigenetic alterations, particularly in constitutive heterochromatic regions (Shumaker et al. 2006) but also in global levels of certain histone marks, as H4K20me3 was increased while H3K27me3 was markedly decreased in HGPS cells compared to healthy controls. Additionally, reduced levels of H3K9me3 were observed as well, which was found to be linked to loss of NURD subunits (RBBP4, RBBP7 and HDAC1), which interact with lamin A; this effect was dependent on the presence of progerin (Pegoraro et al. 2009).

1.8 AIMS OF THIS STUDY

In this thesis, I will conduct an initial exploration of chromatin factors and their potential effect on lifespan regulation by performing an RNAi screen for longevity phenotypes, using lipofuscin accumulation as a biomarker for the initial screen. Further, the secondary screen will be performed to pinpoint which of the factors, when knocked-down, cause lifespan extension in *C. elegans*. Next, I aim to use mutants of the genes pulled out of the RNAi screen to further investigate the effect of the loss-of-function on lifespan in the nematode worm.

Moreover, investigation of the importance of the enzymatic activity as well as the effect of overexpression of the H3K27 methylation regulators on lifespan extension will also be conducted. Exploration of the tissues and molecular pathways involved in lifespan extension by the H3K27 regulators will also be performed.

Finally, I will examine global changes in H3K27 methylation in various longevity mutants as well as examine the changes in H3K27me3 and transcript levels at the *daf-2* locus with age in both wild-type animals and long-lived mutants to help elucidate the mechanisms of lifespan extension.

CHAPTER 2: MATERIALS AND METHODS

2.1 *C. ELEGANS* STRAINS AND MAINTENANCE

A standard in the *C. elegans* field, the Bristol N2 strain as described by Brenner (1974) has been used throughout this thesis as a wild-type strain. All other strains used in this thesis (Table 2.1) have been derived from this and either generated in the Woollard or Salcini lab, or obtained from the *Caenorhabditis* Genetics Center (CGC) or National Bioresource project.

Table 2.1 *C. elegans* strains used in this thesis.

Strain name (if given)	Full genotype	Plasmids in extrachromosomal arrays	Name used for the purpose of this thesis
	mes-2(tm5007) II		*mes-2(tm5007)*
SS186	*mes-2(bn11) unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444) II*		*mes-2(bn11)*
SP127	*unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444) II*		SP127
VC2409	*mes-2(ok2480)/mT1 II; +/mT1 [dpy-10(e128)] III*		*mes-2(ok2480)*
AW1166	*ouEx864[pAW169]*	pAW169=rol-6^{+}	*N2+rol-6*
AW1167 AW1168	*ouEx865[WRM0629dF11+pAW169]*	*WRM0629dF11= fosmid* pAW169=rol-6^{+}	*N2+mes-2*
VC1006	*cbp-1(ok1491) IIIhT2 [bli-4(e937) let-?(q782) qIs48] (I;III)*		*cbp-1(ok1491)*
CF1903	*glp-1(e2141) III*		*glp-1(e2141)*
	wrn-1(tm764) II		*wrn-1(tm764)*
TJ1052	*age-1(hx546) II*		*age-1(hx546)*
MT15795	*isw-1(n3294) III*		*isw-1(n3294)*
MT16012	*isw-1(n3297) III*		*isw-1(n3297)*
	jmjd-3.2(tm3121) X		*jmjd-3.2(tm3121)*
ZR252	*utx-1(tm3118) X*		*utx-1(tm3118)*
ZR254 ZR255	*utx-1(tm3118) zrEx[utx-1p::utx-1::GFP + rol-6^{+}]*	*utx-1p::utx-1::GFP + rol-6^{+}*	*utx-1(tm3118)+ utx-1::GFP*

ZR256 ZR257	*utx-1(tm3118) X zrEx[utx-1p::utx-1DD::GFP + rol-6^{+}]*	*utx-1p::utx-1DD::GFP + rol-6^{+}*	*utx-1(tm3118)+ utx-1DD::GFP*
ZR802	*zrEx264[utx-1p::utx-1::GFP + rol-6^{+}]*	*utx-1p::utx-1::GFP + rol-6^{+}*	*N2+ utx-1::GFP*
ZK856	*zrEx295 utx-1p::utx-1DD::GFP + rol-6^{+}]*	*utx-1p::utx-1DD::GFP + rol-6^{+}*	*N2+ utx-1DD::GFP*
ZR332	*jmjd-3.1(gk384) jmjd-3.2 (tm3121) jmjd-3.3(tm3197) X*		*jmjd-3* triple mutant
ZR693 ZR694	*zrEx231[myo-3p::utx-1::GFP + rol-6^{+} + myo-2::RFP]*	*myo-3p::utx-1::GFP + rol-6^{+} + myo-2::RFP*	*myo-3p::utx-1:: GFP*
ZR695 ZR696	*zrEx233[dpy-7p::utx-1::GFP + rol-6^{+} + myo-2::RFP]*	*dpy-7p::utx-1::GFP + rol-6^{+} + myo-2::RFP*	*dpy-7p::utx-1:: GFP*
ZR697 ZR698	*zrEx235[rab-3p::utx-1::GFP + rol-6^{+} + myo-2::RFP]*	*rab-3p::utx-1::GFP + rol-6^{+} + myo-2::RFP*	*rab-3p::utx-1:: GFP*
ZR699 ZR700	*zrEx237[vha-6p::utx-1::GFP + rol-6^{+} + myo-2::RFP]*	*vha-6p::utx-1::GFP + rol-6^{+} + myo-2::RFP*	*vha-6p::utx-1:: GFP*
ZR701 ZR702	*jmjd-3.2(tm3121) X zrEx239[jmjd-3.2p::jmjd-3.2::GFP + rol-6^{+} + myo-2::RFP]*	*jmjd-3.2p::jmjd-3.2::GFP + rol-6^{+} + myo-2::RFP*	*jmjd-3.2(tm3121)+ jmjd-3.2::GFP*
ZR703 ZR704	*jmjd-3.2(tm3121) X zrEx241[jmjd-3.2p::jmjd3.2DD::GFP + rol-6^{+} + myo-2::RFP]*	*jmjd-3.2p::jmjd-3.2DD::GFP + rol-6^{+} + myo-2::RFP*	*jmjd-3.2(tm3121) + jmjd-3.2DD::GFP*
ZR705	*zrEx243[jmjd-3.2p::jmjd-3.2::GFP + rol-6^{+} +myo-2::RFP]*	*jmjd-3.2p::jmjd-3.2::GFP + rol-6^{+} + myo-2::RFP*	*N2+jmjd-3.2::GFP*
ZR706 ZR707	*zrEx245[jmjd-3.2p::jmjd-3.2DD::GFP + rol-6^{+} + myo-2::RFP]*	*jmjd-3.2p::jmjd-3.2DD::GFP + rol-6^{+} + myo-2::RFP*	*N2+ jmjd-3.2DD::GFP*

C. elegans strains were maintained by standard techniques as described by Brenner (1974) and Sulston and Hodgkin (1988). An *E. coli* OP50 strain from an overnight liquid culture (at 37°C) was streaked onto standard NGM plates and allowed to grow overnight at room temperature. Worms were maintained by picking using a platinum wire, or by chunking a portion of the previous NGM plate and transferring onto a fresh one. Strains were maintained at 20°C and stocks were frozen in liquid nitrogen.

2.2 BACTERIAL STRAINS

2.2.1 *E. coli* strains and maintenance

Two *E. coli* strains were used to maintain and feed *C. elegans*: OP50 and HT115. The OP50 strain was used originally by Brenner (1974), and was selected as the uracil deficiency stops the overgrowth of bacteria on the agar plates used for worm culture. The HT115 (DE3) is a tetracycline-selectable transposon-containing strain (a gift from the Fire lab, University of Stanford). The transposon disrupts the RNase III gene; the DE3 prophage delivers phage T7 RNA polymerase. RNAi-coding constructs in plasmids used for all experiments are under a T7 promoter that can therefore be driven in this strain.

Bacterial strains were retrieved from stocks frozen at -80°C (2YT media, 40% glycerol final concentration), the colonies were streaked onto 2YT plates or inoculated into liquid 2YT media and incubated overnight at 37°C. Where necessary, media was supplemented with antibiotics (50 μg/ml ampicillin, 50 μg/ml kanamycin or 12.5 μg/ml chloramphenicol).

2.2.2 Fosmids

The *mes-2* overexpression experiment (chapter 4.6) was performed using fosmid WRM0629dF11 which contains the *mes-2* gene as part of a ~32kB genomic DNA fragment in the fosmid vector pCC1FOS. The fosmid was obtained from the *C. elegans* fosmid library produced by Don Moerman (see http://www.lifesciences.sourcebioscience.com/clone-products/non-mammalian/c- elegans/c-elegans-fosmid-library/).

To obtain a high copy number of fosmid DNA for injection, a single colony containing the fosmid was grown overnight, with shaking at 37°C in 5 ml LB medium (with 12.5 μg/ml chloramphenicol). The culture was inoculated into a 5 ml of LB medium containing CopyControl solution (Epicentre) and grown overnight. Cells were centrifuged and DNA was purified using the Qiagen miniprep kit according to manufacturer's instructions.

2.3 WORM METHODS

2.3.1 Bleaching

To obtain eggs for subsequent synchronised experiments, plates with gravid adult hermaphrodites were washed using M9 buffer into a 15 ml falcon tube. Samples were centrifuged (2 min, 1500 rpm) and supernatant was removed. 6 ml of M9 buffer was added to the worm pellet and topped up with 6 ml of 2x bleach solution (stock: 12.5 ml 4 M NaOH, 20 ml sodium hypochlorite 15%, 17.5 ml water). The samples were shaken vigorously for 3-5 min and then centrifuged for 2 min at 1500 rpm. Supernatant was removed and the egg pellet was washed twice with M9 buffer. The eggs were then ready to be hatched overnight in M9 buffer or to be put directly onto an NGM plate for subsequent development.

2.3.2 Brood size analysis

Brood sizes of 20 synchronised worms for each strain were assessed. Each worm was picked at the L4 stage onto a separate, OP50-seeded 55 mm NGM plate and moved to a new plate every day until the end of the egg-laying period. Counts of the unhatched eggs and larvae were performed on the plates that had the parent worm removed. Dead eggs were scored 24 hours after removal of the parent, as a viable egg would have hatched by that point.

2.3.3 RNAi by feeding

HT115 *E. coli* clones containing L4440 plasmids coding for appropriate dsRNA were selected either from a genome-wide *C. elegans* library (Kamath et al. 2003) or from the chromatinome library donated by Gino Poulin (for the list of clones see Appendix 1). Sequences of the dsRNA in positive hits from the screen were confirmed by sequencing (Source Bioscience) using T7 primers.

Bacteria were picked from frozen stocks to inoculate 2 ml of liquid 2YT media containing 50 μg/ml each of ampicillin and tetracycline. Bacteria were incubated with shaking, overnight at 37°C. NGM agar plates with additional 25 μg/ml carbenicillin and 1 mM IPTG were

seeded with 100 µl of the bacteria and allowed to dry overnight at room temperature. Three L4 worms were picked onto each seeded plate and were maintained at 20°C. After 4 days, their offspring at the L4 stage were transferred to fresh plates seeded with HT115 expressing the chosen dsRNA. Where necessary, secondary plates were supplemented with FUDR (1 mg/ml in HT115 culture). *C. elegans* were fed empty vector-containing bacteria for negative control of the experiments, while knock-down of the *pop-1* gene (giving an embryonic lethal phenotype) was used as a positive control for uptake and expression of RNAi.

For the design of the RNAi screen for longevity phenotypes see chapter 3.

2.3.4 Lifespan analysis

Analysis of the lifespan of *C. elegans* strains was performed at 20°C using the N2 wild-type strain as a control. All strains were fed with OP50 (unless stated otherwise in RNAi experiments). Where appropriate, the OP50 bacterial culture was supplemented with FUDR (1 mg/ml) prior to seeding the plates. Five hermaphrodites were picked onto each plate at the L4 stage (day 0 of the analysis) and they were assessed every other day and scored as dead when unresponsive to three light taps with a platinum wire. Each experiment was set up using 60 worms per strain and condition, which usually allowed for the final number of worms scored to be around 50 (after discounting worms that crawled off the plate; see individual figures for n numbers for each experiment). All lifespan experiments were done in at least two independent biological replicates. Unless stated otherwise, all of the lifespan analysis in this thesis was done in the presence of FUDR.

2.3.5 Thrashing assay

To analyse healthspan by the documentation of motility change with age, worms of choice were picked into ~20µl M9 buffer (42 mM Na_2HPO_4, 22 mM KH_2PO_4, 86 mM NaCl, 1 mM $MgSO_4$) on an unseeded NGM plate. Worms were allowed to acclimatise in the liquid for 30 seconds. The number of sigmoidal thrashes made by the worm in a subsequent 30 second

period was recorded, 3 times per worm. 10 hermaphrodites per strain per age were analysed, and an average of 30 measurements was used for data comparison.

2.3.6 Stress resistance

To assess stress tolerance, 50 L4 worms per strain were picked onto 30 mm NGM plates (seeded with OP50 or RNAi feeding clones) containing 0 mM, 10 mM or 25 mM of paraquat dichloride (Sigma-Aldrich) to generate reactive oxygen species. All plates were maintained at 20°C. Survival was first assessed after 22 hours and then every 4 hours thereafter (on average). Worms that were unresponsive to three gentle prods of a platinum wire were scored as dead.

2.3.7 Microinjections.

Transgenic *C. elegans* lines were generated using a microinjection method previously described (Mello et al. 1991; Mello and Fire 1995). Prepared fosmid DNA (2ng/µl) was injected into wild-type worms using a dominant *rol-6*$^{+}$ plasmid (pAW169) as a co-injection marker.

2.4 DNA METHODS

2.4.1 Single worm lysis

Individual worms were picked into 2.5 µl of lysis buffer (50 µM KCl, 2.5 µM $MgCl_2$, 10 µM Tris-HCl pH 8.3, 0.45% NP40, 0.45% Tween-20, 0.01% gelatine, 0.1 mg/ml proteinase K) and frozen at -80°C for 1 hour minimum. Samples were then incubated for 1 hour at 60°C, followed by 15 minutes at 95°C. When necessary, PCR reactions were performed using 2.5 µl of the lysis mix as the DNA template.

2.4.2 PCR

DNA template was obtained from either single nematodes lysed as described above, plasmids, or fosmids. Primers were synthesised by MWG (for sequences, see Appendix 2).

A typical PCR reaction using Taq polymerase (NEB) contained: 1 µl Taq polymerase (NEB), 2.5 µl template DNA, 2.5 µl 10x ThermoPol buffer (final concentration 10 mM Tris.HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 4 µl dNTP mix (final concentration 0.4 mM of each of dATP, dCTP, dGTP and dTTP), 2.5 µl of each forward and reverse primers (0.4 µM final), and 10 µl ultrapure water.

Standard cycling parameters, optimised when necessary, were as follows: primary denaturation 95°C for 2 minutes followed by 30 cycles of: denaturation (30 seconds 95°C), annealing (30 seconds at the melting temperature of the primer minus 5°C) and extension at 68°C (1 min/kb). Final extension step: 10 minutes at 68°C. Primer melting temperature was calculated using ApE software.

2.4.3 Agarose gel electrophoresis

DNA fragments were separated by 0.5-2% agarose gel electrophoresis, in TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA). Ethidium bromide (final concentration 0.5 µg/ml) was added to the gel mix cooled to 50°C, before the gel was poured into a gel tank and allowed to set. DNA samples were mixed with loading dye buffers (10 mM Tris.Hcl pH 7.4, 1 mM EDTA, 10% glycerol, 0.1% w/v xylene cyanol or bromophenol blue). Gels were electrophoresed in 1x TBE buffer at 70 V until the desired separation of the fragments was achieved. DNA was visualised by exposure to UV transilluminator and images were collected using camera with AlphaImager software. Molecular weight markers used: 10 kb ladder or 1 kb ladder (NEB).

2.4.4 Gel extraction

Gel bands were excised from the gel under long wavelength UV transillumination. DNA was purified using Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions (Qiagen).

2.4.5 DAPI staining

Worms of choice were picked into 10 µl of M9 in a watch glass. 200 µl of 150 nM DAPI dissolved in 100% ethanol was added to the sample. The watch glass was incubated in a dark chamber for 2 hours. The worms were then washed twice in 2 ml of M9 and allowed to soak overnight at 4°C in a humid chamber. DAPI staining was then visualised as described in section 2.7.

2.4.6 ChIP-qPCR

Worms were cultured on 90 mm NGM plates. 10 full plates of worms were used per sample corresponding to around 0.4 ml of packed worms. Where necessary, worms were synchronised by bleaching three 90 mm plates full of gravid adults (see section 2.3.1) and hatching the eggs by rotating overnight at room temperature in M9 buffer. Next day, hatched L1 larvae were spun down (2 min, 650 g) and resuspended in 500 µl of fresh M9. The worms were distributed between 10 pre-seeded 90 mm plates (50 µl of worm suspension) and grown until the L4 stage (45 hours at 20°C). L4 larvae were washed off the plates with M9 buffer and moved to 10x 90 mm plates containing OP50 with FUDR (1 mg/ml). If worms were cultivated for longer than one day, they were transferred into twice as many plates as the original larvae to assure abundance of food and space (i.e. 20 plates / sample).

On the day of collection, adults were washed off the plates with M9 and spun for 2 min at 650 g. Worm pellets were washed 3 times in PBS and then with 5 ml of CB buffer (1% formaldehyde, PBS). The worm pellet dissolved in CB buffer was transferred into liquid nitrogen (in a mortar) and was ground into powder with the pestlewhilst taking care not to allow liquid nitrogen to evaporate. The progress of worm grinding was observed under the microscope. The worm powder was transferred into 8 ml of CB buffer and incubated rotating at room temperature for 20 min (to allow cross-linking). 400 µl of 2.5 M glycine was used to quench the formaldehyde and the samples were incubated rotating for a further 20 minutes. Samples were then spun down (2 min, 650 g) and washed 3 times in PBS containing protein

inhibitor cocktail (10 µl/ml, Sigma). The supernatant was removed and samples were stored at -80°C.

Next, the samples were defrosted on ice and resuspended in FA150 buffer (0.1% SDS, 1% Triton X-100, 10 mM HEPES pH 7.9, 0.1% sodium deoxycholate, 150 mM NaCl + protein inhibitor) and incubated on ice for 10 min. Samples were transferred to a sonicator (Bioruptor) for 60 min (30 s on, 30 s off, medium power). Samples were then transferred to 2 ml eppendorf tubes and were centrifuged (15 min, 10000 rpm, 4°C). The supernatant was transferred to new tubes and appropriate samples were pooled together (3 samples per condition per strain). Immunoprecipitation (IP) was set up overnight using 1.5 ml of sample with 10 µl of anti H3K27me3 antibody (Abcam, ab6002), 10 µl of anti H3 antibody (Abcam, ab1791) or a control sample with no antibody, and the samples were left rotating overnight at 4°C. For total chromatin, 0.5 ml of each sample was stored at -20°C overnight. Protein G Sepharose CL-4B beads were prepared the same day by taking 500 µl of the bead solution and mixing it with 10 ml of cold PBS. The beads were centrifuged (5 min, 3000 rpm) and resuspended in 1 ml of CL-4B buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 100 µg/ml BSA, 0.1% sodium azide). The beads were centrifuged again (1 min, 3000 rpm) and resuspended in 500 µl of CL-4B. 100 µl of sonicated salmon sperm DNA (1 mg/ml) was added to the beads and they were left rotating overnight at 4°C.

The next day, 'IP' and 'no IP' samples were centrifuged and 50 µl of protein G-Sepharose beads in CL-4B buffer were added to each sample. Samples were incubated rotating at room temperature for 90 min and then spun for 1 min at 2600 rpm. The beads were then washed with 350 µl of TSE-150 buffer (1% Trinton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris-Cl pH 8.0, 150 mM NaCl) allowing 3 min incubation time, 350 µl of TSE-500 (1% Trinton X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris-Cl pH 8.0, 500 mM NaCl) with 3 min incubation time, 350 µl of LiCl wash (0.25 M LiCl, 1% NP-40, 1% sodium dioxycholate, 1mM EDTA, 10 mM Tris-Cl pH 8.0) with 15 min incubation, and twice with 350 µl of TE pH

8.0 (no incubation). Following the washes the beads were resuspended in 100 µl of pre-heated elution buffer (1% SDS, 0.1 M $NaHCO_3$, autoclaved) and incubated at 65°C for 30 minutes. The samples were centrifuged (1 min, 2600 rpm) and the supernatant was transferred to new tubes. 7 µl of 5 M NaCl was added to all the samples, including the 250 µl of total chromatin. Tubes were then incubated at 65°C for 3 hours. Following the decrosslinking, samples were cooled down to room temperature for 10 min and 1 µl of 10 mg/ml RNaseA was added and incubated at 37°C for 1 hour. Finally, 1 µl of proteinase K was aliquoted into each tube and samples were incubated at 65°C overnight.

On the final day, the DNA from the samples was extracted using the QIAGEN PCR purification kit according to the manufacturer's instructions. qPCR was performed using SYBR green and Applied Biosystems kits available according to the manufacturer's instructions. Primers used for each gene can be found in Appendix 2. Quantification was performed using the ΔΔCt method (Livak and Schmittgen 2001) and the results are presented as mean fold enrichment over negative control levels (no antibody), and as the average of three technical repeats.

2.5 RNA METHODS

2.5.1 RNA extraction from whole worm

Whole plate of worms or a minimum of 100 synchronised worms were washed off or picked into M9 buffer. Worms were spun down (2 min, 7000 rpm) and the buffer was removed leaving the worm pellet. 250 µl of Trizol (Invitrogen) was added to the samples and the tubes were vortexed for 10 s each. Eppendorfs were subsequently snap frozen in liquid nitrogen for about 30 min, then transferred into a water bath or heat block at 65°C for 90 s and the samples were topped up to 1 ml with Trizol. The samples were incubated at room temperature for 15 min and transferred to Phase Lock Gel Heavy tubes (5 Prime). 200 µl of chloroform was added to the samples and left to incubate for 3 min, then they were spun down (15 min, 15000

rpm, 4°C) and the clear upper phase transferred to a new tube. 0.5 ml of isopropanol was added to each sample, mixed gently and frozen at -20°C overnight.

The next day, the tubes were spun down (10 min, 14000 rpm, 4°C) and the supernatant discarded. The pellet was washed with ice cold 70% ethanol and then spun down for 5 min at 14000 rpm, 4°C. The pellet was dried at room temperature for 20 min and then resuspended in 10 µl of RNase free water. The samples were treated with DNase I (Invitrogen) according to the manufacturer's instructions and the concentration and quality of RNA was assessed using a Nanodrop micro-spectrophotometer (Thermo Scientific).

2.5.2 qRT-PCR

The reverse transcriptase step was performed using the SuperScript VILO kit (Invitrogen) following manufacturer's instructions and using 1 µg of RNA from the RNA extraction protocol. Finally, samples were treated with RNase H (New England Biolabs) according to the instructions from the manufacturer. Samples were then stored at -20°C. qPCR was performed using SYBR green and Applied Biosystems kits according to the manufacturer's instructions. Primers used for each gene can be found in Appendix 2.

Relative quantification of the transcript abundance was calculated using the ΔΔCt method (Livak and Schmittgen 2001). Every experiment was performed in three technical replicates as well as in three independent biological replicates.

2.6 PROTEIN METHODS

2.6.1 Bradford protein quantification

Protein extract from whole worms was prepared by boiling in 2x SDS loading buffer solution (2% SDS, 125 mM Tris HCl pH 6.8, 12.5% glycerol, 0.5 M DTT) for 10 min at 95°C.

Protein quantification of samples was performed using a Pierce™ Coomassie Plus (Bradford) Assay Kit (ThermoFisher Scientific) following the manufacturer's instructions. A PHERAstar

plate reader with corresponding software was used to assess changes in absorbance and determine changes in protein concentration.

2.6.2 Western Blots

A whole plate or single worms were washed into M9 buffer and set on ice to allow the worms to settle to the bottom of the tube. The supernatant was removed and replaced with a lysis buffer (100 µl of 4x NuPage buffer, 20 µl of 1 M DTT). Worms were boiled in the solution for 10 min at 95°C and then centrifuged for 5 minutes at 13000 rpm. The supernatants were stored at -20°C if necessary.

Protein separation was performed using pre-cast BioRad gels and appropriate BioRad tanks according to the provided instructions. PageRuler Plus protein ladder (ThermoFisher Scientific) was used to determine protein sizes. The transfer to the membrane was performed using a BioRad Trans-Blot Turbo Transfer System and corresponding PVDF membranes (BioRad), using instructions from the manufacturer. After the transfer the membrane was blocked using 5% milk or BSA in PBST (Tween 0.2% in PBS). The membranes were blocked for 1 hour at room temperature or at 4°C overnight. Following blocking, each membrane was washed with PBST three times, then incubated for 2 hours with primary antibody (anti H3K27me2, Millipore 07-452, 1:2000 in PBST and 2.5% BSA). The membranes were then washed with PBST three times and incubated with corresponding secondary antibody conjugated to HRP for 1 hour. The membranes were again washed in PBST three times, and the final wash was done with PBS. Proteins were detected using the chemiluminescent method with Pierce™ ECL Western Blot Substrate (ThermoFisher Scientific) according to the manufacturer's instructions and films were developed using a Konica Film Processor and band intensities were quantified using FIJI software (comparison of the intensity of the pixels) using tubulin as a loading control.

2.6.3 Antibody staining

Worms were picked into 0.2% phenoxypropanol (in M9) and the gonads were dissected out by slicing through the worm using two fresh needles. About 50 worms were dissected per sample. The buffer was washed off and replaced with PBS containing 2% formadehyde and incubated for 5 min. The gonads were then picked up with an eyelash pick and put on a polylysine coated microscope slide. The slide was dipped in liquid nitrogen and then in -20°C methanol for 1 min. Slides were then washed 3 times in PBST (0.1% Tween-20 in PBS) and then covered in blocking solution (0.1% Tween-20, 0.5% BSA in PBS) and incubated for 1 hour in a humid chamber.

Following the blocking step, antibody solution in PBS was put on the slides (MES-2, 1:200) and the slides were left to incubate overnight at room temperature in a humid chamber. The next day, the slides were washed three times in PBST and 100 µl of corresponding secondary antibody diluted in PBST (DyLight 488, anti-rabbit, 1:1000) was left on the slide for 2 hours, in a dark, humid chamber. The slides were then washed 3 times in PBST and mounted using 15 µl of Prolong Gold (Invitrogen), which helps to stop the samples from fading. The slides were then covered with a coverslip, sealed with nail varnish and analysed as described in section 2.7.

2.7 MICROSCOPY

A low power dissection microscope (either 5x or 10x objective, combined with 10x eyepiece) was used during routine worm maintenance and when assessing gross morphological phenotypes, brood sizes or for motility assays. Detailed analysis of gonad phenotypes and immunochemistry was carried out using a Zeiss AxioSKOP2 microscope fitted with differential interference contrast (DIC) and fluorescence optics. Worms were anaesthetised using 10 µl of 10 µM muscimol (Sigma-Aldrich) and then mounted on 2% agar. A Zeiss AxioCamMR digital camera and Axiovision software (release 4.5) was used for digital

photography, using a Zeiss 63x oil immersion objective for tissue and germline analysis, and a 20x Zeiss objective for whole worm microscopy.

For live fluorescence imaging and for maintaining worm stocks labelled with GFP or RFP markers, a Leica MZIII dissection microscope was used. A Hamamatsu camera and HCImage Live (v3.0) imaging software was used for image acquisition of live worms.

2.8 STATISTICAL METHODS

2.8.1 Lifespan – Log Rank Test

The online resource OASIS (http://sbi.postech.ac.kr/oasis; (Yang et al. 2011)) was used to perform statistical analysis on lifespan data obtained in this thesis. Mean lifespan with standard error and age in days at 50% and 100% population mortality are presented in the tables next to each lifespan experiment. Log Rank Test p-values were used to assess the statistical significance of the change in lifespan ($p<0.01$ was deemed significant).

2.8.2 Brood size – One-way ANOVA and Student's T-test

Brood size numbers were analysed using one-way ANOVA to test the null hypothesis that the mean brood sizes of several strains are equal. Post-hoc analysis using Student's T-test was then performed to indicate whether the differences in the brood sizes were statistically significant ($p<0.01$ was deemed significant).

2.8.3 Thrashing – ANOVA and T test

An average number of thrashes per 30 s per strain per condition was calculated from a total of 30 data points (10 worms, 3 technical repeats for each worm). Mean numbers of thrashes were analysed using one-way ANOVA to test the null hypothesis that the mean number of thrashes of various strains are equal. Post-hoc analysis using Student's T-test was then performed to indicate whether the differences in the thrashing were statistically significant ($p<0.01$ was deemed significant).

2.8.4 qRT-PCR

Relative quantification of transcript abundance was performed in at least 3 biological replicates by the ΔΔCt method. An average of the three results and the standard deviation between the replicates were used to assess statistical significance between the strains and ages in question and the wild-type strain at day 1 (using the T test, $p<0.01$ was deemed significant).

2.8.5 ChIP – qPCR

Only one ChIP-qPCR experimental analysis was performed, and the results are presented as the mean fold enrichment over the negative control (no antibody) and as the average of three technical repeats. T test analysis was used to assess statistical significance between the strains and ages in question and the wild-type strain at day 1 ($p<0.01$ was deemed significant).

CHAPTER 3: CHROMATINOME RNAi SCREEN FOR LONGEVITY PHENOTYPES

3.1 INTRODUCTION

3.1.1 RNAi screens help uncover novel factors involved in biological processes.

C. elegans has been proven to be a tremendously useful model organism to study development, ageing and many other biological processes. In fact, studying these processes in other model organisms or cell culture has been made easier thanks to discoveries made in the *C. elegans* field such as RNA interference (Fire et al. 1998) and the pioneering use of Green Fluorescent Protein (Chalfie et al. 1994). The fact that scientists responsible for both these discoveries have been awarded Nobel Prizes speaks volumes about the effect they had on the research community and the impact of basic biology research in model organisms.

RNAi provides a powerful tool to knock-down a single gene (i.e. prevent the translation of the RNA and therefore remove the protein product of the gene) and observe the effect of that manipulation on the whole organism. The ease of using RNAi in worms by feeding them bacterial clones expressing double stranded RNA corresponding to the target gene (Timmons and Fire 1998) led to the first full genomic RNAi screens in *C. elegans* (Kamath et al. 2003; Rual et al. 2004). These screens opened a new era of functional genomics and showed how powerful a tool it can be by identifying hundreds of novel gene functions.

Being able to knock-down rather than knock-out a gene, provides an especially useful method for analysing genes essential for development. Use of RNAi leads to a decrease of the transcript, but usually not to complete removal of the protein product, meaning that some basal amount of the protein might still be functional in the organism. Furthermore, RNAi can be induced at various larval or post-larval stages, allowing the control of the severity of the phenotypic outcome.

First screens for genes affecting longevity followed soon after the initial full genomic screen mentioned above (Hansen et al. 2005; Hamilton et al. 2005). Both of these attempts at discovering the key genes in ageing regulation utilised a semi high-throughput first stage, looking for an increase in maximal lifespan (i.e. assessing viability of the animals treated with RNAi at the day that all of the control animals were dead) across around 16500 gene knock-downs. Both screens were very comprehensive, however they used different approaches to introducing sterility in animals (which is necessary for high-throughput experiments, as the second and subsequent generations of worms that grow on the plate would obscure the primary worms under investigation) and also used different methods for their secondary screens. Hamilton's screen was based on using FUDR in wild-type N2 worms and introducing RNAi at the first larval (L1) stage of the animals to be screened. Then RNAi hypersensitive *C. elegans* strain: *rrf-3(pk1426)* was used for the second round of screening. The screen from the Kenyon lab (Hansen et al. 2005) used temperature sensitive feminised mutants (not producing sperm) at the initial high-throughput stage and induced gene knock-down from the embryo stage onwards (as opposed to L1 in Hamilton's work). The follow up screen was performed using simple RNAi lifespan assay in wild-type strain. These two independent screens covered about 87% of the *C. elegans* genome, revealing 89 and 29 novel lifespan regulating genes respectively. Unsurprisingly, the significant differences in methodology gave rise to two pools of genes with hardly any overlap (Ni and Lee 2010; Yanos, Bennett, and Kaeberlein 2012). This lack of similarity implies that this data set is far from saturated and there are many more genes regulating lifespan to be found.

Since then, more RNAi screens have been used to reveal more longevity genes. One interesting study (Kim and Sun 2007) looked not at longevity itself but at a phenotype connected to longevity – reactive oxygen species (ROS) resistance. It has been shown before that many long-lived mutants are also resistant to various type of stress and this correlation has been exploited in their screening. However, this study showed that most RNAi clones that

induced ROS resistance did not in fact prolong *C. elegans* lifespan. This in turn agrees with the current theory that resistance to reactive oxygen species is not sufficient in itself to increase lifespan (Zhou, Pincus, and Slack 2011).

Another, different approach was used by Samuelson et al. (2007), who performed the first genome wide suppressor screen for a well-characterised long-lived mutant *daf-2,* and identified around 200 suppressors in their primary screen. Until true high-throughput assays are dependable and easy to use (such as microfluidics (Lionaki and Tavernarakis 2013)), the laborious nature of the lifespan assay will continue being a bottleneck in longevity studies. It was therefore suggested that using smaller, focused libraries of factors, where the number of genes targeted was limited, would allow for more stringent screening, thus eliminating some of the false negatives missed in large screens.

Curran and Ruvkun (2007) used this kind of stringent approach to screening for lifespan extension by screening only genes essential for development. These would have been missed in the initial screens by Hamilton et al. (2005) and Hansen et al. (2005) as depletion of the gene products early in development would cause abnormalities and/or early death. In the Curran and Ruvkun screen, worms were subjected to RNAi post-developmentally (at the L4 stage). From around 2700 essential genes screened, the Ruvkun lab identified 64 new longevity genes. The rate of discovery is clearly much higher in this screen, which might be due to its smaller and more precise nature. Other, focussed longevity screens included screening RNAi clones related to proteasome function for enhanced resistance to polyglutamate toxicity, as resistance to this has been correlated with longevity (Mehta et al. 2009) or screening 265 kinases for *daf-18*-dependent lifespan extension (Masse et al. 2008). Finally, screening of putative histone methyltransferases and demethylases (Ni et al. 2012) suggested that enzymes that alter chromatin states might also affect longevity, adding a further layer of complexity to the already seemingly convoluted ageing network that was revealed by the early RNAi screening data.

To date there are over 300 genes annotated as having lifespan regulating effects in the worm. Many approaches have been taken to reveal the molecular details of ageing control in *C. elegans* and many of the screens are still uncovering novel factors. Perhaps we need to alter as many parameters as possible in many separate studies to fill in the blanks in the ageing regulatory network.

3.1.2 Chromatinome RNAi library and biomarkers of ageing

My interest in chromatin regulation combined with the still comparably undeveloped field of ageing initially promped the idea of a "chromatinome" screen. A chromatinome RNAi library of around 400 factors was kindly provided by Dr Gino Poulin from the University of Manchester. The library has been curated manually, using Wormbase annotation of chromatin function (www.wormbase.org), and the full list of genes in the library is attached in Appendix 1. Using an RNAi screening method allowed me to take an unbiased approach to uncover new age-related functions of chromatin regulators.

As mentioned above, there are several ways one can assess longevity, from the 'maximum lifespan' analysis to correlation of phenotype (such as stress resistance) with longevity. One yet unexplored avenue is that of using autofluorescence as a biomarker of ageing. Gut autofluorescent granules were first observed in *C. elegans* by Klass (1977), and it was noted that their properties and spectra are very similar to those of lipofuscin. Lipofuscin is a polymorphous waste material consisting of oxidised proteins and lipid degradation products (Brunk and Terman 2002; Terman and Brunk 2004), and is noted in many organisms. It was first described over 100 years ago by Hannover (1842) and its linear relationship with age was described soon after (Koneff 1886). It was named by Hueck in 1912, and the name comes from the Greek 'lipo' for fat and Latin 'fuscus' for dark. What is interesting is its near-linear relationship with age in vertebrate tissues (Strehler, Mark, and Mildvan 1959), and the fact that the rate of lipofuscin accumulation positively correlates with rate of ageing (Hosokawa et al. 1994; Munnell and Getty 1968).

Actively proliferating cells efficiently dilute lipofuscin during divisions, and stopping proliferation causes the accumulation of the pigment (Brunk et al. 1973). It has also been shown that lipofuscin continues to accumulate in postmitotic cells and it has been shown to be undegradable "due to presence of peptides cross-linked by aldehydes into plastic-like structures" (Kikugawa et al. 1989). This accumulation can lead to a variety of defects in cell function and homeostasis due to the progressive loss of lysosomal function, which can in turn have secondary effects on cell function. This hypothesis aligns well with the evolutionary theory of ageing (Kirkwood 1977) as accumulation of the pigment in somatic cells (post-mitotic) is irrelevant in respect to the selective pressures that drive evolution while the germ line is protected due to the dilution effect (Gray and Woulfe 2005). In nematodes, autofluorescence and lipofuscin have also been correlated, and it has been shown that the gut autofluorescent granules contain acid phosphatase which is a lysosomal enzyme in nematodes. These granules also accumulate exogenous materials taken into the cells by endocytosis and they contain a lysosomal chemical signature (Clokey and Jacobson 1986).

In this chapter I am going to present the development of primary and secondary RNAi chromatinome screen for longevity, based on accumulation of lipofuscin as a biomarker of ageing.

3.2 RESULTS

3.2.1 Optimisation of the RNAi screen conditions

As a screen based on lipofuscin accumulation has not been attempted previously in *C. elegans*, I decided to use this approach to investigate the effects of knock-downs of chromatin-regulating factors on longevity. Firstly, I decided to validate the approach of using lipofuscin as a biomarker and ascertain the best length and endpoint in designing my screen by analysing short- and long-lived mutants described in the literature. Some of the results of the pre-screening are shown in Figure 3.1. Assessing lipofuscin at day 3 and day 6 revealed an increased accumulation before the plateau is reached later in life (Cantwell, Lees, Woollard, personal communication). I chose to analyse a short-lived mutant, *wrn-1(tm764)* (Hyun, Bohr, and Ahn 2008), and a long-lived mutant, *age-1(hx546)* (Friedman and Johnson 1988), to study the changes in accumulation of lipofuscin with age. Both mutant strains showed changes in lipofuscin matching their phenotypes: *wrn-1* had an increased amount of lipofuscin as compared to the wild-type worm at both day 3 and day 6, while *age-1* worms displayed decreased lipofuscin accumulation at the same age points (Figure 3.1). These data suggests that the lipofuscin accumulation does indeed correlate with ageing and longevity in *C. elegans* and therefore might be used as a biomarker for the screen.

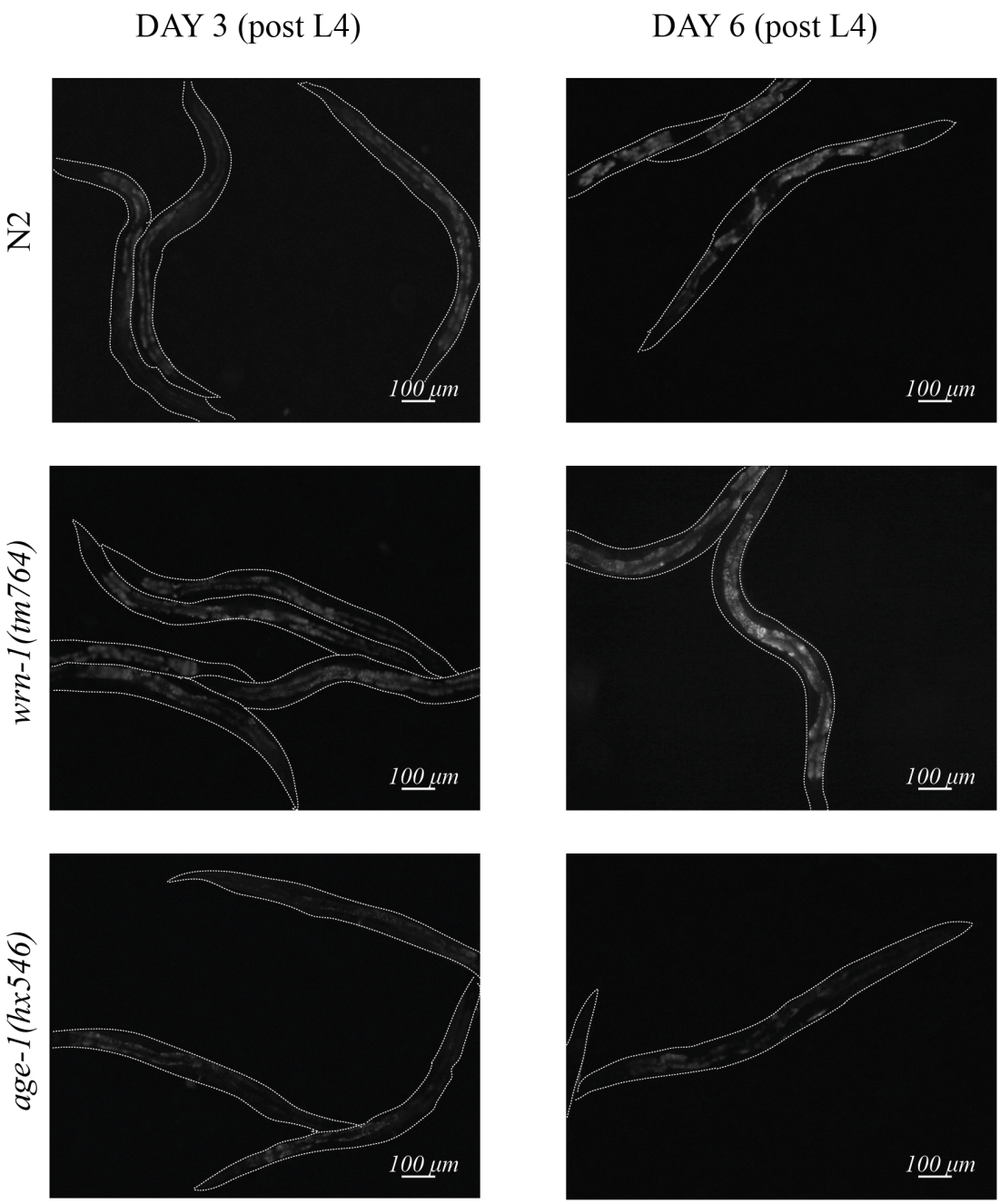


Figure 3.1 Pre-screen of the effect of ageing on the accumulation of the green autofluorescent pigment in *C. elegans* tissue in wild-type worms, short lived *wrn-1(tm764)* mutants and long-lived *age-1(hx546)* mutants. Representative images of differences in degree of autofluorescence accumulation between short and long-lived mutants were scored qualitatively by eye (15 worms imaged per day and genotype, in two biological replicates). White dashed lines are the approximate boundaries of the worm bodies. Image capture: Zeiss AxioSKOP2 microscope with Zeiss AxioCamMR digital camera, FITC filter, 20x Zeiss objective, exposure of 150 ms.

Several further pilot experiments were designed to assess the changes in various parameters. The first factor was the age at which worms were to be exposed to RNAi. The decision to expose the parental generation of worms to RNAi bacteria clones by feeding them from the L4 stage onwards meant that the screened generation (F1) was exposed to only the RNAi-containing bacteria (as a source of food) for their whole life; from gametes through to adulthood. It also limited the likelihood of the phenotypic rescue by maternal stores of the protein as these would be removed by RNAi from the mother too.

The second factor investigated was the necessity of using 5'-fluorodeoxyuridine (FUDR) to prevent the development of second generation of worms. FUDR prevents worm eggs from hatching, however its potential effects on worm lifespan are still controversial (Hosono et al. 1982). It has been argued that there is no effect on wild-type lifespan but that FUDR can cause some changes in, for example, the mitochondrial mutant *gas-1* (Van Raamsdonk and Hekimi 2011). Plates with varying concentrations of FUDR were tested to determine the minimal concentration that stops the eggs from hatching (data not shown). The minimal final concentration of FUDR ensuring lack of progeny was established at 1 mg/ml (final concentration) mixed with OP50 or HT115 bacteria. Finally, the effect of FUDR presence on lipofuscin accumulation was investigated. Wild-type worms were transferred to plates with either pure OP50 or OP50 with FUDR. Figure 3.2 shows that the lipofuscin accumulation at days 3 and 6 does not show changes dependent on the presence or absence of FUDR and the accumulation of lipofuscin with age (day 3 compared to day 6) is still clearly visible.

Taken together, the results of the pilot experiments allowed me to develop appropriate parameters for the final design of the RNAi screen.

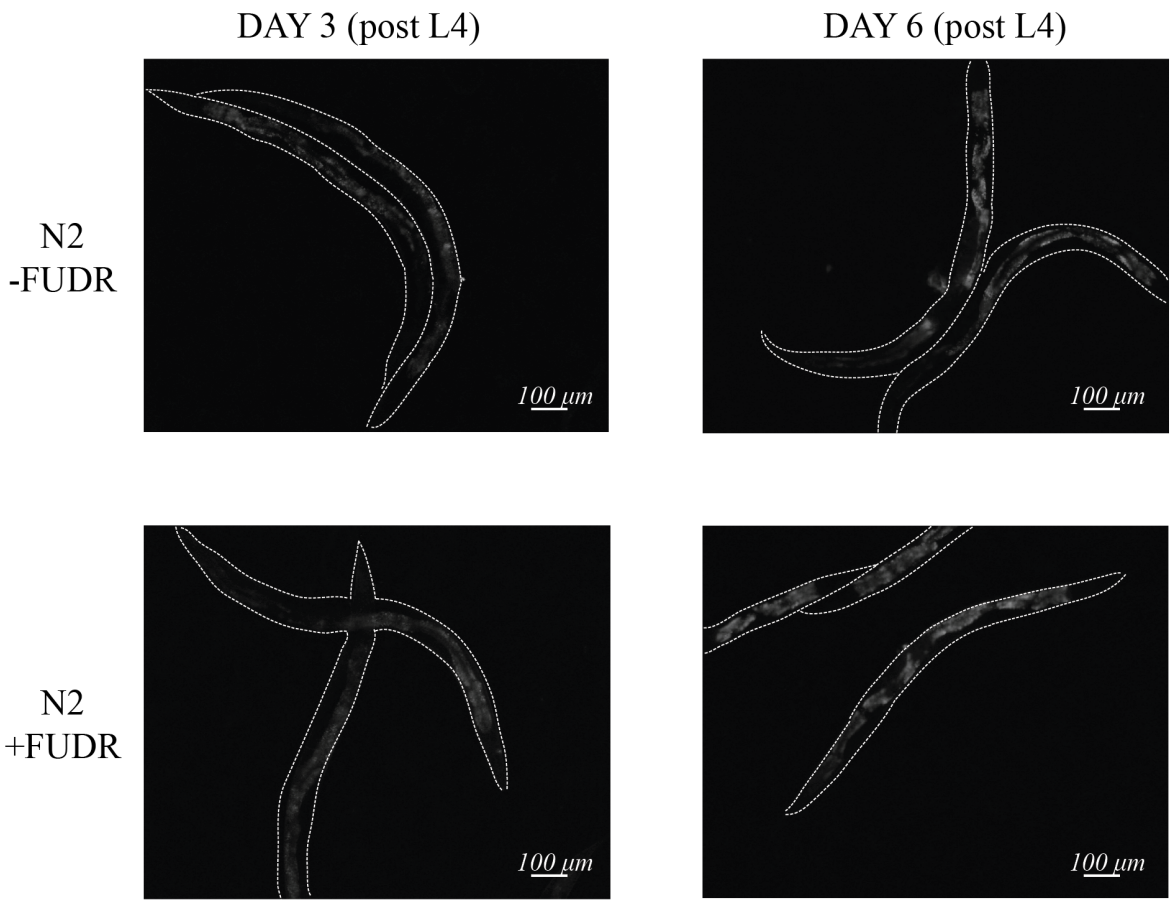


Figure 3.2 Addition of 5-fluorodeoxyuridine (FUDR) to OP50 that prevents egg hatching does not affect lipofuscin accumulation in wild-type worms. Representative images of the degree of autofluorescence accumulation was scored by eye (minimum 15 worms imaged per day and condition, in two biological replicates). White dashed lines are the approximate boundaries of the worm bodies. Image capture: Zeiss AxioSKOP2 microscope with Zeiss AxioCamMR digital camera, FITC filter, 20x Zeiss objective, exposure of 150 ms.

3.2.2 RNAi screen design and results

Figure 3.3 shows the final design of the screen after the data from the pilot screens were taken into consideration. The parental generation of worms (F0, 3 worms per plate, L4 stage) were picked onto plates containing bacteria expressing dsRNA pre-grown on IPTG-containing plates for 24 hours. After 4 days their progeny at the L4 stage were subsequently transferred to new RNAi plates containing FUDR (15 worms/ plate; 2 plates per RNAi). This marked day 0 of the experiment. At day 3, 15 worms were assessed for lipofuscin accumulation, with another 15 being assessed at day 6.

The accumulation of lipofuscin was assessed by eye on a 5 level scale (‘- -’ lipofuscin substantially decreased, ‘-’ small decrease, ‘0’ no change, ‘+’ small increase, ‘+ +’ substantial increase in comparison to empty vector control levels). Figure 3.3 shows examples of the increased and decreased lipofuscin accumulation on both day 3 and 6 compared to control levels. In the case where sterility resulted from the RNAi treatment, the effect of RNAi on lipofuscin accumulation was observed post-embryonically. I.e. the parental generation (F0) was fed ‘empty vector’ control bacteria, and their progeny at L4 stage were transferred to the plates containing bacteria expressing dsRNA of choice supplemented with FUDR (1 mg/ml).

Genes that when knocked-down led to decreased lipofuscin accumulation are the focus of this screen. This assessment is less likely to create false positive results than the reverse. For example, an increase in lipofuscin might be connected to general ‘sickness’ rather than accelerated ageing itself. Furthermore, a decrease in lipofuscin was also easier to identify during the screening process. Table 3.1 shows a list of RNAi clones that were identified as causing a substantial decrease in autofluorescence on both days of screening. These are referred to as ‘confident hits’. Other results from the screen, include the ‘putative hits’ i.e. genes that showed a substantial decrease on one of the screening days (‘- -’ on the scale), or small decreases on both days (‘-’ on the scale), as well the reverse hits i.e. knock-downs

resulting in an increase in lipofuscin accumulation are presented in Appendix 3 and 4 respectively.

One of the confident hits is *F21H12.1*, a homologue of human *RbBP5* known to associate with the COMPASS complex. It was identified in a previous longevity RNAi screen in *C. elegans* (Hamilton et al. 2005). Depletion of members of the COMPASS homologue in *C. elegans* has lifespan-increasing effects as mentioned previously (Greer et al. 2010). Other genes that when knocked-down have lifespan-prolonging effects previously described in the literature are *T26A5.8* (Hamilton et al. 2005) and *mes-2* (Ni et al. 2012). These results confirm the validity of the decrease in lipofuscin accumulation as a proxy for lifespan extension.

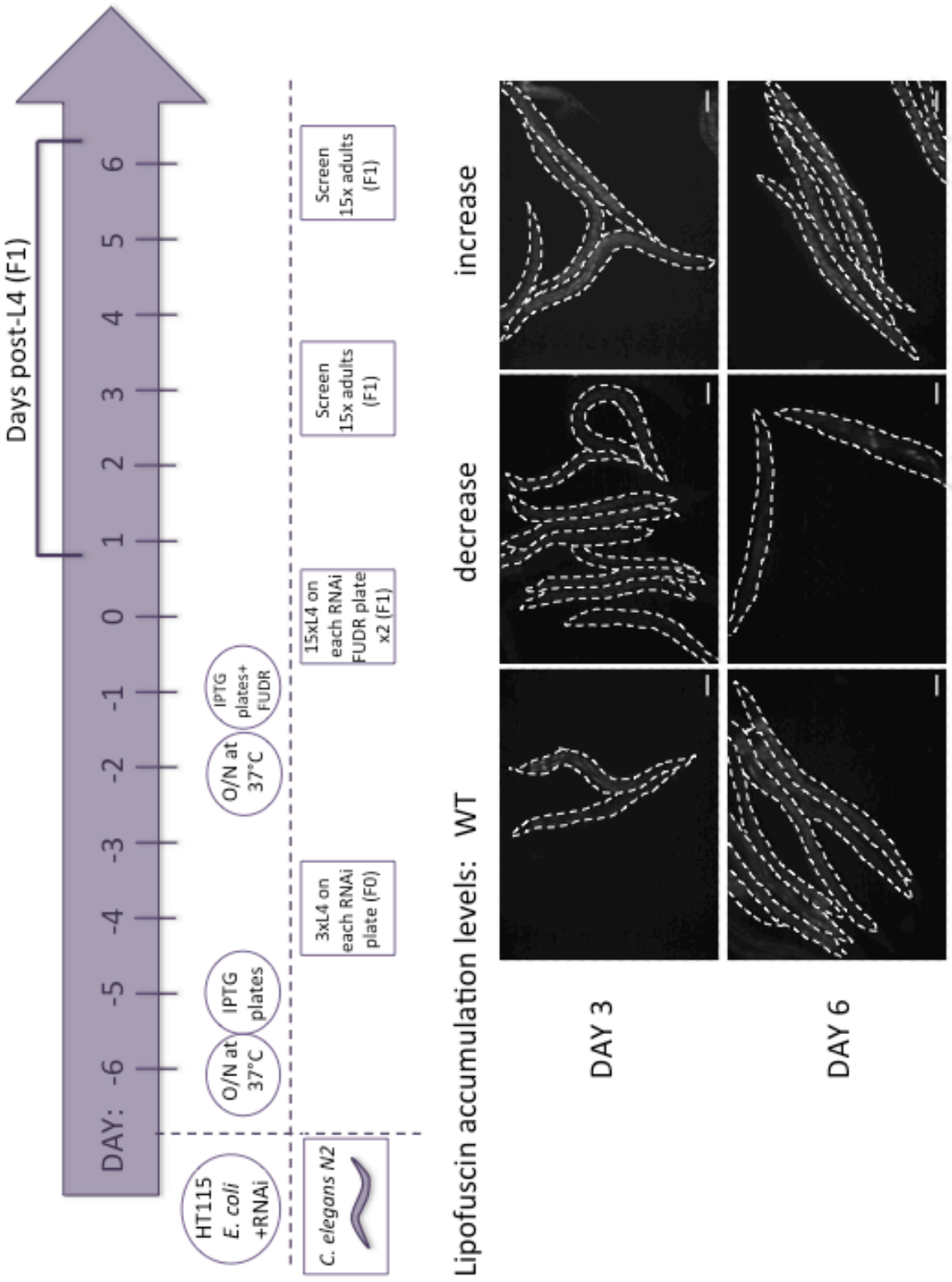


Figure 3.3 RNAi screen for longevity set up and examples of changes in lipofuscin accumulation on chosen days. Panel on the left shows the plan for RNAi screen including worm preparation and screening days. On the right, I show examples of changes in lipofuscin accumulation on both days of screening. Decrease in accumulation equivalent to '- -' score, and increase in accumulation equivalent to '+ +' score. Scale bars shown represents 100 µm. White dashed lines are the approximate boundaries of the worm bodies.

Table 3.1 List of genes which, when knocked-down by RNAi, caused a substantial lipofuscin decrease at both days of the screening i.e. 'confident hits'.

gene name	known function	protein domains	known homologues
mes-2	Upstream of hox genes (*lin-39, mab-5, egl-5, lin-32*). Required for H3K27me2 and H3K27me3. Polycomb- like repressive complex.	SET domain	*Ezh2* in humans *E(z)* in flies
prp-4	Yeast PRP (splicing factor) related	WD-40 repeat, PRP-4-like, splicing factor motif	*prpf4* in humans *prp4* in yeast
lin-53	Class B synMuv, part of DRM and NuRD-like complex	WD-40 repeat, histone-binding protein RBBP4	*RbAp48* in mammals
Y71F9AL.17	Alpha subunit of the coatomer complex	WD-40 repeat, WD-associated region	*alphaCop* in flies
ubl-1	Similar to Drosophila ubiquitin/ribosomal protein S27a	Zinc finger	*RpS27A* in flies
isw-1	Part of NURF-like complex	AT-hook, HAND domain, SLIDE domain, SANT domain, DEAD/H box, ATPase	*Smarca1* in humans *Iswi* in flies *isw2* in yeast
F59E12.1	*uncharacterised*	bromodomain	*muc1* in yeast *Brd8* in humans
F21H12.1	Member of Set1/MLL methyltransferase complex (H3K4me2/me3)	WD40 repeat,	*RbBP5* in mammals Awd1 in yeast
ubc-1	E2 ubq-conjugating enzyme		*rad6/ubc2* in yeast
pis-1	Localised to actively expressed chromatin	Q & N rich N-term, BRCT C-term	*Ptip* in mammals
cbp-1	histone acetyl transferase at H3K27	HAT domain	p300/Cbp in mammals
gad-1	Required maternally for gastrulation	WD- repeat	*lst8* in yeast
mys-1	class C synMuv, putative histone acetyltransferase		*Tip60* in humans *EG0007.7* in flies *Esa1* in yeast
R07B5.8 (***lsy-12***)	histone acetyltransferase, maternally required for deceopmental differentiation of ASEL neurons		*Kat6b* in mammals *enok* in flies *Esa1* in yeast
F23D12.5 (***jmjd-3.2)***	putative H3K27me2 demethylase; expected to antagonise transcriptional repression by Polycomb repressor complex which mark stem cells	JmjC	*jmjd3* and *utx* in mammals
W06D11.4 (***dot-1.3***)	predicted H3K79 methyltransferase		*dot1l* in humans *dot1* in yeast

3.2.3 Secondary RNAi screen

16 primary hits from the RNAi screen (Table 3.1) required confirmation to assess the validity of the lipofuscin-based approach to screening. To do this, I decided to use a secondary RNAi screen where worms were treated in the same way as during the initial screen, however, instead of assessing lipofuscin, I performed a full lifespan assessment (in duplicate), as described in section 2.3.4. The representative lifespan curves are presented in Figure 1.4. Due to sterility effects of *cbp-1*, lifespans of animals with this knock-down was assessed post-developmentally (worms exposed to RNAi bacteria from stage L4 onwards). Out of 16 gene knock-downs tested, 7 caused lifespan increase and 4 of them were statistically significant according to a Log Rank Test: *mes-2*, *isw-1*, *cbp-1* and *jmjd-3.2* (see Table 3.2 for statistics).

In the next chapter I will discuss each one of these genes in detail and try to elucidate the mechanism through which they extend lifespan.

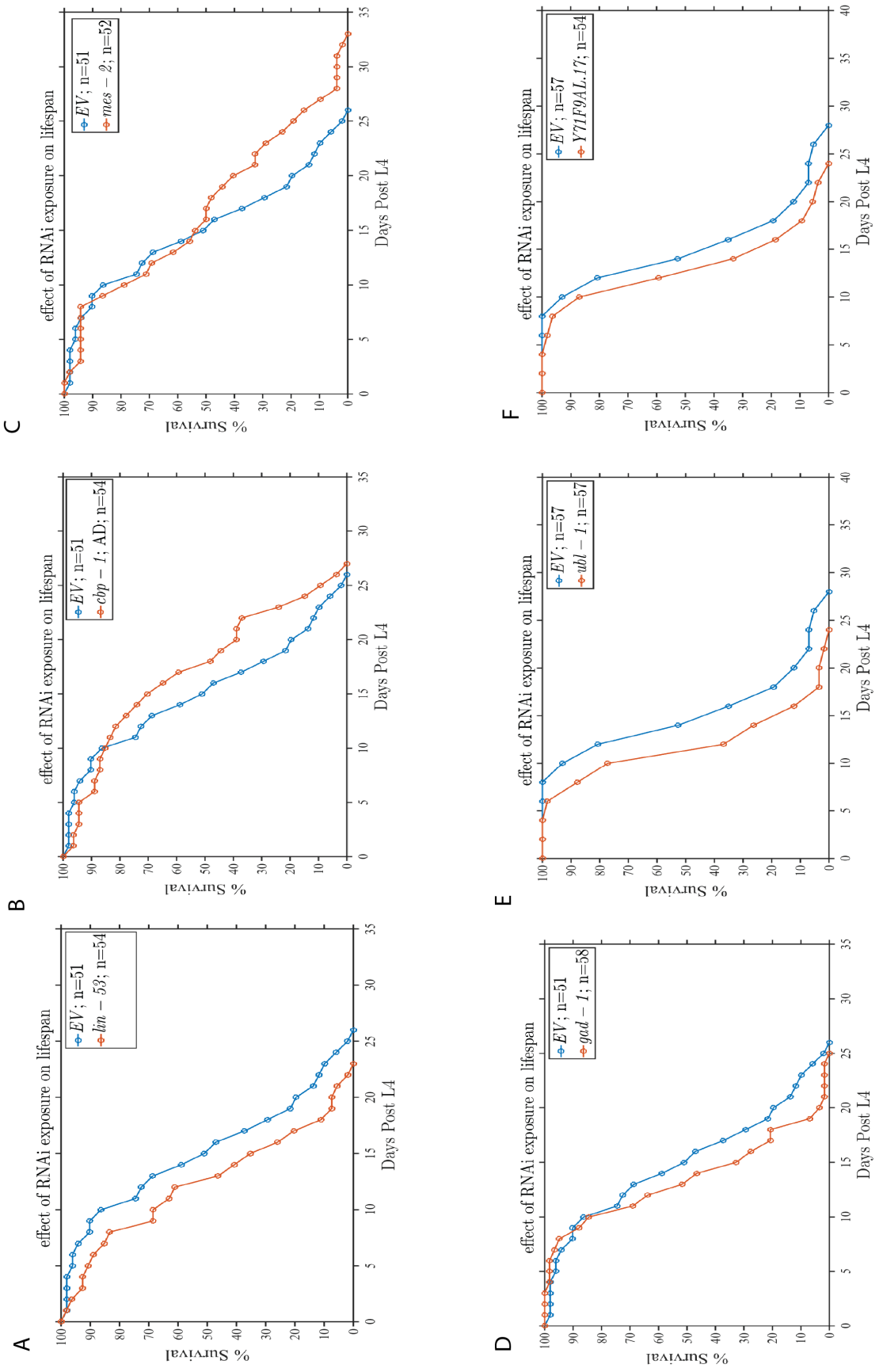


Figure 3.4 (part 1/3) Secondary RNAi screen of 'confident hits' that decreased lipofuscin accumulation on both days of primary screen (A-F)

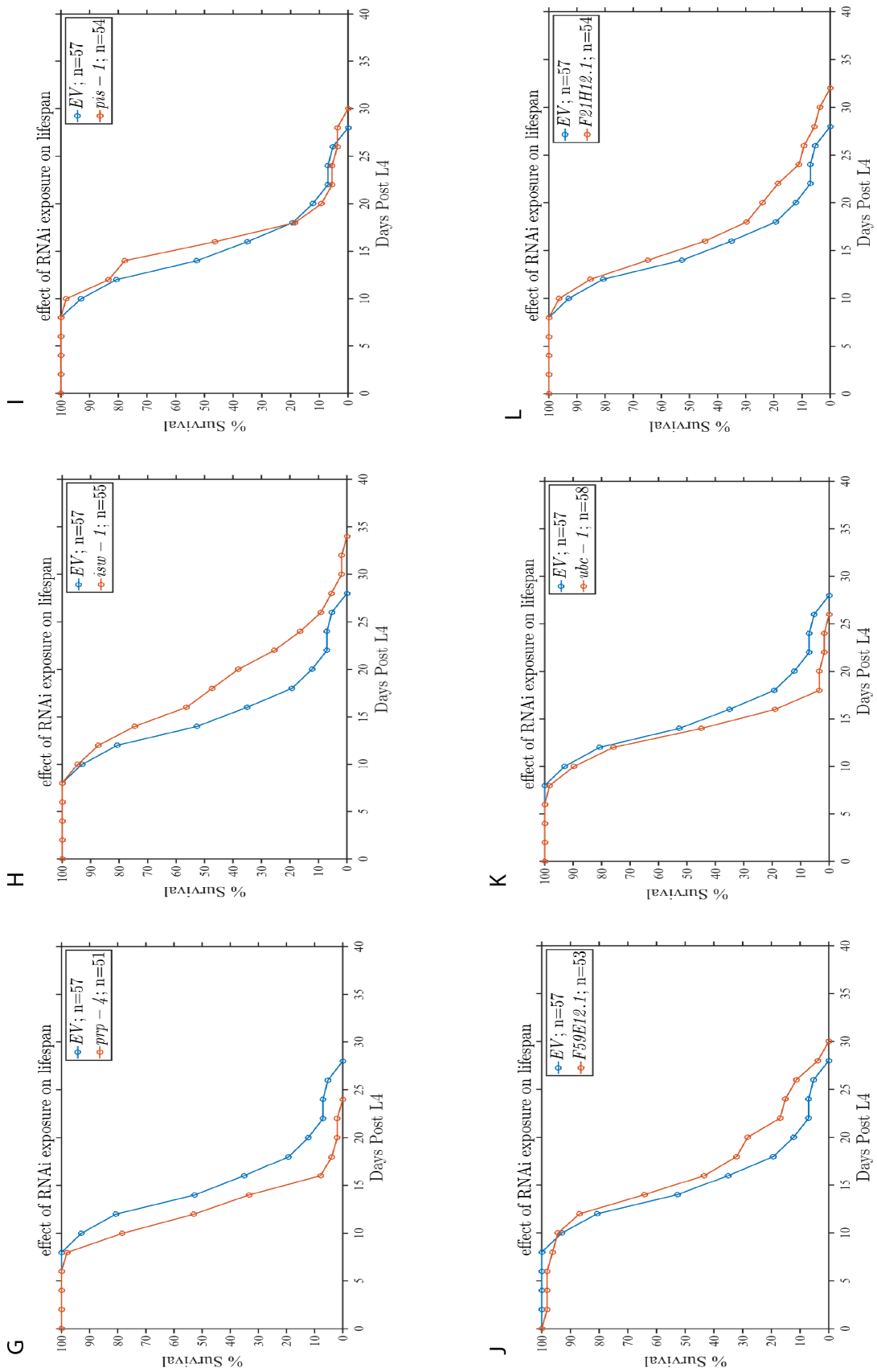


Figure 3.4 (part 2/3) Secondary RNAi screen of 'confident hits' that decreased lipofuscin accumulation on both days of primary screen (G-L)

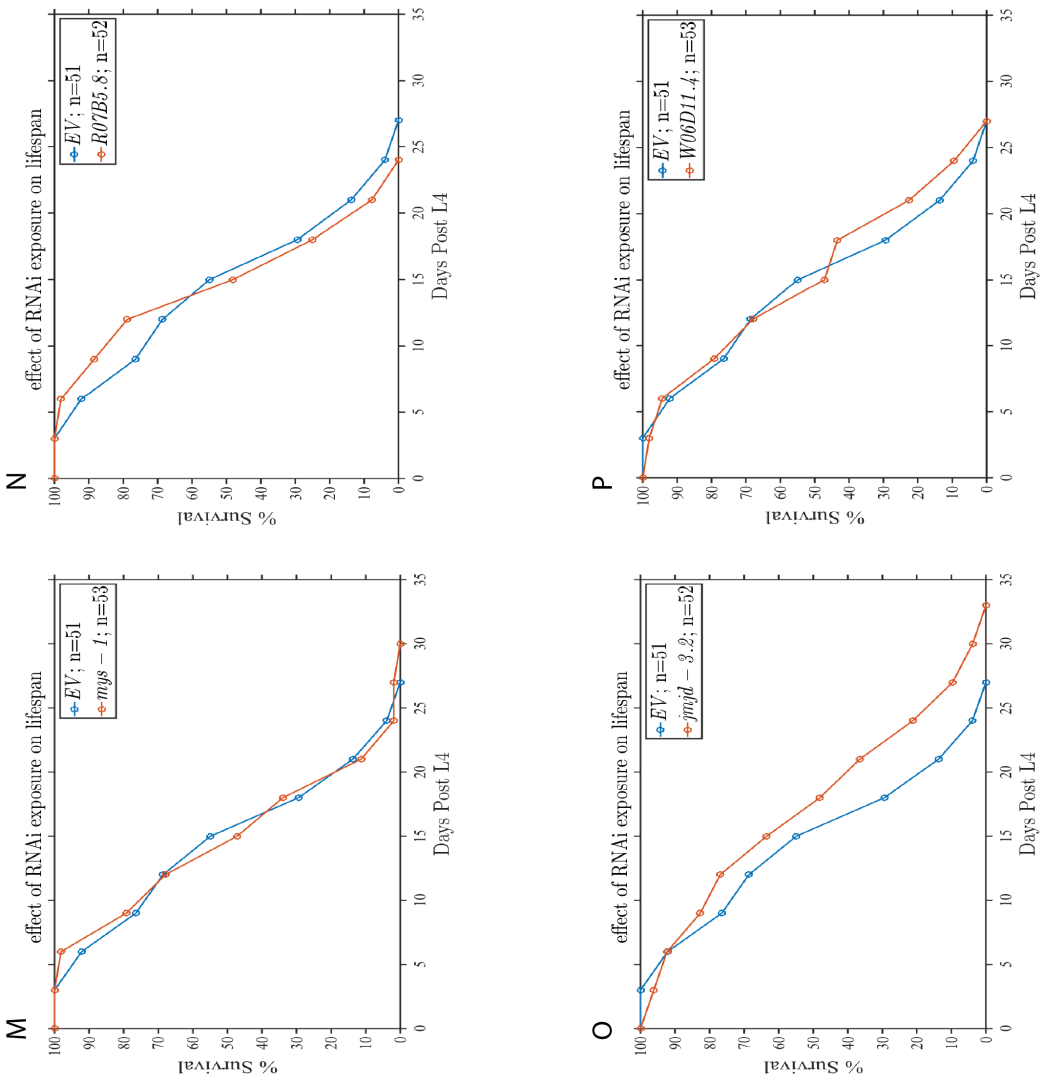


Figure 3.4 (part 3/3) Secondary RNAi screen of 'confident hits' that decreased lipofuscin accumulation on both days of primary screen (M-P). (A-P) show lifespan curves of 16 factors as compared to a negative RNAi control, empty vector (EV). RNAi experiments were performed as outlined in the methods, apart from *cbp-1* RNAi, which was assessed post-embryonically due to sterility effect. Log-Rank Test p values can be found in the Table 3.2. 4 knocked-down genes caused statistically significant lifespan extension: *isw-1, cbp-1, mes-2, jmjd-3.2*.

Table 3.2 Log-Rank statistics and overview of the experiment shown in Figure 3.4. Lifespan change: '+' depicts lifespan extension, '-' lifespan shortening and '0' no apparent change from empty vector control.

Figure 3.4 reference	RNAi #1	RNAi #2	lifespan change vs EV	Log- Rank Test p value	statistical significance (*p<0.05; ***p<0.01)
A	EV	*lin-53*	-	0.0023	***
B	EV	*cbp-1 (post larval)*	+	0.0073	***
C	EV	*mes-2*	+	0.0384	*
D	EV	*gad-1*	-	0.0122	*
E	EV	*ubl-1*	-	4*10^-5	***
F	EV	*Y71F9AL.17*	-	0.0092	***
G	EV	*prp-4*	-	0.0003	***
H	EV	*isw-1*	+	0.003	***
I	EV	*pis-1*	0	0.2559	
J	EV	*F59E12.1*	+	0.0649	
K	EV	*ubc-1*	-	0.0296	*
L	EV	*F21H12.1*	+	0.0803	
M	EV	*mys-1*	0	0.9882	
N	EV	*R07B5.8*	0	0.5311	
O	EV	*jmjd-3.2*	+	0.0067	***
P	EV	*W06D11.4*	+	0.2998	

3.3 DISCUSSION

3.3.1 RNAi screen

I have shown that by using lipofuscin as a biomarker of ageing I could distinguish several genes from the chromatinome library that had a decreased accumulation of autofluorescence on both day 3 and day 6 of adulthood. This decrease was correlated with statistically significant increase in lifespan in the case of 4 RNAi clones at 20°C.

To compare the effectiveness of my RNAi screen to other peer-reviewed screens for longevity, I calculated the enrichment in positive hits at primary (lipofuscin assessment) and secondary (lifespan assessment) screen stage as well as final identification rate (Table 3.3). The comparison shows that my screen efficiency lies somewhere in between the original whole-genome longevity screens (Hamilton et al. 2005; Hansen et al. 2005) and the smaller RNAi screen performed with essential genes (Curran and Ruvkun 2007). What is noticeable is the significant difference in the primary screen enrichment in Curran and Ruvkun (2007) screen in contrast to the other three experiments. The reason for this discrepancy is the low

stringency of the initial screen, leading the group to perform not just a secondary but also a tertiary analysis. If, in my calculations, I include not only my 'confident hits' list but also the further 28 putative hits, then the primary enrichment of my screen is comparable to that of Curran and Ruvkun (2007) – see 'primary screen enrichment' for low stringency.

Table 3.3 Comparison of the previous RNAi screens for longevity and this work with regards to enrichment in positive hits and final identification rate

RNAi screen	Primary screen enrichment	Secondary screen enrichment	Identification rate
Hamilton et al. (2005)	600/16475=3.6%	89/600=15%	89/16475=0.5%
Hansen et al. (2005)	94/16757=5.6%	29/94=31%	29/16757=0.1%
Curran and Ruvkun (2007)	470/2698=17.4%	Secondary: 134/470=29% Tertiary: 64/134=48%	64/2698=2.4%
this thesis	16/330=4.8% low stringency: (16+28)/330=13.3%	4/16=25%	4/330=1.2%

Also noteworthy is the fact that both my screen and the screen from Curran and Ruvkun (2007), both of which had limited pool of primary targets, were overall 2.5-20 times more effective in identifying longevity target genes (based on comparison of the identification rates). That could mean that either the small screens are better suited to finding hits, as the initial approach can use lower stringency, and so ensure fewer false negatives, or that both of the gene groups investigated in those screens are simply highly enriched for genes affecting longevity. Both of these could be true, especially considering that the screens looked at crucial cellular regulators in both cases (genes essential for regulating development (Curran and Ruvkun, 2007) and chromatin modifiers (this thesis)).

The use of lipofuscin as a biomarker has been validated previously in my pre-screening approaches using short- and long- lived mutants. As mentioned before (section 3.1.2) lipofuscin has been described in various organisms and its correlation with ageing is well proven. There is still a little controversy in using it as a biomarker of ageing, as a different

form of autofluorescence was recently identified which occurs during the death of *C. elegans* (Coburn and Gems 2013). The fluorescence spectra signature of the two observations are found at different wavelengths (green vs blue), and have a different dynamic at different times in the worm's life (accumulation with age vs sudden wave at the time of death). Therefore, it seems that this secondary fluorescence is the sign of unrelated phenomena. It should not therefore be seen as an argument against studying the accumulation of green autofluorescence with age, although precautions should obviously be taken to differentiate the two spectra when reporting on age-related autofluorescence.

Measuring lipofuscin in a more quantitative way than the 5 point qualitative scale used in this thesis has proven difficult. Rotation students in the Woollard and Cox labs have unsuccessfully attempted to measure autofluorescence and correlate it with ageing (Cox and Woollard, personal communication). The problem was usually a high variability between biological replicates. Due to high usage of the fluorescent bulbs on the Zeiss Microscope, even if the same exposure was used for different experiments, inherent variety due to bulb efficiency was introduced. This problem was overcome in this screening as samples screened at different days were not compared to each other but rather, every screening day, worms at the same age were only compared to the control (EV) sample. Finally, an additional control was introduced by introducing a pre-established set of five RNAi knock-downs at random points during the blind screen to ensure consistency in the screening approach.

Using the RNAi as a genetic screen approach has many advantages: genes are immediately identified, libraries are widely available, knock-downs are easy to introduce in worms and large populations can be evaluated for changes. However, there are also some obvious points that have to be addressed. Firstly, a classical forward genetic screen has some advantages over RNAi as random mutations introduced can cause various disruptions to the gene causing misregulation, functional point mutations or gain-of-function mutations. Using RNAi allows us to only look at the effects of the loss of the transcript and therefore a decreased amount of

functional protein, so some genes that might still affect the phenotype in question will therefore not be picked up by RNAi screening. Further, some RNAi clones can have weak effects in *C. elegans*, not functionally affecting protein levels and therefore creating false negatives. An interesting example of such a problem is the knock-down of neuronal genes, which, using standard RNAi protocols, has proven difficult (Kamath et al. 2003; Timmons, Court, and Fire 2001). One response to this problem has been to develop *C. elegans* strains with increased neuronal sensitivity to RNAi (Calixto et al. 2010).

The choice and design of an RNAi screen as a way of discovering novel functions of the genes will always come at the cost of finding some false positive and losing some false negative genes. In the case of my screen, the false positives could have come from genes affecting protein turnover, as a decrease in lipofuscin could be connected to, for example, increased cellular autophagy. Conversely, false negative results could arise if gene knock-downs extend lifespan without having any effect on the accumulation of autofluorescence. An improvement on my screen, in order to decrease the number of potential false negatives, could have been the use of either an RNAi hypersensitive strain such as *rrf-3* (Simmer et al. 2003) or neuronally sensitive RNAi strain (Calixto et al. 2010) to ensure responsiveness to the dsRNA.

3.3.2 'Confident hits'

Interestingly, the 'confident hits' list contains several genes that code for proteins that have been shown in the literature to be interacting. For example, *lin-53* is a synMuv B gene which is a member of one of the three groups of proteins (synMuv A, B and C) that negatively regulate vulval cell fate (Ferguson and Horvitz 1989). Both *isw-1* and *mes-2* have been categorised as synMuv B suppressors (Andersen, Lu, and Horvitz 2006; Cui, Kim, and Han 2006). Further, homologues of LIN-53 were shown to associate with ISW-1 homologues in flies and human cells with use of co-purification assays (Blagoev et al. 2003; Poot et al. 2000).

Additionally, both *mes-2* and *isw-1* were identified as High Temperature Arrest (HTA) suppressors genes. HTA is induced at the L1 larval stage of the worm in synMuv B mutants grown at high temperatures (25°C) (Petrella et al. 2011). Expression of germline genes in somatic cells is enhanced at elevated temperature, causing developmental arrest. This suggests that the synMuv B mutants fail to erase the germline chromatin state in somatic cells during development, which results in a mixed cell fate. The fact that the intestine is involved in this phenomenon is noteworthy, as this tissue is involved in lifespan control as well, for example through dietary restriction pathways that can cause lifespan and health-span extension in many model organisms (Kenyon 2005). For example, expression of *daf-16* only in the intestine of the worms deficient in both *daf-2* and *daf-16* (short-lived) animals is able to reconstitute most of the *daf-2* lifespan extension (Libina, Berman, and Kenyon 2003).

Finally, I used an online tool STRING (http://string-db.org/) to visualise known and predicted protein-protein interactions between the 16 confident hits (Figure 3.5). Interestingly, many of the proteins identified from the primary screen associate together, either by being in proximity in physical protein complexes or by co-expression experiments. This perhaps, should not be that surprising, as the proteins are functionally connected by the fact that they are all chromatin modifiers and hence, often need to work together to ensure adequate gene regulation in cells.

Overall in this chapter, I have shown that lipofuscin-accumulation based RNAi screening is a valid approach to identify genes that regulate longevity in *C. elegans*. Following from that, in the next chapter I will investigate whether the mutants of the four genes identified in this screen also affect lifespan regulation.

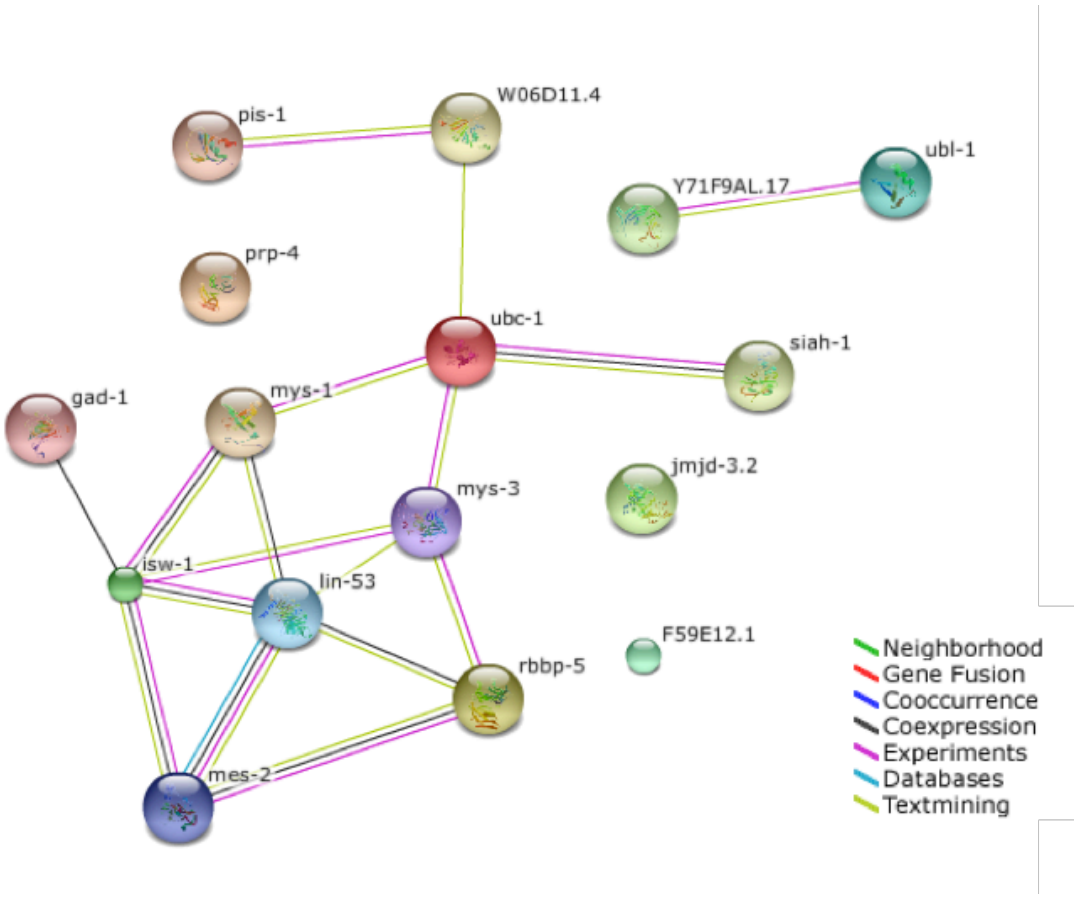


Figure 3.5 Network of known and predicted protein-protein interaction between the 'confident hits' gene list, compiled using an online tool STRING. The colours of the connections describe the type of evidence for association.

CHAPTER 4: LIFESPAN EXTENSION IN 'CONFIDENT HITS' FROM RNAI SCREEN IS VALIDATED BY MUTATIONS IN THE GENES

4.1 INTRODUCTION

In the RNAi screen described in Chapter 3, I uncovered four genes, whose effect on longevity has not been studied in detail by other groups: *isw-1, cbp-1, mes-2* and *jmjd-3.2*. Interestingly, two of those genes, *mes-2* and *jmjd-3.2,* have predicted opposite enzymatic functions at the same histone lysine residue. MES-2 is the enzymatic part of the worm's PRC-2 complex and is a methyltransferase acting on H3K27, whereas JMJD-3.2 is one of the enzymes in the family of demethylases acting at the same lysine.

In this chapter I will investigate whether mutations in the genes pulled out of RNAi screen affect lifespan and concentrate on the interesting point of the contrasting enzymatic activities of the H3K27 methylation modifiers.

4.1.1 Use of mutants for the final validation of the RNAi screen

Following the confirmation that knock-down of any of the four genes from the screened library consistently lengthens lifespan in *C. elegans* WT strain, the next step was to investigate mutations in those genes, to ascertain, whether the mutant phenotypes correlate with the RNAi response. Mutations in genes may have different effects to those of RNAi knock-down for several reasons. Firstly, nucleotide changes can cause both gain-of-function and loss-of-function phenotypes. Secondly, reduced levels of protein as a result of RNAi treatment do not necessarily have the same effect as presence of substitutions or truncations to a protein, which could still participate in complexes, or have dominant negative effects. Finally, levels of RNAi-induced knock-down is not quantitative, and can be inconsistent between experiments, different degrees of depletion could be achieved.

Below I introduce the known functions of the genes identified in my RNAi screen to have effect on *C. elegans* lifespan.

4.1.2 ISW-1

ISW-1 is a catalytic subunit of several ATP-dependent nucleosome remodelling enzymes. Its homologues in other organisms have several roles in transcription, chromosome organisation and DNA replication and hence are essential proteins for cell viability (Dirscherl and Krebs 2004). Inactivation of the *Iswi* gene causes global transcription defects and leads to significant changes in chromatin structure in *Drosophila* including chromosome decondensation (Corona et al. 2007), while in humans loss of its homologue is implicated in several melanocytic tumours and a multi-systemic disease Williams-Beuren syndrome (Mellor and Morillon 2004). Despite its varied roles, *S. pombe* does not have an *Iswi* gene, instead it uses a CHD1 protein as a chromatin remodelling enzyme. ISWI has been shown to interact with a broad range of factors (see Figure 4.1 from Längst and Becker (2001)); given its likely participation in a number of different pathways it has proven a difficult protein to study.

In *C. elegans isw-1* was identified in a screen for synthetic Multivulvae (synMuv) gene suppressors (Andersen, Lu, and Horvitz 2006). The *isw-1* was shown to supresses all known synMuv B genes phenotypes including High Temperature Arrest (HTA), soma-to-germline transformation and RNAi responses (Andersen, Lu, and Horvitz 2006; Curran and Ruvkun 2007; Petrella et al. 2011; Cui, Kim, and Han 2006; Wang et al. 2005)

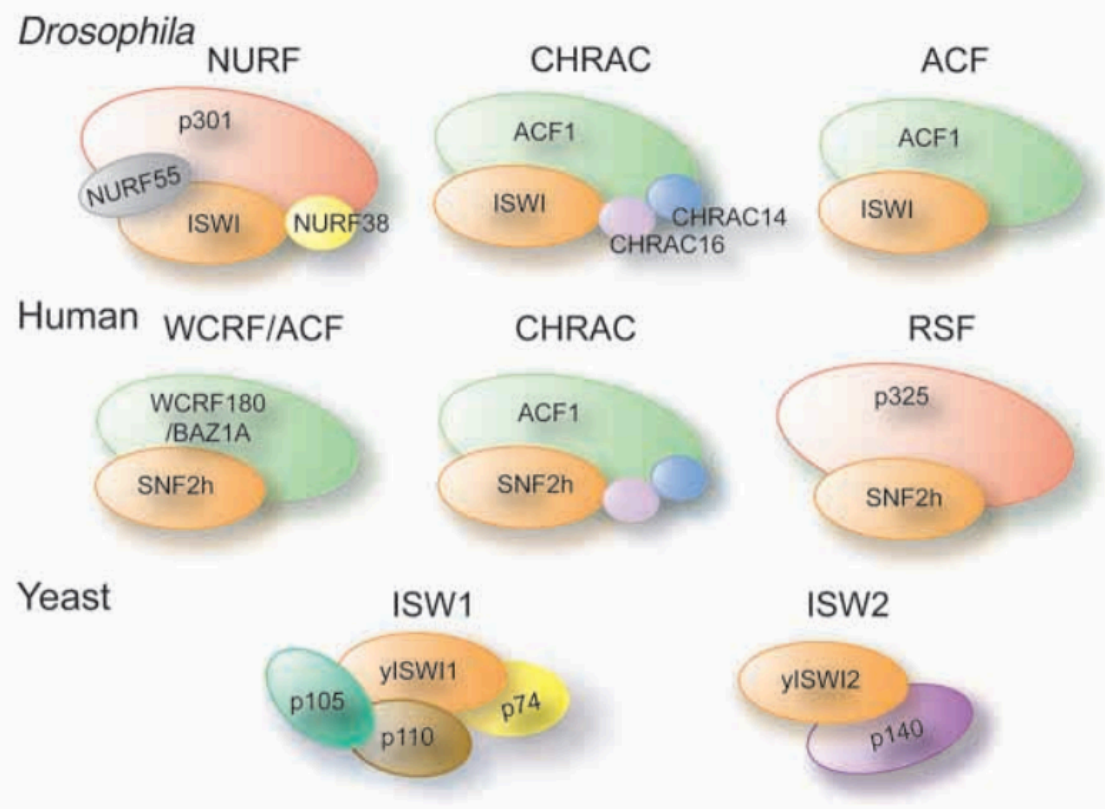


Figure 4.1 Variety of complexes that ISWI and its homologues are known to participate in, from Längst and Becker (2001)

4.1.3 CBP-1

Worm CBP-1 is a protein with high sequence homology to mammalian p300 and CBP transcription cofactors known to be involved in integration of transcriptional and cell signalling events controlling cell growth and differentiation. p300/CBP was shown to have a lysine acetyl transferase (KAT) activity (Bannister and Kouzarides 1996; Ogryzko et al. 1996) and also to associate with other KATs (Yang et al. 1996; Chen et al. 1997). The p300/CBP family of factors are very versatile transcription regulators and can act either as scaffolds or adaptor proteins on the promoters or through their KAT activity on histones and other transcription factors.

4.1.4 H3K27 methylatransferase complex and its regulation of gene expression

H3K27 trimethylation is a histone modification associated with repressed chromatin and is known to be positively regulated by Polycomb complex proteins. The core of the complex is conserved from Drosophila to vertebrates and consists of EZH1/2, SUZ12, EED and RbAp46/48 (Margueron and Reinberg 2011). The part of the complex that actually contains the KMT enzymatic activity is EZH1 or EZH2. Either of these two proteins can be found in mammalian PRC2 complexes. This choice is likely to provide specific function to PRC2 as EZH1 and EZH2 vary in expression patterns, and they have been shown to modulate the efficiency of PRC2 (Margueron et al. 2008). Polycomb proteins have been shown to have a fundamental role during development, which is emphasized by the fact that deletions of some of the members of the complex in mice result in early embryonic lethality (*Eed*, *Ezh2*, *Suz12* and *Ring1B*) (Margueron and Reinberg 2011).

The complete mechanism through which Polycomb-mediated repression is achieved is not fully understood, however, an interesting study by Kanhere et al. (2010) suggested that some of the PRC2 control is achieved at the elongation rather than the initiation step of transcription. They showed that RNA polymerase II complex can still be recruited at polycomb-regulated genes, however it encounters early blocks during the elongation phase. In fact, they suggested that a poised RNA pol II carrying a short newly-synthesised RNA transcript can further recruit the PRC2 complex.

Around 10% of the genes in human embryonic stem cells have been estimated to be PRC2 targets (Mohn et al. 2008). It is also proposed to particularly affect the control of Hox genes and developmental regulators (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). Additionally PRC2 has been shown to function in the establishment of bivalent chromatin domains in ES cells that allow for developmental plasticity. In these domains, PRC2 and PRC2-dependent H3K27me3 have been shown to co-localise with H2.AZ (Creyghton et al.

2008) and H3K4me3 (Mikkelsen et al. 2007; Mohn et al. 2008; Zhao et al. 2007), both usually associated with active transcription.

4.1.5 H3K27 methyltransferases in *C. elegans*

MES-2 protein is a homologue of the Enhancer of Zeste - E(z) *Drosophila* polycomb repressive complex (PRC2) protein. In *C. elegans*, this complex consists of MES-2 as the enzymatic core (containing a SET domain responsible for the methyltransferase activity) that together with MES-3 and MES-6 induces transcriptional repression by deposition of H3K27 di- and tri-methylation marks on target gene regulatory regions (Capowski et al. 1991; Garvin, Holdeman, and Strome 1998; Holdeman, Nehrt, and Strome 1998; Bender et al. 2004). MES-6 is a homologue of another protein in the mammalian PRC2 complex called EED, while MES-3 is a nematode-specific protein with no known homologues.

MES proteins are known to function in silencing the X chromosome in the hermaphrodite germline (Gaydos et al. 2012), and also in large-scale reorganisation of chromatin between 2E (endodermal stage) and 8E embryonic stages during the transition from developmental plasticity into differentiation in *C. elegans* (Yuzyuk et al. 2009).

There is some preliminary evidence for *mes-2*-mediated longevity control in worms from a recent small scale RNAi screen of histone methyl transferases and demethylases (Ni et al. 2012). Further supporting this are data from *D. melanogaster*, where heterozygous mutations in the PRC2 complex in also increase lifespan (Siebold et al. 2010). However, neither of these studies uncovered a mode of action of this KMT in expanding lifespan.

4.1.6 H3K27 demethylases and their functions

H3K27 demethylases have a function opposite to the factors described above and cause derepression of the chromatin state and can work together with other chromatin-associated factors that promote formation of active chromatin. UTX, which is a vertebrate demethylase is

mostly connected to epigenetic regulation of development, differentiation and maintenance of cell fates.

The crucial function of UTX in control of the cell cycle has been confirmed by Wang, Tsai, Poulin et al. (2010) who showed that UTX is both necessary and sufficient to mediate cell cycle arrest of primary human fibroblasts. This work identified 49 gene targets for UTX that encode RB-binding proteins. This group of proteins regulates the G_1 to S transition, so UTX-mediated demethylation of their promoters resulted in inhibition of the cell cycle and promoted differentiation.

Conversely, the other mammalian demethylase JMJD3 has been shown to be involved in control of inflammation and cellular senescence; together they are known to counteract the actions of the PRC2 complex (Salminen et al. 2014).

JMJD3 activity in human cells has been shown to be induced by various cellular stresses (De Santa et al. 2007; Ishii et al. 2009; Shan et al. 2012; Lee et al. 2014) and to upregulate transcription of inflammatory genes by NFkB, STAT, SMAD and T-bet transcription factors (Salminen et al. 2014). JMJD3 also has an important role in senescence by regulating the *Ink4a/Arf* locus. In normal growing cells, this locus, encoding $p16^{INK4A}$ and $p14^{ARF}$, is transcriptionally silenced by Polycomb-mediated H3K27me3. In response to oncogene- and stress-induced senescence this locus is derepressed (loss of H3K27me3), $p16^{INK4A}$ and $p14^{ARF}$ expression is induced which drives cellular senescence (Gil and Peters 2006). $p16^{INK4A}$ inhibits CDK4/6, while $p14^{ARF}$ inhibits MDM2 resulting in p53 upregulation (Gil and Peters 2006). JMJD3 can bind upstream of *Ink4a* and assist in loss of H3K27me3 at the locus (Barradas et al. 2009). In accordance with this, overexpression of JMJD3 leads to increased *Ink4a* activation and inhibition of cell proliferation.

Interestingly, studies by Miller, Mohn, and Weinmann (2010) show that some of the functions of UTX and JMJD3 might be independent of their demethylase activity, in particular,

regulation of gene expression dependent on several T-box transcription factors (via interaction with the Brg-1-containing SWI/SNF complex to promote general chromatin remodelling). It may be very important to elucidate which UTX function is important on which promoter: the H3K27 demethylase activity or general chromatin remodelling. One further point to support their hypothesis is the fact that JMJD3 and UTX mutations have been shown to occur in certain forms of cancer (van Haaften et al. 2009), however the mutations are not concentrated around the enzymatic JmjC domain, but also occur at the N terminus, which might be used for binding to other factors, suggesting that it is not just the enzymatic activity of these proteins that is important.

JMJD3 in human cells can have seemingly antagonistic pro- and anti-inflammatory effects depending on cellular context and it may be involved in both developmental regulation and the induction of cellular senescence. This makes this protein a master regulator for cellular decisions.

Various chromatin regulators implicated in lifespan control in *C. elegans* are summarised in Table 4.1.

In this chapter I will investigate whether the mutations of the four 'confident hits' from my RNAi screen (*isw-1, cbp-1, mes-2* and *jmjd-3.2*) also extend lifespan in *C. elegans,* concentrating my efforts on the H3K27 regulators.

Table 4.1 Summary of histone modifying enzymes affecting lifespan in *C. elegans*

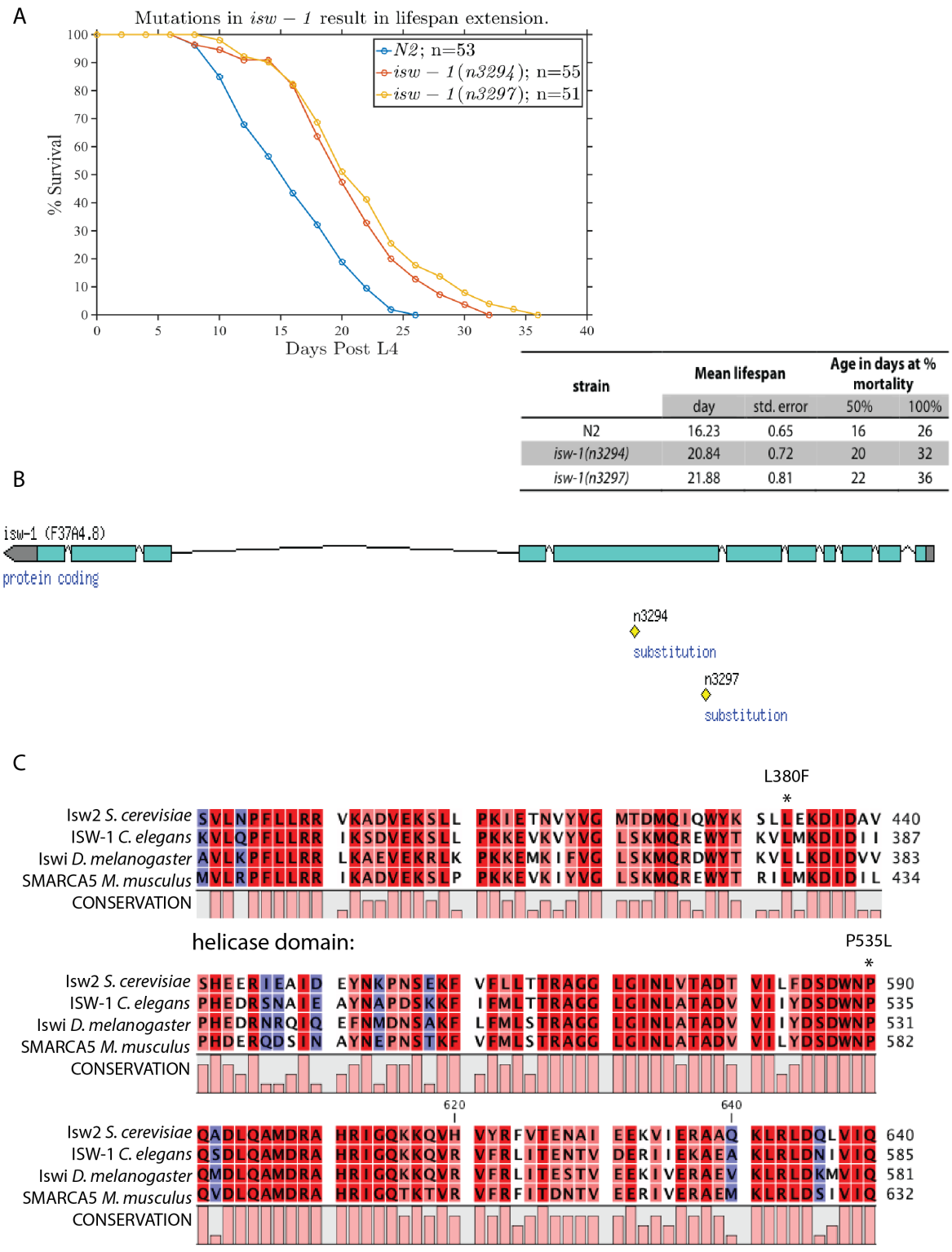
Histone residue	modifier type	*C. elegans* protein	mammalian homologue	effect on chromatin
H3K27	methyltransferase	MES-2	EZH	repression
	demethylase	UTX-1, JMJD-3.1, JMJD-3.2, JMJD-3.3	UTX, UTY, JMJD3	activation
	acetyltransferase	CBP-1	p300/CBP	activation
H3K4	methyltransferase	COMPASS (ASH-2, SET-2 and WDR-5)	SET1/ASH2	activation
	demethylase	LSD-1, RBR-2	KDM1A, KDM5B	repression
H3K9	demethylase	JMJD-2	KDM4A	activation
	deacetylase	SIR-2.1	SIRT1	repression
H3K36	methyltransferase	MET-1	SETD2	activation

4.2 RESULTS

4.2.1 Mutations in *isw-1* cause lifespan extension

Following my experiments that showed lifespan extension upon *isw-1* knock-down (Figure 3.4 (H)), I decided to investigate *isw-1* mutant worms to confirm the role of *isw-1* in ageing. Figure 4.2(A) shows that two separate strains of *C. elegans* with two different mutations in the *isw-1* gene both have significantly longer lifespans than that of the wild-type N2 strain. The strains used are *isw-1(n3294)* and *isw-1(n3297)* (see Figure 4.2(B)). Both strains have point mutations in *isw-1* gene; *isw-1(n3294)* results in L380F substitution and *isw-1(n3297)* in a P535L substitution. Both of these substitutions are present in two parts of the protein that are highly conserved from yeast to vertebrates (Figure 4.2(C)) which suggests they could disrupt functionality of the protein.

Further investigation of the mode through which ISW-1 extends lifespan was hindered by the extreme variety and sheer number of functions performed by this enzyme, meaning that it is difficult to design experiments to tease out a single function of this protein. Lack of potential target genes and lack of other work on ISW-1 in *C. elegans* made it a difficult gene to investigate further and therefore I concentrated my efforts on other genes pulled out of my RNAi screen, knock-down of which also resulted in lifespan extension.

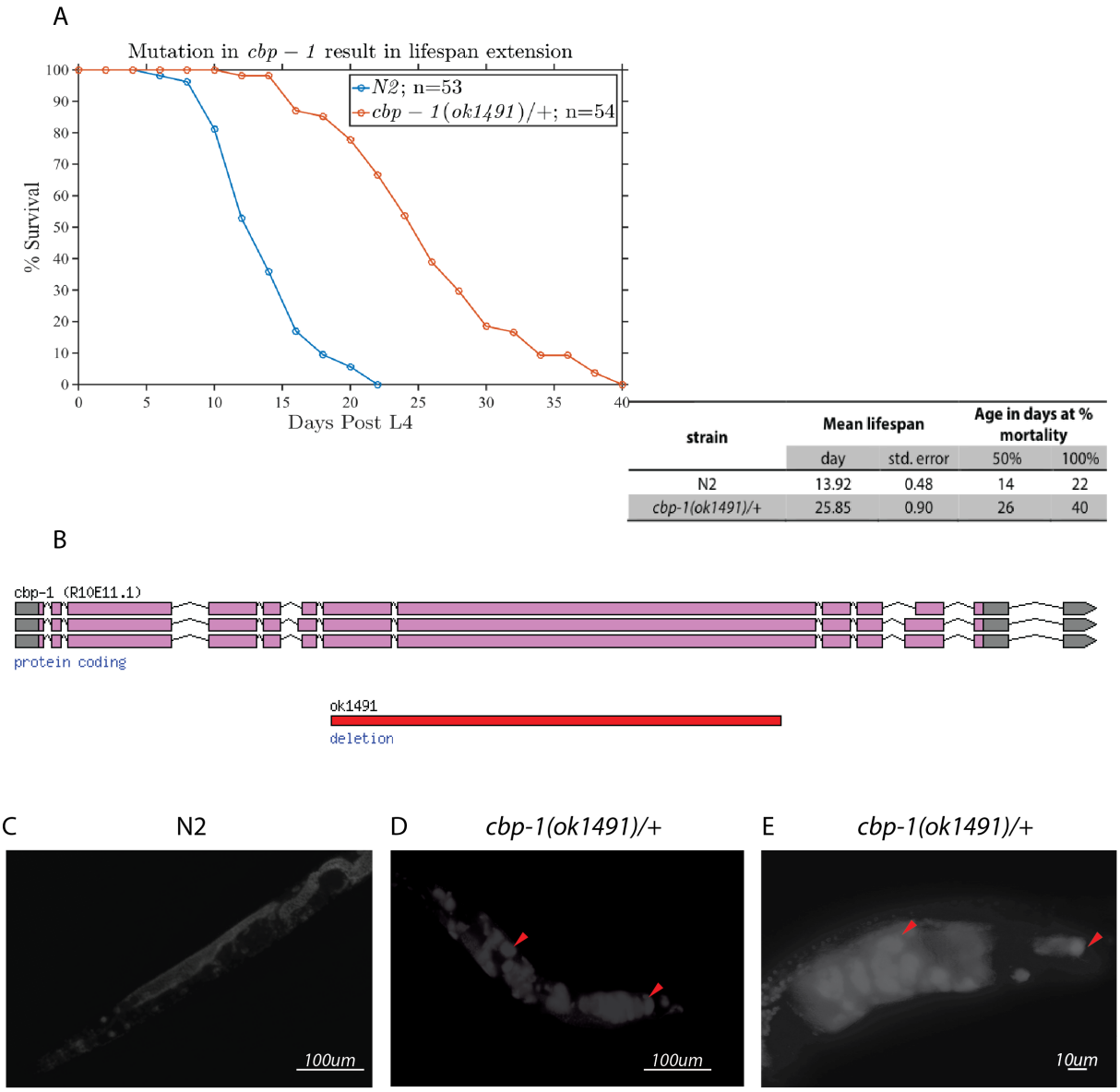


strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	16.23	0.65	16	26
isw-1(n3294)	20.84	0.72	20	32
isw-1(n3297)	21.88	0.81	22	36

Figure 4.2 Point mutations in *isw-1* increase lifespan. (A) Lifespan of both *isw-1(n3294)* and *isw-1(n3297)* strains is significantly extended when compared to an N2 WT strain. (Log rank test, p <0.01). All three strains were cultured on OP50 plates supplemented with FUDR (B) Gene organisation of *isw-1* with site of point mutations in each strain. (C) Sequence alignment of *isw-1* homologues in *S. cerevisiae*, *D. melanogaster* and *M. musculus*, indicating the sites of the substitutions in *C. elegans*. Both of the amino acids ('*') as well as the domains in which they are found have been strongly conserved through evolution.

4.2.2 Heterozygous mutation in *cbp-1* causes lifespan extension

One of the phenotypes of knocking down *cbp-1* is sterility (lack of a gonad) and therefore the RNAi-mediated lifespan extension I observed (Figure 3.4 (B)) was a consequence of a post-developmental RNAi treatment (L4 stage worms were transferred to the bacteria expressing dsRNA corresponding to *cbp-1*). Not surprisingly, *cbp-1* homozygous mutation is lethal in early development, with severe developmental defects (CGC database, https://cbs.umn.edu/cgc/home). To assess how changes in *cbp-1* affect longevity, I needed to use a heterozygous mutant crossed with a balancer chromosome, allowing me to select for the *cbp-1* heterozygotes using a pharyngeal GFP marker (*cbp-1(ok1491) III/hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)).* Figure 4.3 (B) shows the location of the deletion in the *cbp-1(ok1491)* allele and its 3 splice variants as well as the sizeable lifespan extension in heterozygote mutants (Figure 4.3 A). This observation is somehow surprising considering the previous reports from Zhang et al. (2009) who showed that *cbp-1* RNAi causes shortened lifespan and attenuation of the DR longevity effects. The observed extended lifespan in heterozygous worms is also interesting, as gonads of this strain show many signs of abnormalities, including overproliferation and accumulation of chromatin masses (Figure 4.3(C)) which could be evidence for poor health and tumour-like phenotype.



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	13.92	0.48	14	22
cbp-1(ok1491)/+	25.85	0.90	26	40

Figure 4.3 Mutation in *cbp-1* extends lifespan and causes gonadal abnormalities.

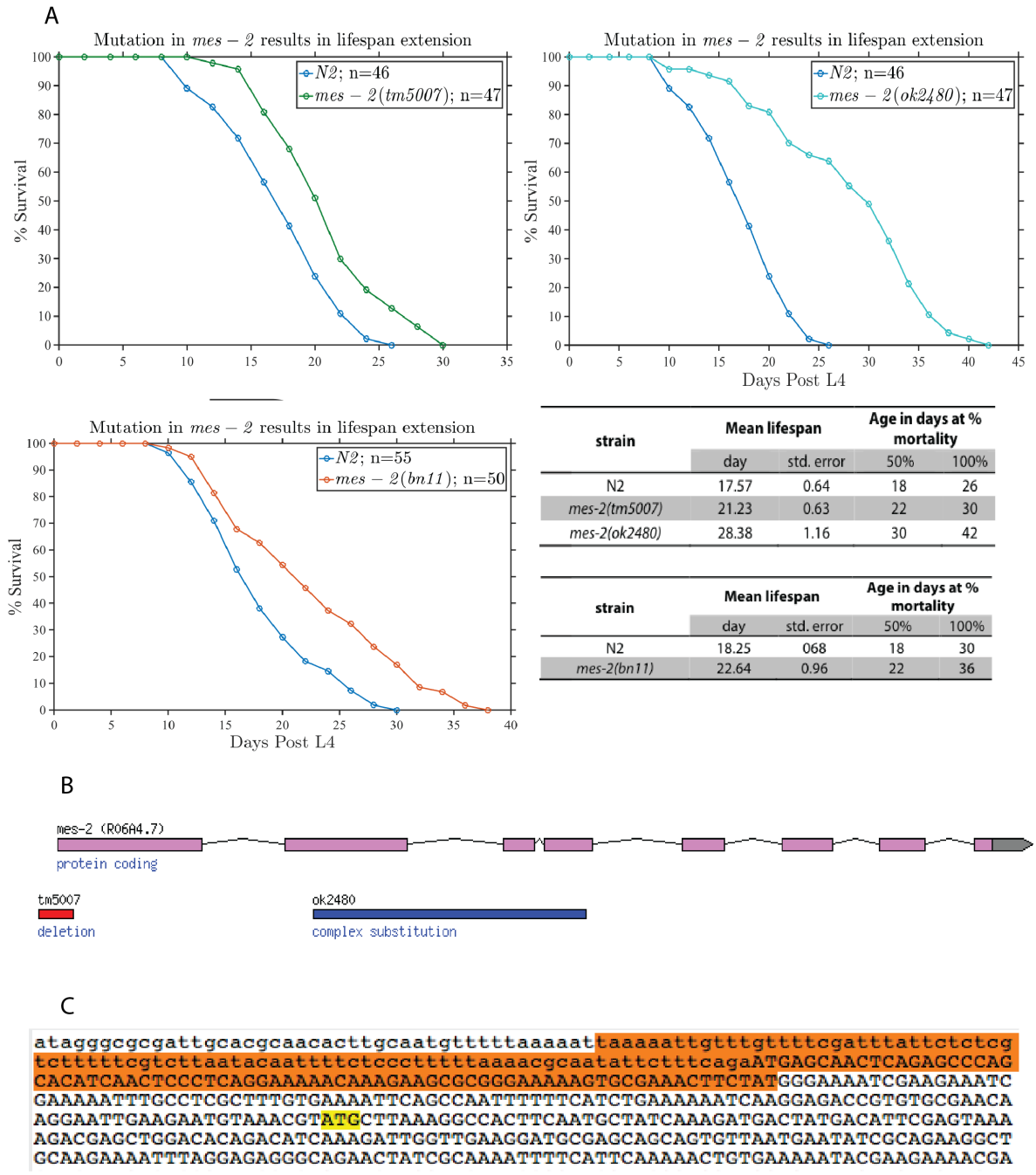
(A) The heterozygous mutant *cbp-1(ok1491)* has a statistically significant longer lifespan than N2 worms (Log Rank test, $p<0.01$). Both strains were cultured on OP50 plates supplemented with FUDR (B) The organisation of *cbp-1* gene and the mutation under investigation. (C-E) DAPI-stained DNA of the gonad in (C) WT worms, (D-E) heterozygous *cbp-1(ok1491)* mutants. Evident gonadal abnormalities (red arrows) in the mutant strain. Image capture: Zeiss AxioSKOP2 microscope with Zeiss AxioCamMR digital camera, UV filter, 20x or 63x Zeiss objective

4.2.3 Mutation of *mes-2* causes lifespan extension

According to the results of my RNAi screen, knock-down of *mes-2* results in lifespan extension (Figure 3.4 (C)). To further investigate this finding, I decided to use a variety of *mes-2* mutant strains; Figure 4.4 shows the alleles used as well as the finding that all of the mutants have significantly extended lifespan.

The first allele under investigation was *tm5007*, which is a deletion consisting of 87 bp upstream of the transcription start site plus the first 192 bp of the first exon (Figure 4.4 (B), allele marked in red and (C) – deletion highlighted in orange). This allele has not been very well studied before, and considering the placement of the deletion it might well affect the *mes-2* regulatory region. I hypothesise that this allele results in use of an alternative, in frame start codon as highlighted in yellow in Figure 4.4(C). This is due to several lines of evidence, as detailed below.

Firstly, the *mes-2(tm5007)* strain of *C. elegans* has a statistically significant lifespan extension compared to WT strain, however this is not as sizeable as one in a known null *mes-2* allele *ok2480* (Figure 4.4 (A)). Therefore *tm5007* is unlikely to code for a null mutation. Furthermore, the *ok2480* allele causes sterility, as homozygous *ok2480* mutants lack the whole gonad. In Figure 4.5, one can compare DAPI staining and brood size of various *mes-2* mutants, showing the complete loss of gonad in the *ok2480* allele. On the other hand, the nematodes with *tm5007* allele shows a functional gonad and no significant difference in brood size in comparison to the wild-type N2 strain. This evidence points towards a hypothesis that *tm5007* is a hypomorphic mutation of *mes-2*. Considering its positioning at the promoter and part of the first exon of the gene, as well as lack of described functional domains on the N-terminus of the MES-2, there is a high probability that the mutation exerts some effect on regulation of the *mes-2* gene expression.



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	17.57	0.64	18	26
mes-2(tm5007)	21.23	0.63	22	30
mes-2(ok2480)	28.38	1.16	30	42

strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	18.25	068	18	30
mes-2(bn11)	22.64	0.96	22	36

Figure 4.4 Mutations in *mes-2* result in lifespan extension. (A) 3 different mutations in *mes-2* cause significant lifespan increase (Log Rank Test, *mes-2* mutants vs N2, p<0.01 for all mutants). (B) Organisation of the *mes-2* gene and location of mutations studied. The *bn11* allele is not documented in the literature and its exact effect on the gene sequence is not yet determined (C) Sequence of the first exon of *mes-2* gene (shown in capital letters) and 130 nucleotides upstream of the open reading frame. Highlighted in orange is the region deleted in the *tm5007* allele. Highlighted in yellow is the potential, alternative, in frame TSS.

A strong correlation between sterility and longevity, as suggested by the "resource allocation" theory of ageing (Kirkwood 1977) has been widely reported. For example, loss of germline cells of the gonad by laser ablation of gonadal precursor cells (Z2 and Z3) causes lifespan extension (Hsin and Kenyon 1999). Therefore it was important to dissect the effect on longevity that *mes-2*-related sterility could have from any other pathways that it could be working through. As mentioned above, the fact that *tm5007* shows a wild-type brood size and yet displays lifespan extension (albeit not as striking as the null mutant- *ok2480*) suggests that *mes-2* could be working through at least 2 different pathways in controlling longevity. Further supporting this hypothesis is the case of the third allele investigated, *mes-2(bn11).* This allele, when homozygous also causes sterility in *C. elegans* (Figure 4.5) and substantial lifespan extension (Figure 4.4). Interestingly, the heterozygous mutation has the same degree of lifespan extension (Appendix 9) while not having any effect on broodsize or visible gonad structure (Figure 4.5). MES-2 is essential for germline but not somatic development, as *mes-2* null homozygous mutants develop correctly except for the lack of gonad proliferation (Garvin, Holdeman, and Strome 1998). This correct development is facilitated by maternal stores of MES-2 in the embryo that are detectible in homozygous mutants until the L2 stage (Gaydos et al. 2012).

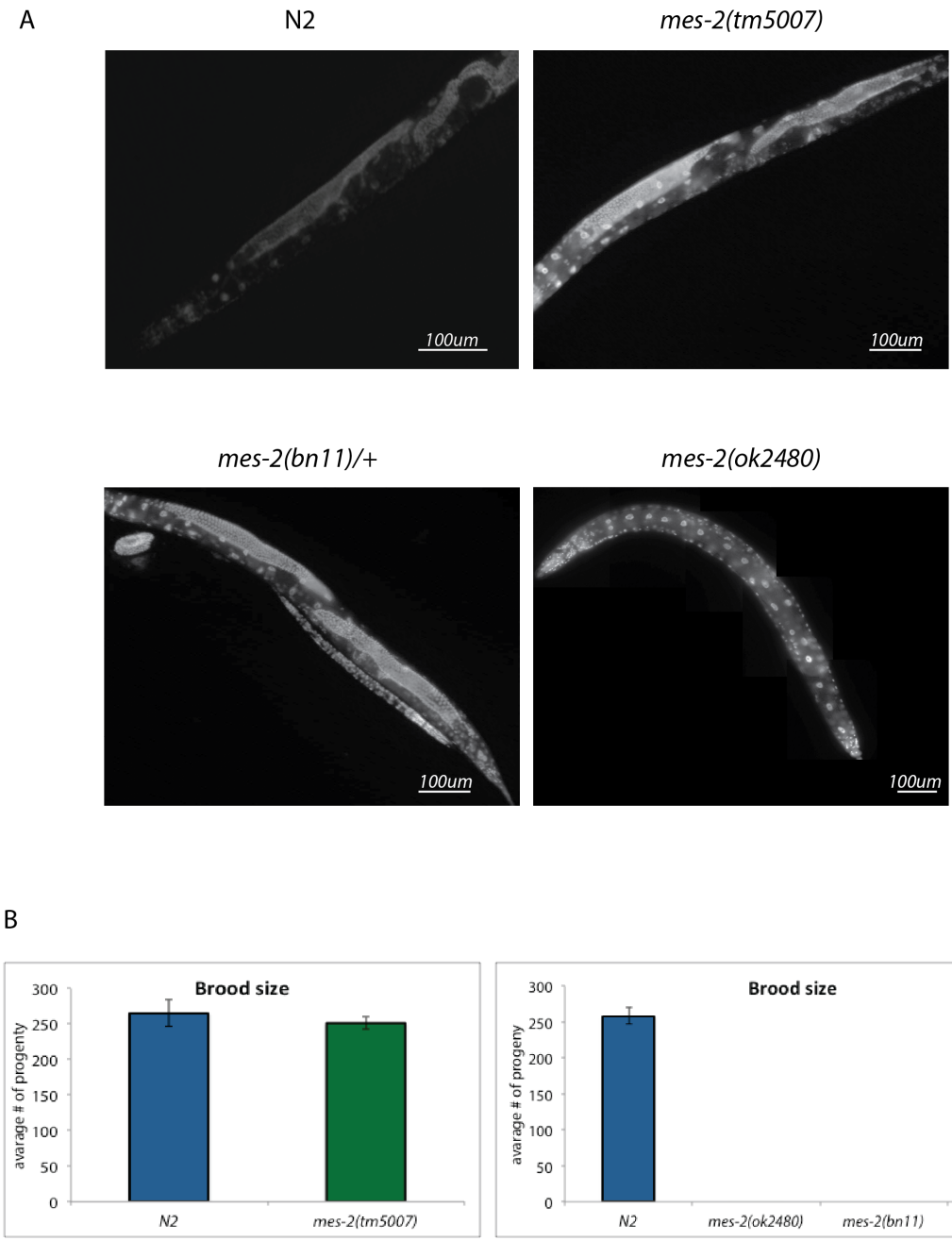


Figure 4.5 Null mutation of *mes-2* leads to loss of gonad while the heterozygous mutation and presumptive hypomorphic mutation do not affect the phenotype of the gonads. (A) DAPI staining of nuclei in whole mount *mes-2* mutant worms. The homozygous mutation *mes-2(ok2480)* leads to lack of gonad. Both *mes-2(tm5007)* and heterozygous *mes-2(bn11)* mutants have a wild-type gonad phenotype. (B) The brood size of *mes-2* mutants shows no statistical difference between a wild-type strain (N2) and *mes-2(tm5007)* or *mes-2(bn11)* heterozygote. (Student's t-test, p=0.58 and p=0.89 respectively).

To address the question of potential changes in *mes-2* regulation in the strain containing *tm5007* allele, I performed immunofluorescence in isolated gonads of different mutants using MES-2 antibody, shown in Figure 4.6. In wild-type worms (A) MES-2 is visible in all nuclei throughout the germline (which can also be seen in the DAPI staining). On the other hand, the *tm5007* allele (B) seems to be lacking MES-2 in the early (mitotic) region of the gonad, only acquiring it late in pachytene and through the loop of the gonad, which might suggest abnormal localisation and regulation of MES-2 in the gonad. Using the online resources from the modENCODE project (http://www.modencode.org/), which aims to "identify all of the sequence-based functional elements in the *Caenorhabditis elegans* and *Drosophila melanogaster* genomes", I investigated transcription factor binding sites within the 100 bp of the *mes-2* promoter that is deleted in *tm5007* allele.

There are several factors that may bet putative regulators of *mes-2* expression including EFL-1, DPL-1, LIN-54 and LIN-35 in the embryo and KLE-2 in *C. elegans* adults. As most of the effect of misregulation seen is in the developed gonad, KLE-2 is the most likely condidate. GFP-tagged reporter of *kle-2* shows transient localisation to condensing chromosomes in metaphase (Gunsalus et al. 2005), suggesting it is mostly present in actively dividing cells, which could correlate with transcriptional regulation of *mes-2* in the early gonad.

Further investigation into how loss of MES-2 affects lifespan was done by subjecting all three of the *mes-2* mutants to *mes-2* RNAi. Due to the partial presence of MES-2 in the *tm5007* allele, I hypothesized that exposing it to *mes-2* RNAi would alter its phenotype to resemble that of the null mutant. As predicted, *mes-2* RNAi causes additional and highly significant lifespan extension in *tm5007* mutant (Figure 4.7) similar to that of the null allele ok2480, as well as increased sterility (data not shown). However, RNAi knock-down of *mes-2* in worms carrying homozygous *bn11* or *ok2480* alleles had no further impact on lifespan, confirming these as *mes-2* null strains. An observed increase in sterility of the *tm5007* strain on *mes-2* RNAi (not shown) is further evidence supporting the idea that *mes-2* affects lifespan via two

different pathways. This suggests that by using two different alleles of *mes-2* (*tm5007* and *ok2480*), I can dissociate *mes-2* effects on longevity into gonad-dependent and gonad-independent.

Finally, I investigated whether other members of the PRC2 complex are involved in the *mes-2*-dependent longevity phenotype. In Figure 4.8(A) I show that *mes-3* causes a small average lifespan decrease (not significant), while *mes-6* RNAi knock-down result in very modest lifespan extension as compared to an empty vector control (mean lifespan in days: EV 13.69, *mes-3* RNAi 12.28, *mes-6* RNAi 14.61; Log rank test EV vs mes-3 RNAi, p=0.09; EV vs mes-6 RNAi, p=0.24). Interestingly, the effect of *mes-6* RNAi in a *mes-2* mutant background gives distinctive results dependent on the *mes-2* allele used. Knock-down of *mes-6* in the *tm5007* strain (Figure 4.8 (B)) causes a significant additional lifespan extension, while on the other its depletion in *mes-2(ok2480)* null mutants causes lifespan decrease (Figure 4.8(C)). These worms were also very sick and many of them exploded or had prolapsed intestine and therefore had to be removed from the experimental set (hence the low n number in the lifespan experiment).

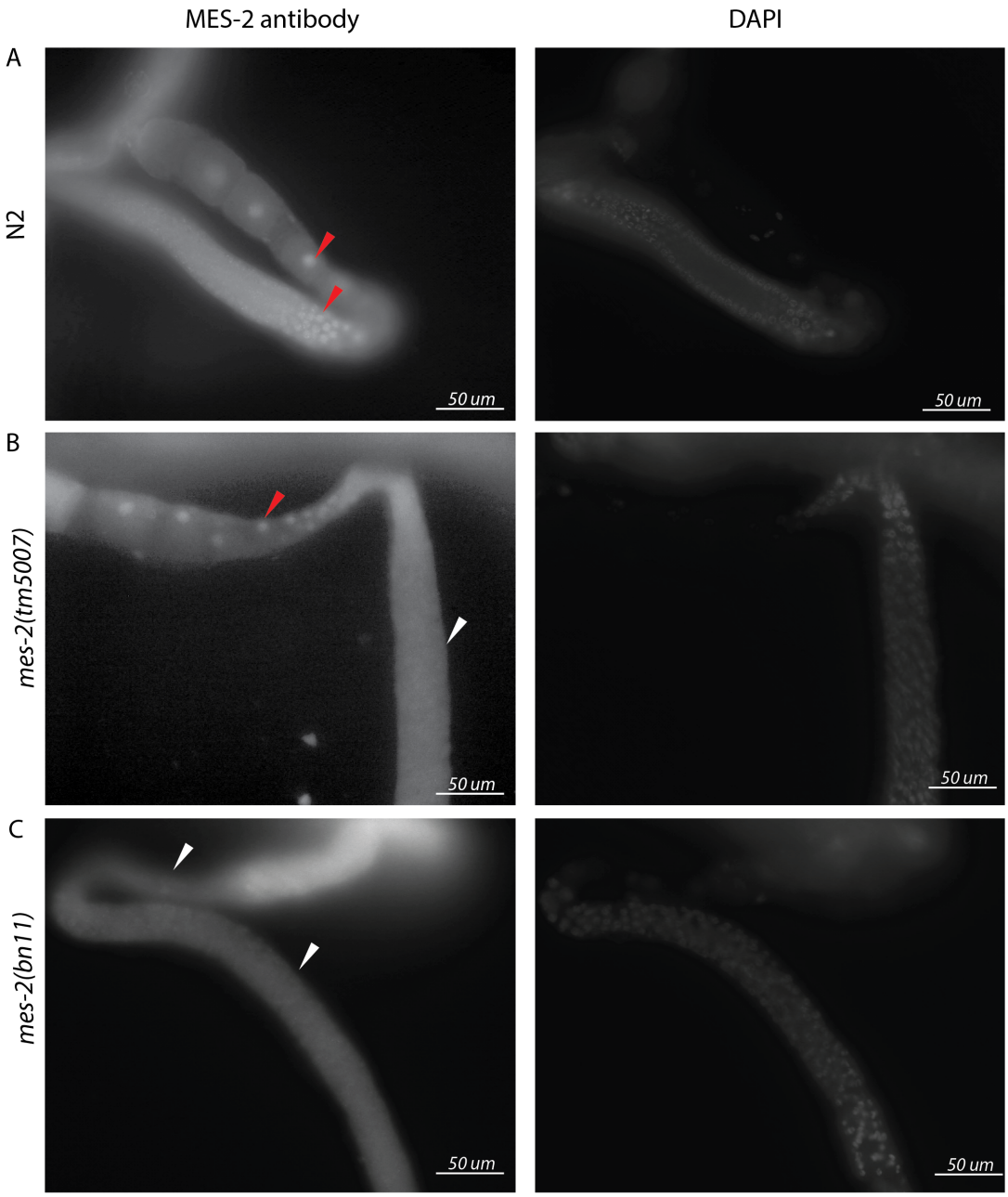
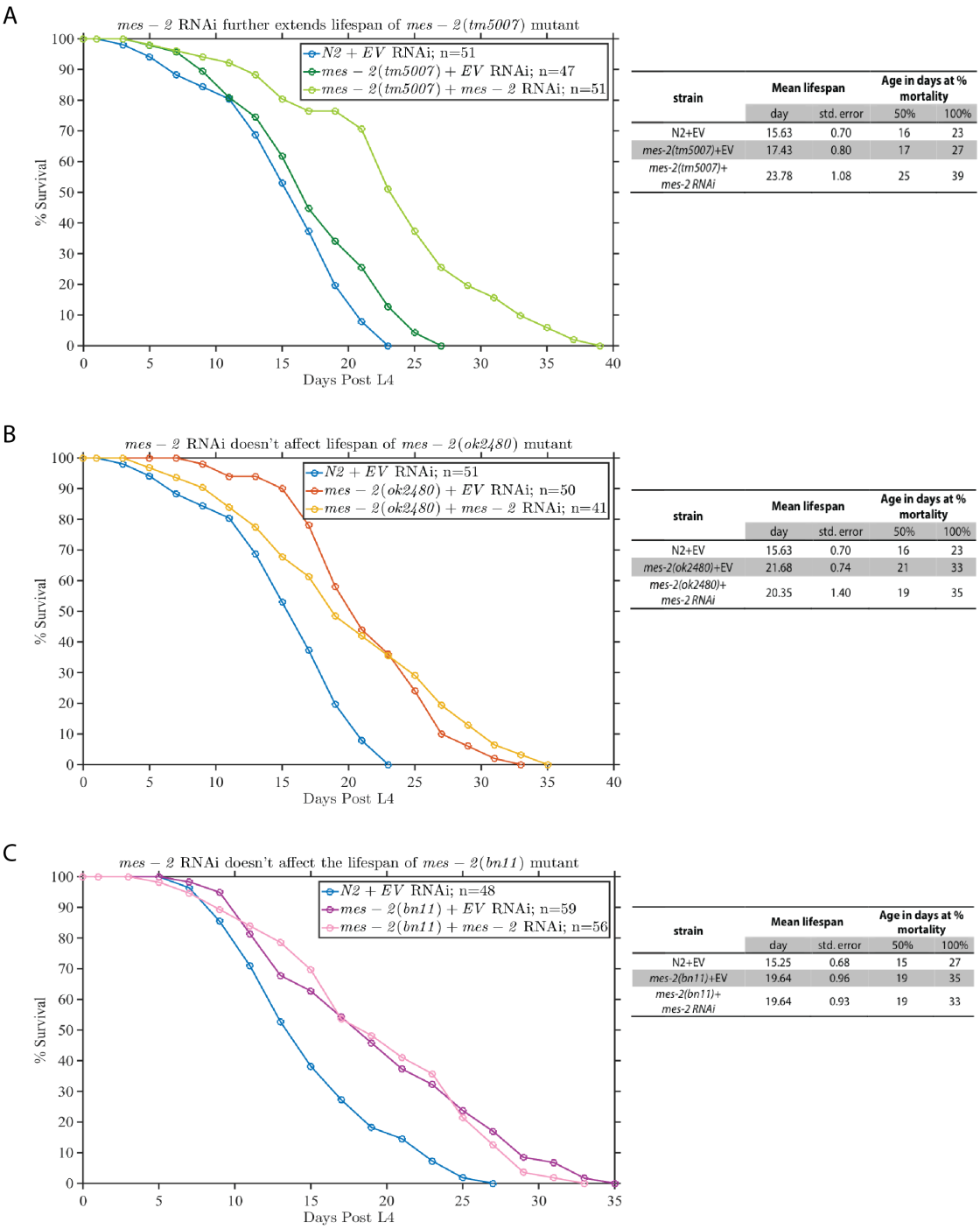


Figure 4.6 Immunofluorescence staining using antibody against MES-2 in the isolated gonads. Gonads were isolated in the watch glass and fixed in 1% formaldehyde followed by -20°C methanol and prepared on polylysine slides. 1° antibody: MES-2 Novus Biologicals, 1:200, 2° antibody: DyLight 488, anti-rabbit, 1:1000. DAPI staining: ProLong Gold. (A) Localisation of MES-2 in the WT worm is visible throughout the whole gonad. (B) a *mes-2(tm5007)* mutant shows changes in staining, lacking it in early stage nuclei and acquiring MES-2 around the transition zone. (C) Loss of MES-2 staining in homozygous *mes-2(bn11)* worms. Red arrows indicate nuclei with MES-2 signal present, white arrows point to nuclei that do not contain MES-2 (as compared to the wild-type phenotype, where MES-2 is present). Image capture: Zeiss AxioSKOP2 microscope with Zeiss AxioCamMR digital camera, GFP and UV filter, 63x Zeiss objective.



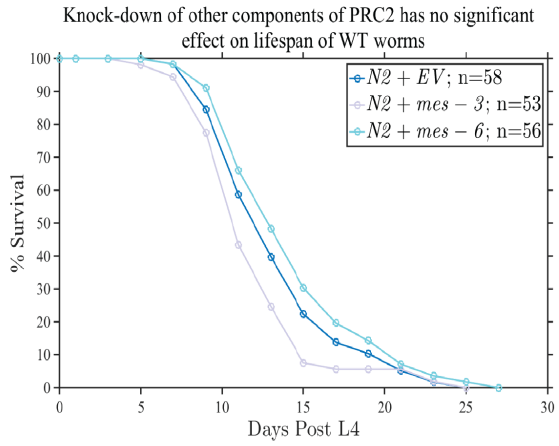
strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2+EV	15.63	0.70	16	23
mes-2(tm5007)+EV	17.43	0.80	17	27
mes-2(tm5007)+ mes-2 RNAi	23.78	1.08	25	39

strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2+EV	15.63	0.70	16	23
mes-2(ok2480)+EV	21.68	0.74	21	33
mes-2(ok2480)+ mes-2 RNAi	20.35	1.40	19	35

strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2+EV	15.25	0.68	15	27
mes-2(bn11)+EV	19.64	0.96	19	35
mes-2(bn11)+ mes-2 RNAi	19.64	0.93	19	33

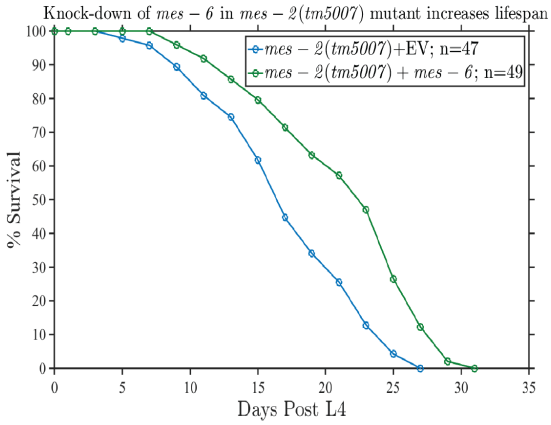
Figure 4.7 Knock-down of MES-2 further extends lifespan only in the non-null mutant of *mes-2*. (A) Non-null mutant *mes-2(tm5007)* upon MES-2 knock-down has statistically significant further lifespan extension as compared to both N2 strain, and *mes-2(tm5007)* on EV control (Log Rank Test, N2+EV vs. *mes-2(tm5007)*+EV, $p<0.05$; *mes-2(tm5007)*+EV vs. *mes-2(tm5007)+mes-2* RNAi, $p<0.01$). (B) and (C) Null (sterile) mutants: *mes-2(ok2480)* and *mes-2(bn11)* do not show any further lifespan extension after treatment with *mes-2* RNAi (Log Rank Test: N2+EV vs. *mes-2(ok2480)*+EV, $p<0.01$; *mes-2(ok2480)*+EV vs. *mes-2(ok2480)+mes-2* RNAi, $p=0.81$; N2+EV vs. *mes-2(bn11)*+EV, $p<0.01$; *mes-2(bn11)*+EV vs. *mes-2(bn11)+mes-2* RNAi, $p=0.68$)

A



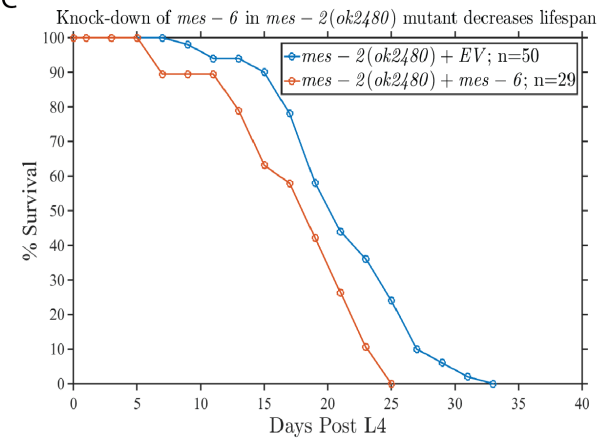
strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2+EV	13.69	0.52	13	25
N2+*mes-3 RNAi*	12.28	0.51	13	25
N2+*mes-6 RNAi*	14.61	0.58	13	27

B



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
mes-2(tm5007)+EV	17.43	0.80	17	27
mes-2(tm5007) +mes-6 RNAi	21.65	0.84	23	31

C



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
mes-2(ok2480)+EV	21.68	0.74	21	33
mes-2(ok2480) +mes-6 RNAi	17.95	1.20	19	25

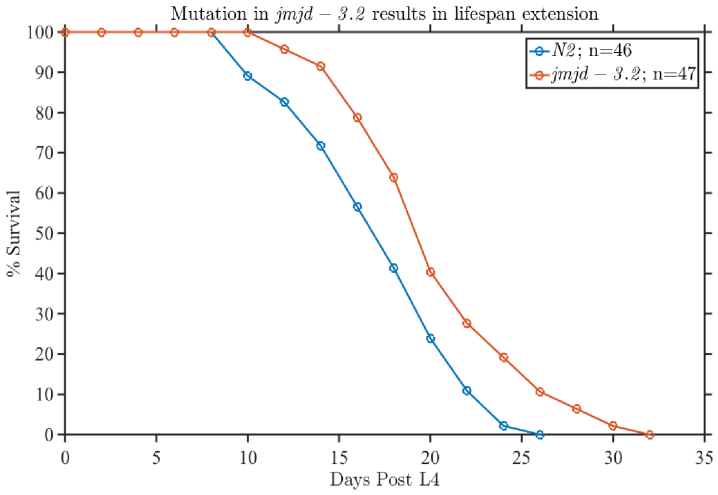
Figure 4.8 Depletion of other components of PRC-2 complex affects has variable effects on lifespan. (A) Knock-down of *mes-3* in wild-type worms results in minor lifespan decrease. *mes-6* RNAi causes modest lifespan increase. Neither of the effects is statistically significant (Log Rank Test, p=0.09 and p=0.24 respectively). (B) Knock-down of *mes-6* in *mes-2(tm5007)* worms results in significant lifespan extension (Log Rank Test, p<0.01). (C) Knock-down of *mes-6* in *mes-2(ok2480)* worms results in shortening of the lifespan (Log Rank Test, p<0.01).

4.2.4 Mutation in *jmjd-3.2* causes lifespan extension

Considering the lifespan extension of worm strains that have mutations in *mes-2* which is a H3K27me3 methyl transferase, is was rather surprising to find that JMJD-3.2 was also identified in the RNAi screen. This protein has been named and described recently as an H3K27 demethylase. It contains a JmjC demethylase domain at its C terminus and has been shown to be part of larger family of factors that all act on the same histone lysine modification (H3K27).

To further elucidate JMJD-3.2's function in lifespan extension (see Figure 3.4 (O)), I utilised the mutant strain *jmjd-3.2(tm3121).* Mutation of the *jmjd-3.2* locus results in similar lifespan extension effects to those of the RNAi knock-down of the gene (compare Figure 4.9 (A) with Figure 3.4 (O)). Figure 4.9(B) shows the allele used, which introduces a complex substitution destroying a splice site at the beginning of the third intron and introducing a codon shift and thus likely causes a null mutation. This mutant does not have any other reported phenotypes, presumably because *jmjd-3.2* expression in adult worms is restricted to several head neurons; it is however expressed in most cells during embryo development (Vandamme et al. 2012). Intriguingly, head neurons have been strongly connected to longevity regulation, for example in DR, where two ASI neurons in the head have been shown to be responsible for mediating responses to the environment such as food abundance (Bishop and Guarente 2007).

A



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	17.57	0.64	18	24
jmjd-3.2(tm3121)	21.65	0.74	20	30

B

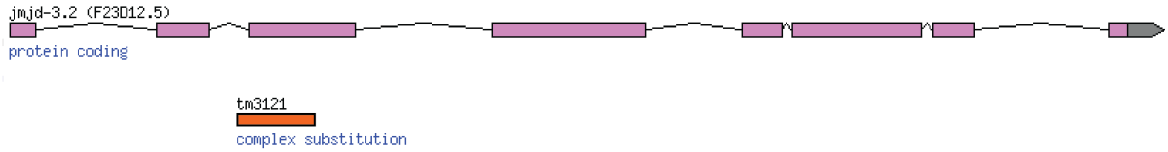


Figure 4.9 Mutation in *jmjd-3.2* causes extension of lifespan. (A) *jmjd-3.2(tm3121)* strain has statistically significant longer lifespan than that of WT strain (Log Rank Test, $p<0.01$). (B) Organisation of the *jmjd-3.2* gene and the position of the *tm3121* mutation.

4.3 DISCUSSION

4.3.1 *isw-1* mutants have extended lifespan

ISW-1 is a ubiquitous, ATP-dependent chromatin remodelling enzyme, which is expressed in most if not all cells at all developmental stages in *C. elegans* (Andersen, Lu, and Horvitz 2006). Due to its presence in several potential complexes, its functions are extremely broad and include: maintenance of chromosome structure, DNA replication, chromatin assembly, cohesin loading, rDNA silencing and transcription (Dirscherl and Krebs 2004; Mellor 2006). Further, global changes in chromatin structure have been linked to the loss of ISWI in *Drosophila* (Corona et al. 2007). Significant reductions in levels of histone H1 in ISWI mutants could suggest an important role of this protein in chromatin compaction and loading of the linker H1 (Corona et al. 2007).

A decrease in ISW-1 levels in *C. elegans*, similarly to other synMuv B suppressors, was also shown to rescue the synMuv B mutant phenotype of germline P-granule expression in somatic cells (Wang et al. 2005; Cui, Kim, and Han 2006). The synMuv B and synMuv suppressors act together to create a balance during formation of the somatic tissues. This might be the source of the effects on longevity in the worms with knocked-down *isw-1*. Experiments on other long-lived mutants show that their expression profiles exhibit a degree of soma-to-germline transformation which might allow them to become protected from genotoxic stress (Curran et al. 2009). The balance of soma-germline transcription programmes is affected by *isw-1* RNAi, which could potentially cause life extension by the same process. Interestingly, in the same work it was shown that *isw-1* RNAi can partially suppress *daf-2*-mediated longevity, and, on its own, does not produce lifespan extension which is in contrast to the data presented in Figure 3.4 (H) and Figure 4.2 (A). It must be noted that the experimental set up in the work of Curran et al. (2009) varies from mine as the worms in their experiments have been exposed to RNAi only from adulthood onwards so this might be the reason for the

difference in the outcome of *isw-1* knock-down. This may suggest that the role of *isw-1* specifically during development is necessary to promote longevity.

4.3.2 Mutation in *cbp-1* extends lifespan

CBP-1 protein has a lysine acetyl transferase activity and its homologues (CBP and p300 in vertebrates) are involved in transcription regulation that include both enzymatic function and a protein-binding scaffold function. It has also been shown that p300/CBP can regulate p53 stability (Lambert et al. 1998; Shi et al. 2009). It can also itself be regulated by post-translational modification e.g. phosphorylation or conformation-altering cofactor binding in order to change its activities (Chan and La Thangue 2001). Further, expression of p300/CBP was shown to be altered in various human tumours (Petrij et al. 1995; Borrow et al. 1996), which is consistent with *Cbp*+/- mouse studies that suggest that CBP is a tumour suppressor (Kung et al. 2000).

In worms, CBP-1 functions in the early embryo as a general activator necessary for proper specification of nearly all somatic cell fates. Blocking *cbp-1* expression results in extra cell divisions and ectopic neuronal differentiation in the embryo (Shi and Mello 1998). Victor et al. (2002) showed that the KAT activity is crucial for its biological function. Finally, Eastburn and Han (2005) used a semidominant allele of *cbp-1* to assess its role in antagonising Ras signalling in vulval development.

The role of *cbp-1* and its homologues in longevity promotion is a complicated one. Zhang et al. (2009) showed that hypothalamic expression of *cbp1* and *satb1* are highly positively correlated with lifespan in mice. Furthermore, in *C. elegans* CBP-1 levels are increased in long-lived *daf-2* mutants as well as in worms undergoing DR, while *cbp-1(RNAi)* blocks its upregulation in response to DR. Interestingly, in this study, Zhang et al. report that the longevity effects of DR are attenuated by *cbp-1* RNAi and that knock-down of *cbp-1* causes

overall lifespan shortening and produces dye-filling defects in *C. elegans* amphid neurons suggestive of loss of structural integrity of the neuronal network.

Curiously, the only difference between the experiments of Zhang and collegues and mine is the in use of an *rrf-3* RNAi hypersensitive strain for their lifespan assay, otherwise the protocols are very similar and include culture of worms at 20°C and RNAi induction post-developmentally. Both methods include FUDR as a method of sterilisation. This could suggest that the exact dosage of the *cbp-1* gene is important in how it affects the lifespan. This hypothesis is consistent with my observation that the heterozygous mutant of *cbp-1* has significant lifespan extension (Figure 4.3).

There are many potential pathways through which CBP-1 could affect longevity in a positive manner. The most interesting, and perhaps worth pursuing in *C. elegans*, is a connection to p53, by an observation made by Shi et al. (2009) in human cells where compartmentalisation of p300/CBP allows for different functions in regulating p53. In the nucleus, p300/CBP activates p53 by acetylation. Conversely, in the cytoplasm, the E4 polyubiquitin ligase activity of p300/CBP causes p53 destabilisation. Considering the variable roles of the *C. elegans* p53 homologue (CEP-1) in lifespan control (Arum and Johnson 2007; Ventura et al. 2009; Baruah et al. 2014), this could be an interesting avenue for exploration.

4.3.3 *mes-2*-dependent lifespan extension

In this chapter, I investigated three different alleles of *mes-2* and demonstrated that all of them cause statistically significant lifespan extension. Two of these affect the germline (causing sterility when homozygous) and result in larger lifespan extensions than the presumptive hypomorphic *tm5007* mutation. The large deletion in the *ok2480* allele might give rise to truncated and probably misfolded protein, that is likely to be degraded leading to loss of functional MES-2. The *tm5007* allele contains a deletion that covers approximately 100 bp of the promoter and part of the first exon of the gene, which I predict still allows for expression

of functional mRNA. Experiments described in this chapter suggest that this deletion causes misregulation of MES-2 in the germline, while keeping its enzymatic function intact (considering that the SET domain is at the unaffected C terminal of the protein, Bender et al. (2004)).

Taken as a whole these observations suggest that *mes-2*-dependent lifespan extension can work through two pathways, one of which involves induction of sterility and is probably conveyed by the PRC2 complex, as loss of one of the other members of the complex (MES-6) shortened lifespan and increased sickness in *mes-2(ok2480)* mutants. Given that *tm5007* worms subjected to *mes-6* RNAi a have wild-type brood size and increased lifespan suggests that this lifespan extension it is likely to be both germline- and *mes-6*-independent.

The hypothesis that there is a germline-independent role for *mes-2* in lifespan regulation is supported by the observed expression of *mes-2* outside of the germline. Holdeman, Nehrt, and Strome (1998) claim that there is faint staining of MES-2 in the intestine, which I investigated using a *mes-2::GFP* integrated strain (data not shown). This potentially increases the complexity of how *mes-2* regulates lifespan, as it is known that intestine is a major lipid store in *C. elegans* and a tissue through which a lot of lifespan signalling has been shown to occur (Libina, Berman, and Kenyon 2003; Portal-Celhay, Bradley, and Blaser 2012). The *C. elegans* intestine is also the major site for the worm stress response to chemicals as well as to oxidative or heat stress. This has been confirmed by demonstrating the upregulation of intestinal markers in response to various environmental challenges (An and Blackwell 2003).

Noticeably, the *mes-2(bn11)* strain behaves somewhat differently to the *mes-2(ok2480)* strain, despite both being sterile and being described in the literature as null alleles (Capowski et al. 1991; Garvin, Holdeman, and Strome 1998; Holdeman, Nehrt, and Strome 1998). The phenotypes of the *bn11* allele have been defined in the literature (loss of MES-2 in germline nuclei resulting in maternal effect sterility) but the exact type of mutation and its positioning

is not described. My hypothesis is that the *bn11* allele causes a deletion in the SET domain of MES-2, which however still allows for the production of a partially functional protein which cannot be detected by the antibody used (Figure 4.6 C). Interestingly, the *bn11* strain, despite being a null mutant, does not live as long as *ok2480* strain (Figure 4.4), in fact the *bn11* heterozygote lifespan is actually indistinguishable from that of the homozygotes (Appendix 9). This could be due to a dominant negative phenotype of misfolded mutant MES-2, which would therefore be responsible for the longevity phenotype in both heterozygotes and in homozygotes (for example it could work by artificially stabilising MES-2 containing complexes).

The fact that the *ok2480* null allele subjected to *mes-6* RNAi results in sicker and faster-dying worms might mean that the lifespan extension achieved when MES-2 is absent (or sterility) is somehow dependent on *mes-6*, perhaps via a signalling cascade or through alternative PRC2 complex formation (as it is known that complete loss of MES-2 does not cause a loss of all of H3K27me3, which suggests there could be other enzymes also responsible for lysine 27 methylation (Bender et al. 2004).

4.3.4 *jmjd-3.2*-dependent lifespan extension

Mutations in the *jmjd-3.2* lysine demethylase identified in my RNAi screen confirmed that loss-of-function of this protein increases lifespan (Figure 4.9). The lifespan effect of *jmjd-3.2* was relatively modest but statistically significant and replicable over several biological repeats. Interestingly, JMJD3 in human cells has been shown to be mostly activated under particular stress conditions (Agger et al. 2009), which could suggest that the response to loss of *jmjd-3.2* might be greater when *C. elegans* is challenged by difficult environmental conditions. Furthermore, Miller, Mohn, and Weinmann (2010) have proposed that in human cells, demethylase activity of the enzymes might only be critical during developmental transition, eventually becoming superfluous in the committed lineages. This correlates well

with the expression of JMJD-3.2 seen in *C. elegans,* which is ubiquitous in the early embryo then restricted to several head neurons in the adult (Vandamme et al. 2012).

In summary, in this chapter I have shown that mutations in all four genes identified as strong hits in my RNAi screen result in lifespan extension. In the next part of my work, I will concentrate on the importance of the enzymatic activity of these enzymes for lifespan extension, as well as investigate the effects of overexpression of the H3K27 modifiers on both lifespan and healthspan of *C. elegans.*

CHAPTER 5: MODIFIERS OF H3K27 METHYLATION PROMOTE LIFESPAN EXTENSION

5.1 INTRODUCTION

5.1.1 H3K27 demethylases in *C. elegans*

Considering the work done on human cells (Miller, Mohn, and Weinmann 2010) who reported the possibility of non-enzymatic function of the demethylases, Vandamme et al. (2012) looked into the importance of the demethylase activity in *C. elegans* UTX-1 during development. UTX-1 has been found to be expressed in most, if not all nuclei of early to late stage embryos as well as all larval stages and into adulthood. Of the whole family of H3K27 demethylases, UTX-1 and JMJD-3.1 are the most widely expressed, while the other two proteins (JMJD-3.2 and JMJD-3.3) are spatially or temporally regulated (Vandamme et al. 2012). Knock-down of *utx-1* by RNAi or the *tm3118* mutation both cause tail and gonadal defects that can be fully rescued by the genomic *utx-1* construct (*utx-1p::utx-1::GFP*) shown in Figure 5.1(A, B and D). The rescue of the gonad phenotype led the authors to conclude that UTX-1 is likely to play a role in the somatic gonad as the transgene is not expressed in the germline and hence would not be able to rescue the phenotype. The most striking discovery, however, was the fact that both phenotypes could also be rescued with a demethylase-dead (DD) construct of *utx-1* which generates substitutions at crucial amino acids in the demethylase domain (*utx-1p::utx-1DD::GFP*) (Figure 5.1 C and D). It has been additionally shown that both enzymatically active and inactive UTX-1 could be recovered from large complexes that included SET-16 (MLL), suggesting that this complex could be involved in UTX-1 role in development (Vandamme et al. 2012).

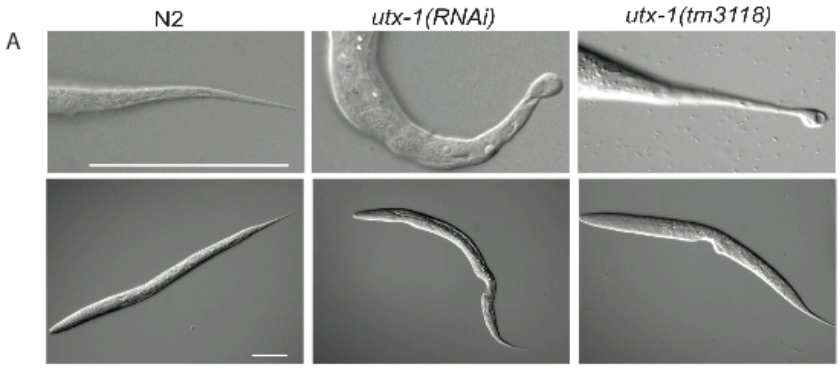
Interestingly, there is some overlap in function between various histone demethylases as the germline phenotype is shared between *utx-1* and *jmjd-3.1* mutants, however depletion of both

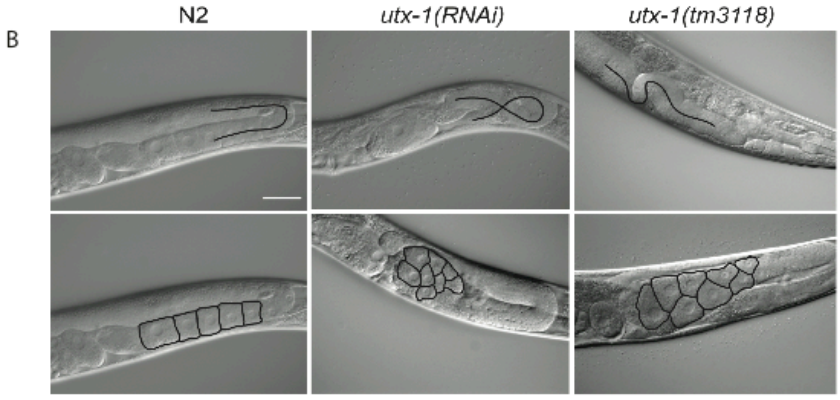
of those genes did not worsen the phenotype suggesting that they might be working in the same pathway. Additionally, worms subjected to *utx-1* RNAi, or *utx-1* mutants seemed to have decreased transcript levels of *jmjd-3.1*, but increased expression of *jmjd-3.2* suggesting a degree of co-regulation between the genes (Vandamme et al. 2012). According to published experiments (Vandamme et al. 2012), global levels of H3K27me3 have only been rescued by *utx-1::GFP* but not *utx-1DD::GFP* (as predicted), supporting the idea of a *utx-1* demethylase-independent role in development. Further, the fact that transcript levels of other members of the *jmjd-3* family were comparable between the enzymatically active and DD rescue lines implies that none of the *jmjd-3* genes was compensating for the global H3K27me3 loss and therefore suggesting their specific functions in chromatin regulation.

A role of *utx-1* in regulating longevity in *C. elegans* has also been reported by Jin et al. (2011) and Maures et al. (2011). Both report that *utx-1* loss of function extends lifespan which is dependent on the IIS pathway. Loss of UTX-1 function was shown to increase H3K27 trimethylation at the *daf-2* locus, resulting in *daf-2* transcriptional downregulation that in turn causes localisation of DAF-16 to the nucleus and activates anti-ageing genes in the IIS pathway. H3K27me3 levels were additionally shown to decrease in somatic cells while *utx-1* mRNA levels increase with age (Jin et al. 2011; Maures et al. 2011) suggesting that UTX-1 could be responsible for the loss of H3K27me3.

5.1.2 Aims

Following the investigation into mutations in the genes I identified as potential ageing regulators in the RNAi screen (chapter 3) and my observation that mutation in *jmjd-3.2* increases lifespan, I decided to investigate whether the other members of the family of demethylases also affect lifespan, and to explore the importance of the enzymatic activity of UTX-1 and JMJD-3.2. Finally, I set out to examine if the lifespan extension phenotypes can be correlated with healthspan *C. elegans*.





C

UTX-1 demethylase domain (JmjC), 863-1026aa:

820 840 860 880
AEYQSESFKHTLKGEAEKIKKNVGGTRGPSPKRMKLYKRASSPPKHLKSIKYGTNIDLSDDTKWGKQINELSKLPAFCRL
900 H914A D916A 940 960
IAGSNMLSHLGHQVHGMNTVKLFMKVPGCRTPAHQDSNHMASININIGPGDCEWFAVPYEYWGKMHKLCEKNGVDLLTGT
980 1,000 1,020 1,040
FWPIIDDLLDAGIPVHRFTQKAGDMVYVSGGAIHWVQASGWCNNISWNVAPLNFQQLSISLLSYEYNKLRRFKSEVPMQL

D

Genotype	% tail defects		% gonadal defects	
N2	0	n=50	0	n>100
N2 *control (RNAi)*	0	n=50	0	n=124
N2 *utx-1(RNAi)*	43	n=98	48	n=215
utx-1(tm3118)	68	n=74	100	n=23
utx-1(tm3136)	58	n=43	100	n=26
utx-1(tm3118)+UTX-1::GFP	2	n>100	1	n>100
utx-1(tm3118)+UTX-1DD::GFP	1	n>100	1	n>100

Figure 5.1 UTX-1 is an essential protein required for development, however the phenotypes can be rescued by a demethylase dead (DD) version of the protein (from (Vandamme et al. 2012))(A) Representative DIC images of L1 larvae tails (upper panel) and L1 larvae (lower panel) of N2, *utx- 1*(RNAi) and *utx-1(tm3118)*/+ animals. Animals are oriented anterior to the left. Scale bar is 50 µm. (B) Representative DIC images of gonads in adults N2, *utx-1(RNAi)* and *utx-1(tm3118)*/+ animals. Scale bar is 25 µm. In the upper panels, blacks lines indicate the migration of the gonad arm. In the lower panels, black lines indicate the contours of the oocytes. (C) Amino acid sequence of UTX-1 JmjC domain. '*' in red shows the sites of point mutations in DD strain. (D) Percentages of posterior (% tail defects) and gonad (% gonadal defects) defects in the indicated strains are shown. For RNAi, F1 larvae and adults from at least three independent experiments were analysed.

5.2 RESULTS

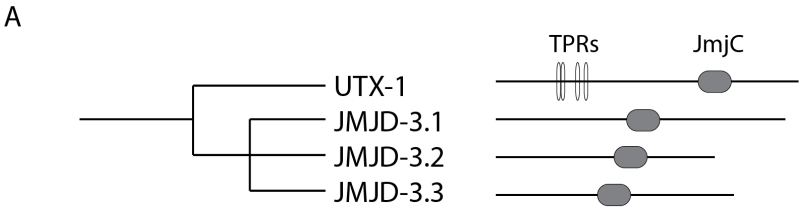
5.2.1 Analysing other members of the JMJD-3.2 family of demethylases

JMJD-3.2 is part of a larger family of factors (Agger et al. (2007); Figure 5.2(A)), which include JMJD-3.1, JMJD-3.2, JMJD-3.3 and UTX-1. Intriguingly, UTX-1 has been previously linked to control of longevity, as its depletion extends lifespan (Jin et al. 2011; Maures et al. 2011). I decided to investigate whether other proteins in the family share this function. In Figure 5.2(B), I show that knock-down of either *jmjd-3.1* or *jmjd-3.3* has no effect on longevity: mean lifespan of the worms is comparable to that of an empty vector control for *jmjd-3.1* RNAi and is marginally shortened for *jmjd-3.3* RNAi (Log Rank Test p=0.43 and p=0.28 respectively). Conversely, *utx-1* RNAi results in lifespan extension as seen in Figure 5.2(C).

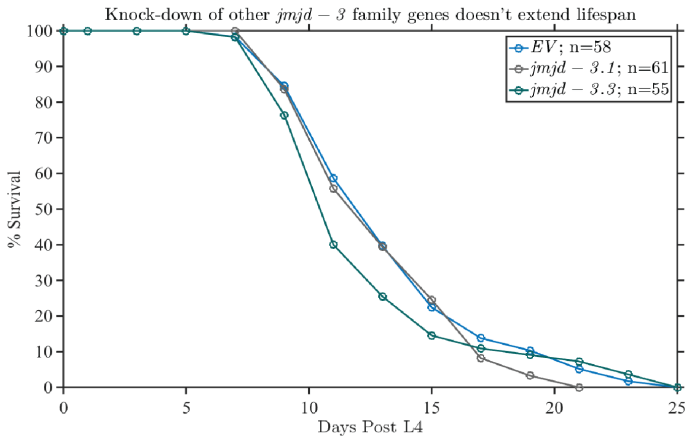
Due to the high degree of homology between the *jmjd-3* genes, I hypothesised a possible redundancy, and therefore we used a *jmjd-3* triple mutant to investigate if there was any additional effect on lifespan as compared to the single *jmjd-3.2(tm3121)* allele. Figure 5.3(A) illustrates that there is no significant difference between the lifespan of the *jmjd-3* triple mutant and *jmjd-3.2(tm3121)*. It is worth noting, though, that both *jmjd-3.1(gk384)* and *jmjd-3.2(tm3121)* alleles are known null mutations, while the status of *jmjd-3.3(tm3197)* has not yet been confirmed. This is an interesting observation especially in the light of other published data (Vandame et al. 2012), which shows a degree of redundancy in methyltransfease function between these genes (as H3K27me3 increases in triple mutant in comparison to any of the strains with single *jmjd-3* mutation; while both H3K27me2 and me1 are lower in triple mutant).

Another step to clarify the potential roles of the H3K27 demethylase family in longevity regulation was to determine whether UTX-1 and JMJD-3 proteins have overlapping functions by performing a combinatorial analysis. I tested *utx-1* RNAi in combination with

jmjd-3.2(tm3121) as well as the *jmjd-3* triple mutant: *utx-1* has an additive effect on longevity in both cases (Figure 5.4). This could suggest that despite being part of the same enzymatic family, JMJD-3.2 and UTX-1 control lifespan via different pathways. Taken together with the fact that *utx-1* is fairly ubiquitously expressed in *C. elegans*, while *jmjd-3.2* expression is very specifically spatially and temporarily controlled (Vandamme et al. 2012), it provides an interesting insight into this demethylase family.

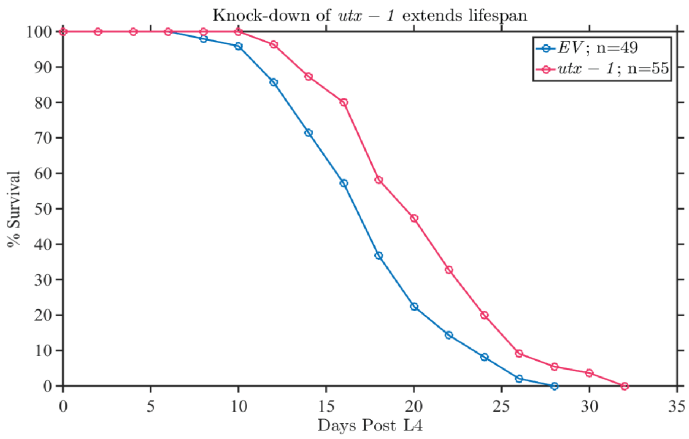


B



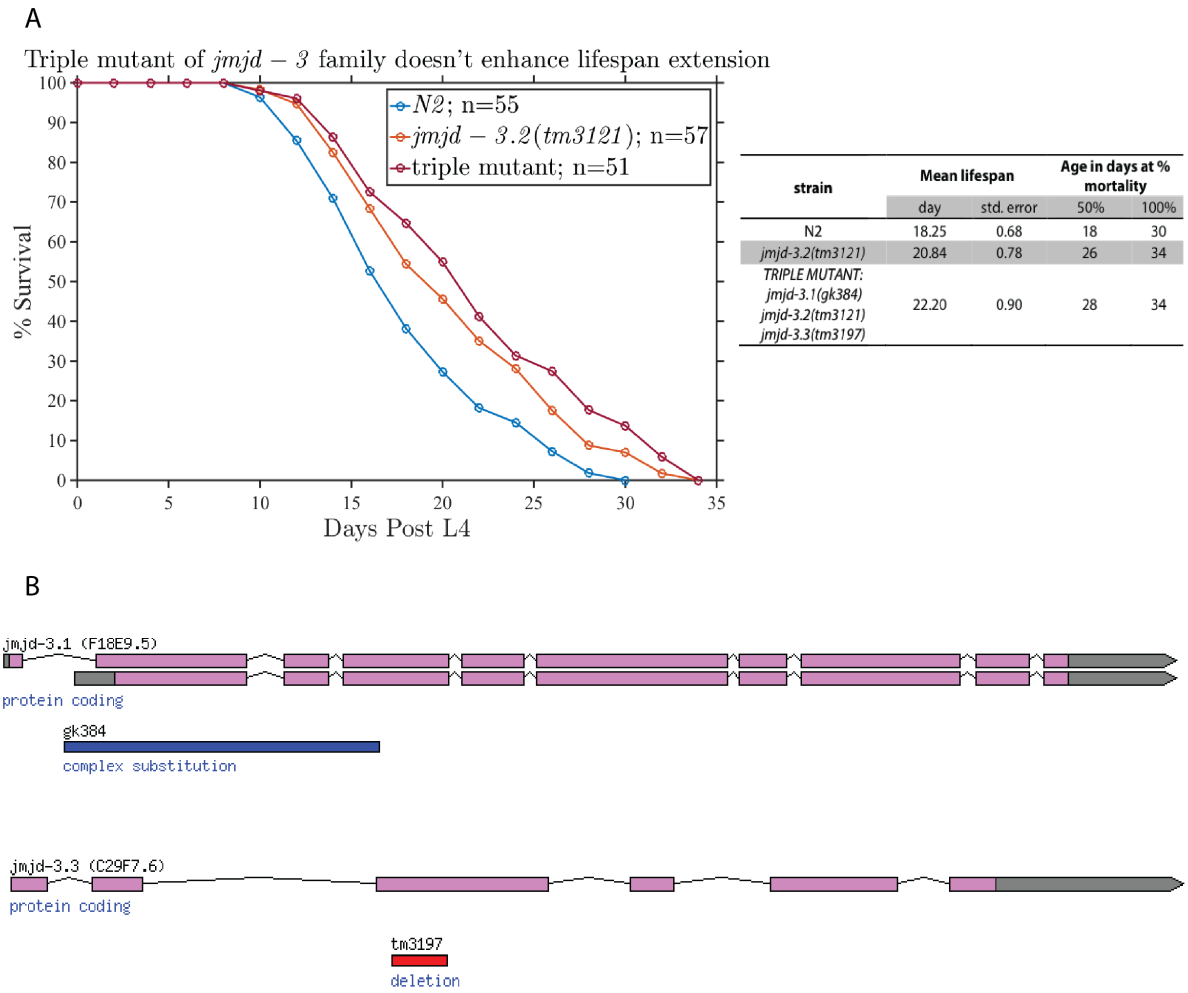
strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2+EV	13.69	0.52	13	25
N2+*jmjd-3.1 RNAi*	13.30	0.42	13	21
N2+*jmjd-3.3 RNAi*	12.71	0.56	13	27

C



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2+EV	17.84	0.64	18	28
N2+*utx-1 RNAi*	20.80	0.65	20	32

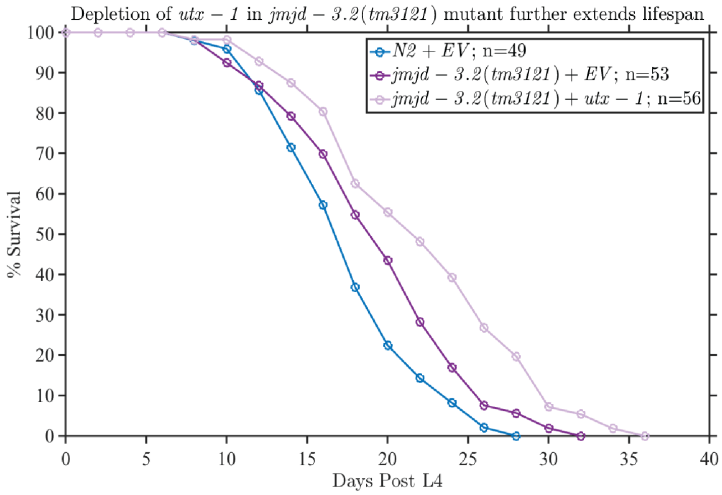
Figure 5.2 *utx-1* is the only member of the demethylase family, apart from *jmjd-3.2,* that affects lifespan. (A) *jmjd-3.2* is part of the family of H3K27me3 demethylases in *C. elegans*. JmjC – Jumonji C domain, TPRs – tetratricopeptide repeats. (B) Knock-down of either *jmjd-3.1* or *jmjd-3.3* does not cause significant change to lifespan (Log Rank Test, p=0.43 and p=0.28 respectively). (C) Knock-down of *utx-1* results in lifespan extension (Log Rank Test, p<0.01).



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	18.25	0.68	18	30
jmjd-3.2(tm3121)	20.84	0.78	26	34
TRIPLE MUTANT: jmjd-3.1(gk384) jmjd-3.2(tm3121) jmjd-3.3(tm3197)	22.20	0.90	28	34

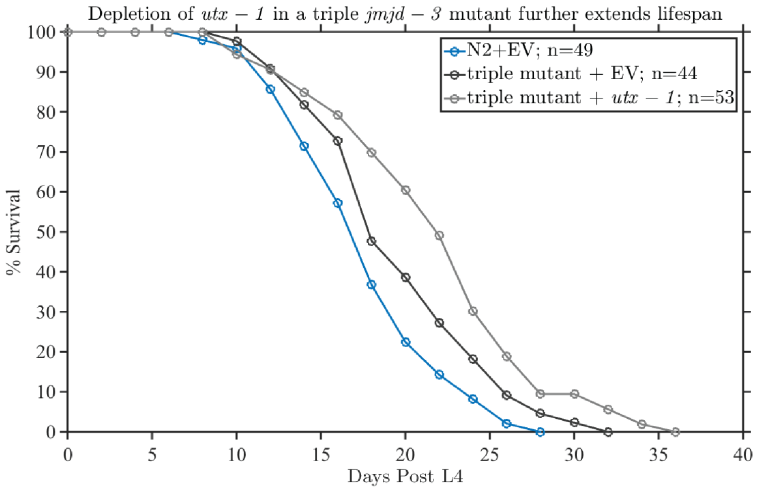
Figure 5.3 Triple mutation in *jmjd-3* family of genes does not result in additional lifespan increase. (A) Lifespan of triple mutant *jmjd-3.1(gk384) jmjd-3.2(tm3121) jmjd-3.3(tm3197)* is not significantly increased over the *jmjd-3.2(tm3121)* single mutant (Log Rank Test: N2 vs *jmjd-3.2(tm3121)*, p=0.01; *jmjd-3.2(tm3121)* vs the triple mutant, p=0.19). (B) Gene organisation and the lesions identified in *jmjd-3.1* and *jmjd-3.2* mutants.

A



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	17.84	0.64	18	28
jmjd-3.2(tm3121) + EV	19.70	0.76	20	32
jmjd-3.2(tm3121) + *utx-1 RNAi*	22.46	0.86	22	36

B



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	17.84	0.64	18	28
triple mutant + EV	19.82	0.77	18	32
triple mutant + *utx-1 RNAi*	22.08	0.85	22	36

Figure 5.4 Combination of *utx-1* RNAi and *jmjd-3.2* single or *jmjd-3* triple mutant results in additive lifespan. (A) Depletion of *utx-1* by RNAi in a *jmjd-3.2(tm3121)* mutant background causes further lifespan extension (Log Rank Test, *jmjd-3.2(tm3121)* EV vs *utx-1* RNAi, p<0.01). (B) Depletion of *utx-1* by RNAi in the *jmjd-3* triple mutant background (*jmjd-3.1(gk384) jmjd-3.2(tm3121) jmjd-3.3(tm3197)*) also causes further lifespan extension (Log Rank Test, triple mutant EV vs *utx-1* RNAi, p<0.01).

5.2.2 UTX-1 and longevity in *C. elegans*

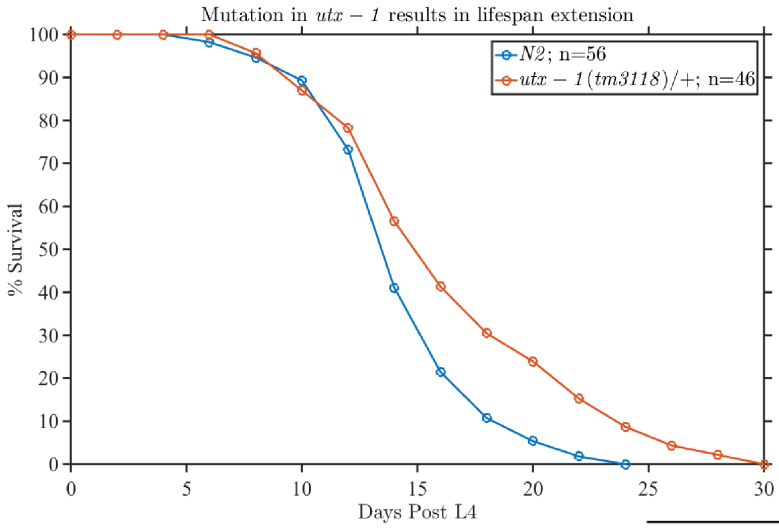
C. elegans UTX-1 is a homologue of the vertebrate UTX and UTY proteins and is shown to be antagonistic to H3K27me3 mark deposited by the PRC2 complex as in human cells, JMJD3 and UTX are specific H3K27me3/2 demethylases (Agger et al. 2007). In worms, UTX-1 has been shown to be necessary for development, as null mutants without maternal stores of UTX-1 show significant developmental defects, dying mostly in the late embryonic stages (Vandamme et al. 2012). UTX-1 has also been implicated in vulval development, acting in complex with SET-16 (MLL) (Fisher et al. 2010).

For preliminary testing, I decided to confirm the results previously published by Jin et al. (2011) and Maures et al. (2011) which showed that heterozygous mutations in *utx-1* cause extension of lifespan. In Figure 5.5, I show that *utx-1(tm3118)* heterozygous mutant has got a statistically significant increased lifespan in comparison to a wild type strain (Log Rank test, $p<0.01$). Maures et al. (2011) have shown that loss of UTX-1 causes global changes in chromatin, namely an increase in H3K27me3. Considering the evidence for age-related decrease in H3K27me3 in somatic tissues across different species (Bennett-Baker, Wilkowski, and Burke 2003; Lee, Weindruch, and Prolla 2000; Lu et al. 2004; Lund et al. 2002; Scaffidi and Misteli 2005, 2006), this suggests a general "rejuvenation" or retention of chromatin in its "younger" state. It has also been suggested that *utx-1*-dependent lifespan extension is independent of the germline pathways (*glp-1*) but is dependent on DAF-16 (Maures et al. 2011), as *utx-1* depletion in a *glp-1* mutant resulted in additional lifespan increase, while depletion of *daf-16* in the *utx-1* mutant results in a lifespan comparable to that of the *daf-16* mutant.

This is also confirmed by the work of Jin et al. (2011), who investigated H3K27me3 at *daf-2* locus and hypothesises that the alteration of epigenetic landscape of *daf-2* is a major pathway through which *utx-1* extends lifespan. Loss of UTX-1 activity (either by mutation or RNAi) causes an increase of the H3K27me3 repressive mark, and therefore lowers *daf-2* expression

during later life, mimicking a long-lived *daf-2* mutant (or a young worm), and ultimately causing DAF-16 nuclear localisation and upregulation of DAF-16 targets involved in longevity (Jin et al. 2011).

A



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	14.71	0.46	14	20
utx-1(tm3118)/+	18.87	0.78	18	26

B

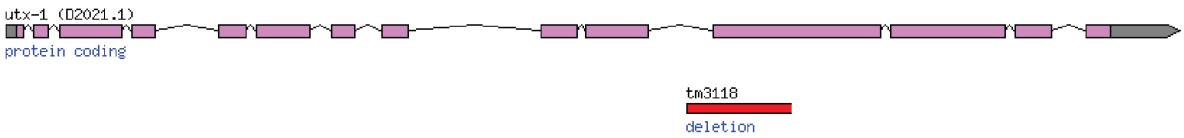


Figure 5.5 Heterozygous mutation in *utx-1* causes lifespan extension. (A) A heterozygous mutant *utx-1(tm3118)* has statistically significant lifespan extension (Log Rank Test, $p<0.01$). (B) Structure of the *utx-1* gene and the site of the mutation studied.

Mindful of the published work from Vandamme et al. (2012) showing the demethylase-independent roles of UTX-1 in development, we established a collaboration with the Salcini lab to investigate whether a *utx-1DD::GFP* construct lacking the demethylase activity can also rescue the longevity phenotype of the *utx-1* heterozygous mutant. Figure 5.1 (C) shows position of amino acid substitutions resulting from the point mutations in the *utx-1* JmjC demethylase domain made by the Salcini lab to create the *UTX-1DD::GFP* construct: two amino acids thought to be involved in Fe(II) binding are substituted for alanine (based on constructs used by Agger et al. (2007)).

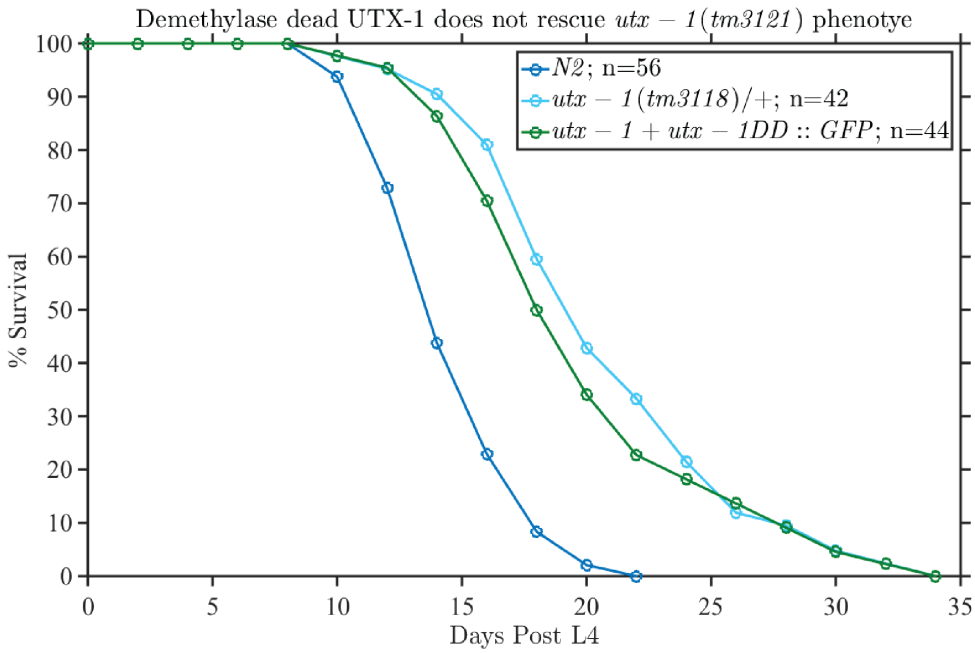
In Figure 5.6, I compare the longevity of the wild type strain to *utx-1(tm3118)* heterozygous mutant and to a *utx-1(tm3118)* mutant recued with the enzymatically inactive UTX-1. I show that both the heterozygous mutant and strain overexpressing *utx-1DD* have got longer lifespan when compared to the N2 strain, and there is no significant difference between the mutant and the strain expressing the *utx-1DD* array. This suggests that despite the fact that *utx-1DD::GFP* can rescue the *developmental* phenotypes of the *utx-1(tm3118)* mutant, it cannot rescue its *longevity* phenotype (which would result in the *utx-1(tm3118)+utx-1DD* strain to have lifespan comparable to that of wild-type strain). To control for this experiment, I also decided to investigate the full genomic rescue of *utx-1* with *utx-1::GFP*. Remarkably, *utx-1::GFP* in a *utx-1(tm3118)* background also does *not* restore normal lifespan; instead, it actually causes a marked lifespan extension with almost a 100% increase in mean lifespan between the *utx-1(tm3118)* mutant and *utx-1(tm3118)+utx-1 strain* (Figure 5.7).

We attributed this effect to an overexpression of the *utx-1* gene, due to its injection as an extrachromosomal array. This was confirmed using quantitative Reverse Transcriptase PCR (qRT-PCR) and assessing *utx-1* transcript levels following RNA extraction from mixed stage worms; levels of *utx-1* (in both enzymatically active and DD rescue strains) were 14-16 times higher than in wild-type controls (Figure 5.8(D)). To confirm that the difference in longevity phenotypes between the *utx-1::GFP* and *utx-1DD::GFP* worm was not due to difference in

expression, I checked the strains for expression of GFP using a fluorescence microscope (Figure 5.8(A)), as well as by Western blot; quantification shows that UTX-1 protein levels are similar in the two rescue strains - Figure 5.8 B and C.

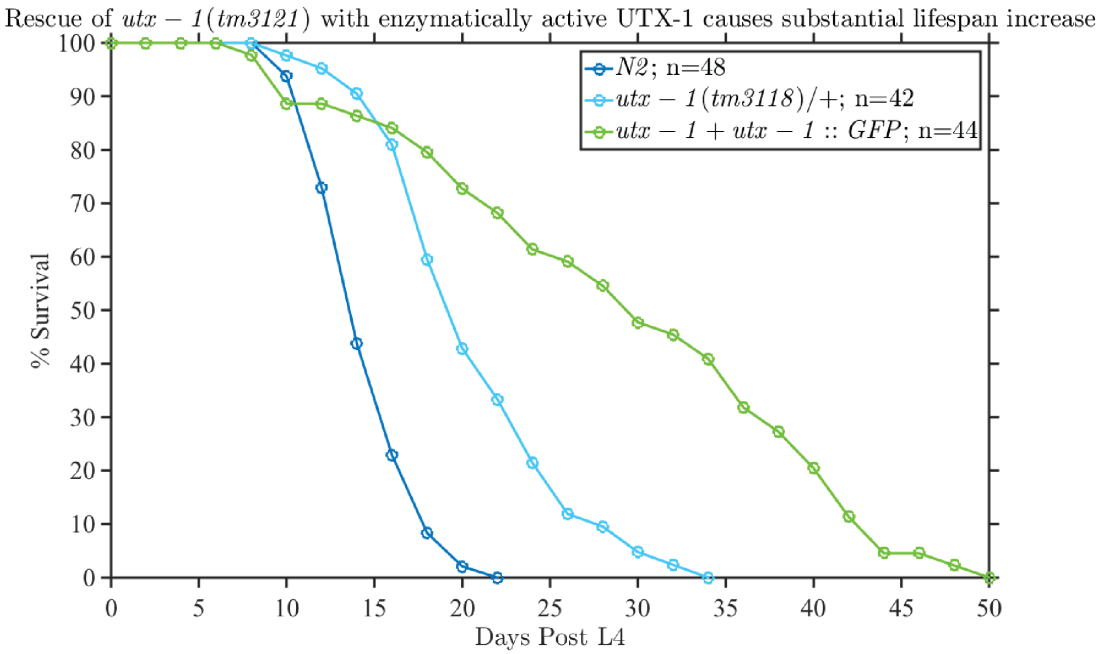
The next step to confirm that longevity could be conferred by overexpression of *utx-1* was to inspect the *utx-1::GFP* and *utx-1DD::GFP* transgenic constructs in a wild-type background.

Results presented in Figure 5.9 show that overexpression of *utx-1::GFP* but not *utx1DD::GFP* causes an increase in lifespan. The levels of transcript and protein were quantified using qRT-PCR and Western blot against GFP. Overexpression of *utx-1* results in similar levels of *utx-1* mRNA and UTX-1 protein in the two strains; the levels are also comparable to the overexpression in the *utx-1* mutant background. Noticeably, however, lifespan extension via *utx-1::GFP* in the N2 background is not as substantial as that in the mutant background. One possible explanation for this is the fact that high levels of UTX-1 extend lifespan via a unique mechanism separate to that of *utx-1* mutation. If that is the case, then the combination of the two might give greater lifespan extension than either of them on their own.



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	14.88	0.40	14	22
utx-1(tm3118)/+	21.00	0.80	20	34
utx-1(tm3118)+ utx-1DD::GFP	20.09	0.80	18	34

Figure 5.6 Overexpression of demethylase dead (DD) UTX-1 does not affect the lifespan of *utx-1* worms. Heterozygous mutation in *utx-1* extends lifespan (Log Rank Test, $p<0.01$). The DD mutant UTX-1 in a *utx-1(tm3118)* background does not rescue the longevity phenotype (Log Rank Test, $p=0.49$).



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	14.88	0.40	14	22
utx-1(tm3118)/+	21.00	0.80	20	34
utx-1(tm3118)+ utx-1::GFP	29.55	1.74	30	50

Figure 5.7 Overexpression of enzymatically active UTX-1 results in substantial lifespan extension. Heterozygous mutation in *utx-1* extends lifespan (Log Rank Test, p<0.01). (Log Rank Test, p<0.01). The full genomic UTX-1 in *utx-1(tm3118)* background resulted in mean lifespan increase of almost 100% when compared to wild-type worms: mean N2 lifespan: 14.88 days, mean *utx-1+utx-1::GFP* lifespan: 29.55 days (Log Rank test: *utx-1+utx-1::GFP* vs N2, p<0.01; *utx-1+utx-1::GFP* vs *utx-1(tm3118)/+,* p<0.01). The experiment was performed at the same time as experiment in Figure 5.6 and therefore the control strains (N2 and *utx-1(tm3118)/+* were used for both experiments).

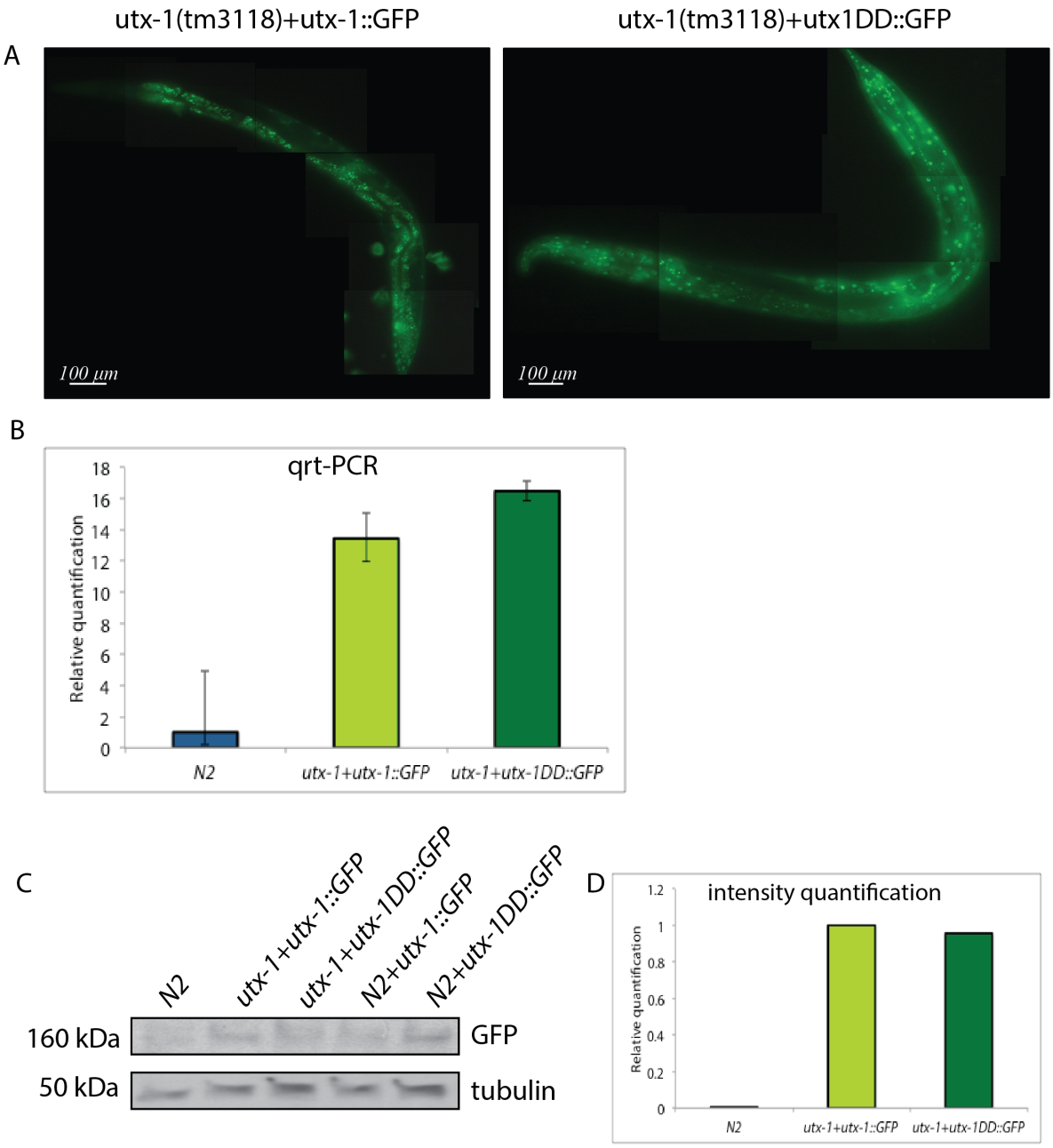


Figure 5.8 Measurement of *utx-1* transcript and UTX-1::GFP protein level in transgenic strains. (A) UTX-1::GFP and UTX-1DD::GFP levels are comparable in staged animals. (B) Levels of *utx-1* transcript in mixed stage animals, measured by qRT-PCR, normalised to levels of *cdc-42* mRNA as internal control and to levels of *utx-1* in day 1 in wild-type animals (C) levels of GFP measured by Western blot, this blot is reused in Figure 5.9 for *N2+utx-1::GFP* and *N2+utx-1DD::GFP* (D) Quantification of Western blot using FIJI software, normalised to tubulin, lack of error bars are due to the fact that the quantification is based on only one sample.

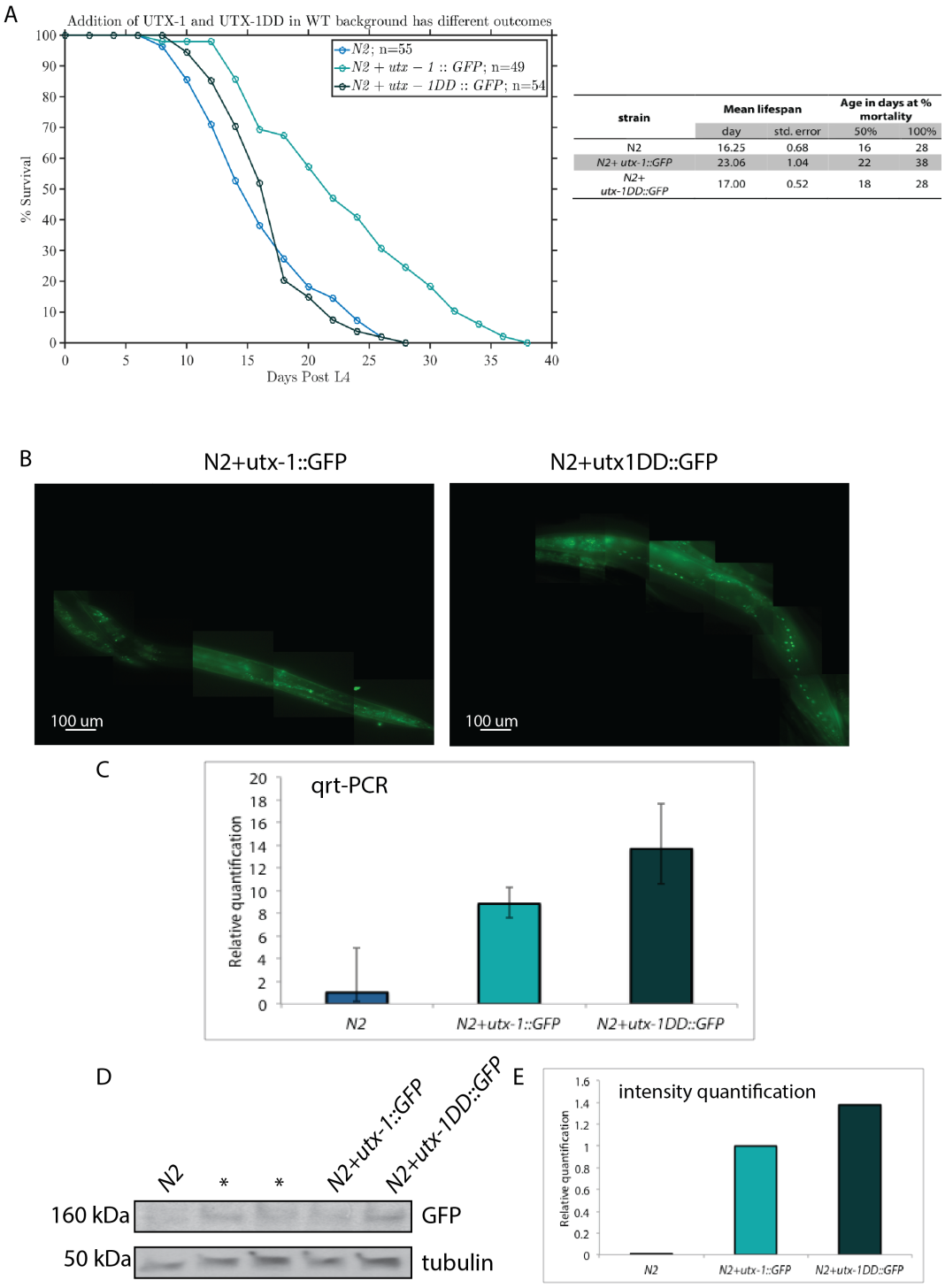


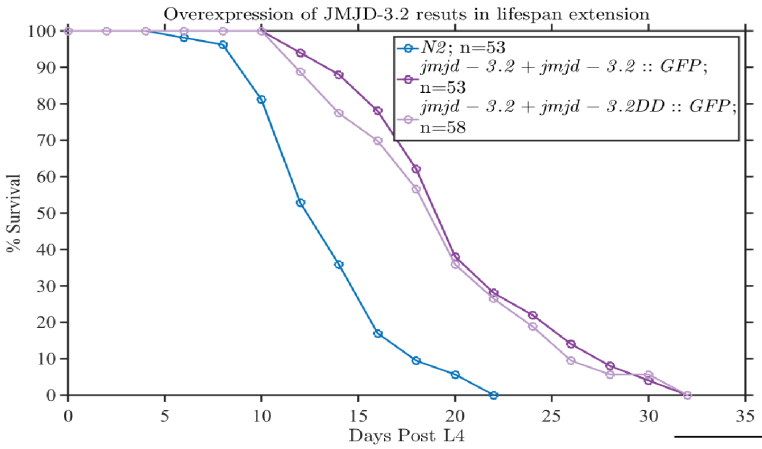
Figure 5.9 Expression of UTX-1 in wild-type background. (A) Expression of *utx-1DD::GFP* in WT strain does not affect lifespan (Log Rank Test, p=0.68). However expression of enzymatically active *utx-1::GFP* increases lifespan (Log Rank Test, p<0.01) (B) UTX-1::GFP and UTX-1DD::GFP levels are comparable in animals at the same stage (C) Levels of the *utx-1* transcript in mixed stage animals correlate with the levels of protein expression, measured by qRT-PCR, normalised to levels of *cdc-42* mRNA as internal control and to levels of *utx-1* in day 1 in wild-type animals (D) Levels of GFP measured by Western blot ('*' - , Western blot reused in **Figure 5.8**) (E) Quantification of Western blot using FIJI software, normalised to tubulin. Lack of error bars are due to the fact that the quantification is based on only one sample.

5.2.3 Overexpression of *jmjd-3.2* demethylase also leads to lifespan extension

We decided to follow up the observation of lifespan extension via *utx-1* overexpression by investigating what happened when another member of the demethylase family, *jmjd-3.2* is overexpressed (Figure 5.10). Interestingly, as mentioned before, the expression pattern of *jmjd-3.2* differs significantly to that of *utx-1*: JMJD-3.2 is abundant during egg and early larval development, however in adult worms it is only expressed in several head neurons (Appendix 5, Figure 5.11). Therefore, overexpression of *jmjd-3.2* under the *jmjd-3.2* promoter might not have resulted in as striking a phenotype of overexpression as that of ubiquitously expressed *utx-1*. Figure 5.10, however, shows that overexpression of *jmjd-3.2* in either *jmjd-3.2(tm3121)* or N2 backgrounds results in significant lifespan extension. Interestingly, in contrast to overexpression of *utx-1,* this lifespan extension is *not* dependent on the enzymatic activity of the protein, as *jmjd-3.2DD::GFP* prolongs lifespan to the same extent as the enzymatically intact version of the protein (in both N2 and mutant backgrounds).

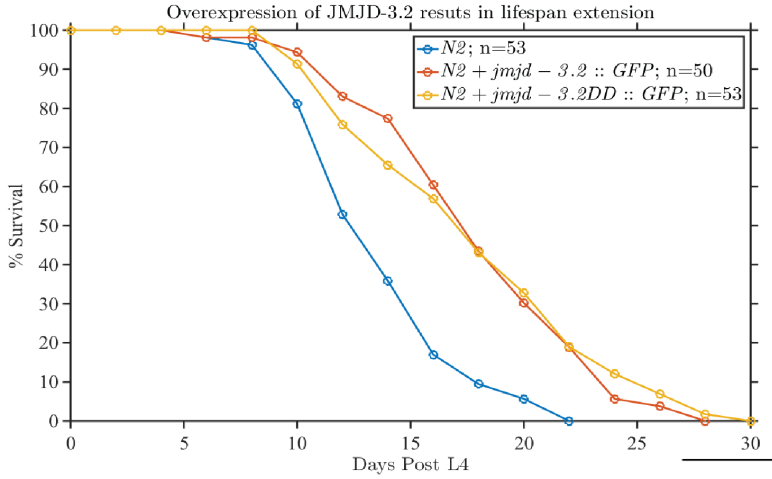
This observation taken with the observation that the lifespan extension difference between *jmjd-3.2* overexpression strains in N2 and mutant background is not statistically significant, suggests that both mutation and overexpression of JMJD-3.2 protein works through the same, non-enzymatic mechanism. All of the lines examined were confirmed to contain detectably higher levels of *jmjd-3.2* expression compared to wild-type measured both by qRT-PCR. Furthermore Western blot against GFP compared the levels of protein expression between the four strains (Figure 5.11) and the levels of transcript correlate well with the levels of protein. It is also indicated that there is some variability in expression between the lines, this might be due to mosaicism of the extrachromosomal arrays.

A



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	13.92	0.48	14	22
jmjd-3.2(tm3121)+ jmjd-3.2::GFP	18.26	0.66	18	28
jmjd-3.2(tm3121)+ jmjd-3.2DD::GFP	18.10	0.71	18	30

B



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	13.92	0.48	14	22
N2+ jmjd-3.2::GFP	20.72	0.72	20	32
N2+ jmjd-3.2DD::GFP	19.89	0.73	20	32

Figure 5.10 Overexpression of JMJD-3.2 in *jmjd-3.2(tm3121)* mutant and wild-type backgrounds results in lifespan extension independent of demethylase activity.

(A) Extrachromosomal expression of *jmjd-3.2* gene (both enzymatically active and DD) in a *jmjd-3.2(tm3121)* background results in lifespan extension (Log Rank test, $p<0.01$), the extension is independent of demethylase activity (*jmjd-3.2(tm3121)+jmjd-3.2::GFP* vs *jmjd-3.2(tm3121)+jmjd-3.2DD::GFP*, p= 0.81). (B) Extrachromosomal expression of the *jmjd-3.2* gene (both enzymatically active and DD) in a wild-type background results in lifespan extension (Log Rank test, $p<0.01$), the extension is independent of the demethylase activity (*N2+jmjd-3.2::GFP* vs *N2+jmjd-3.2DD::GFP*, p= 0.61).

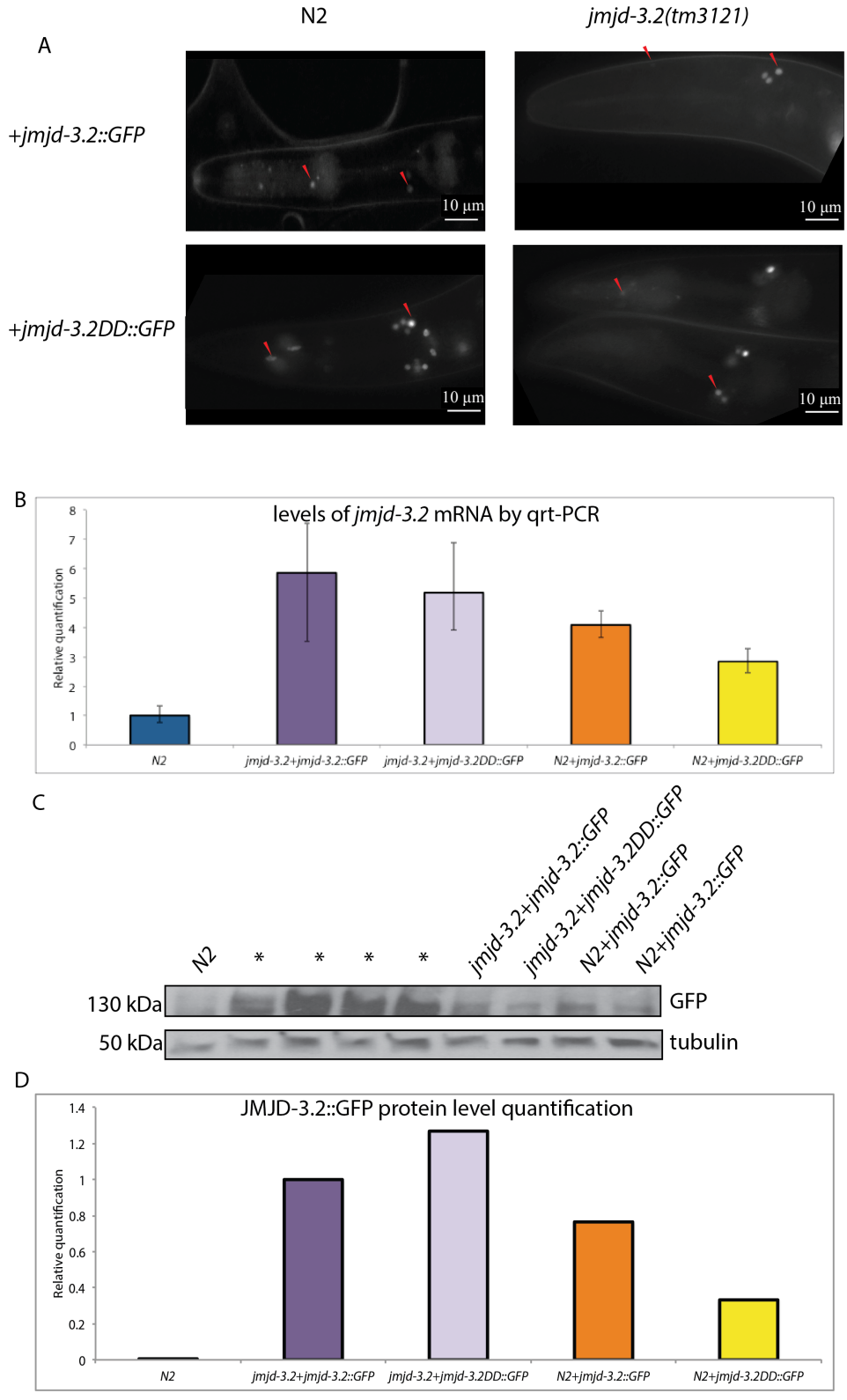


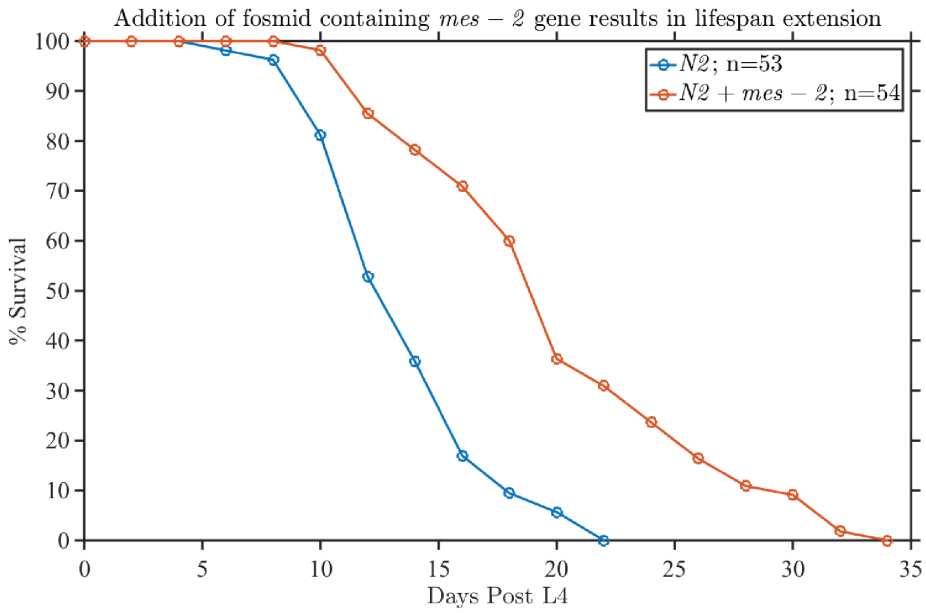
Figure 5.11 *jmjd-3.2* overexpression in transgenic lines. (A) expression of GFP in four transgenic lines, present in head neurons: red arrows point to GFP positive nuclei (B) *jmjd-3.2* transcript levels measured by qRT-PCR, normalised to levels of *cdc-42* mRNA and to N2 strain (C) levels of GFP measured by Western blot ('*' – Western Blot reused in **Figure 5.8**) (D) Quantification of Western blot using FIJI software, normalised to tubulin, lack of error bars are due to the fact that the quantification is based on only one sample.

5.2.4 A *mes-2*-containing fosmid induces lifespan extension

Overexpression or loss of function of either of the H3K27 demethylases causes lifespan extension as does mutation of *mes-2*. I therefore decided to investigate whether overexpression of *mes-2* could also have the same phenotype. I chose to use a fosmid to induce expression of *mes-2* at higher level because of difficulties I encountered in cloning the gene. Appendix 6 and Figure 5.12 (B) shows details of the fosmid chosen and the other genes present on it. Intriguingly, expression of this fosmid did, in fact, induce longevity (Figure 5.12 A), however this did *not* correlate to high levels of *mes-2* transcript (Figure 5.12 C). The obvious reason for lack of overexpression is the fact that *mes-2* is known to be mostly expressed in the germline cells, while extrachromosomal arrays are thought to be silenced in the *C. elegans* germline (Kelly and Fire 1998).

The question thus arises: what is the potential pathway through which the lifespan extension is induced in these fosmid-containing transgenic lines? There are several potential explanations for this observation. Firstly, it could be an array-mediated RNAi effect that induces repression of the genes on the array, and therefore also endogenous copies of *mes-2* causing lifespan extension as shown earlier. This repression should however be confirmed by reduction in *mes-2* levels measured by qRT-PCR which is not the case. Alternatively, the construct might be overexpressed in non-germline cells enough to cause phenotypic lifespan extension, but due to the volume and mix of cells used in analysis (whole animal extracts) this is not revealed by qRT-PCR. Finally, the lifespan extension could be due to overexpression of any of the other genes on the fosmid. None of these has a known lifespan function, however they might still affect one of the genetic pathways or act in a hitherto unknown mechanism.

A



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	13.92	0.48	14	22
N2+*mes-2*	20.19	0.80	20	32

B

gene on WRM0629dF11 fosmid	function
imb-2	reproduction, germline development
acr-20	ligand-gated ion channel
sdz-26	E-lineage specific gene
F26H11.8	unknown
kbp-3	kinetochore-binding protein; germline development
nurf-1	NURF chromatin remodeling complex, vulva development
mir-5595	miRNA;

C

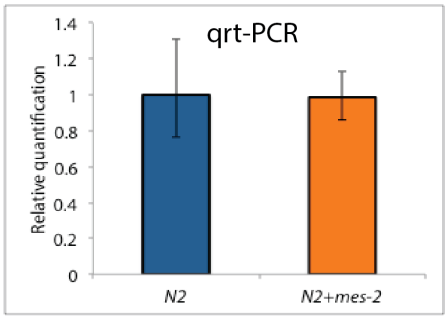


Figure 5.12 Worms expressing fosmid containing the *mes*-2 gene have a longer lifespan. (A) injection of a WRM0629dF11 fosmid (see Appendix 6) into a wild-type background results in significant lifespan extension (Log Rank Test, $p<0.01$). (B) List of genes covered by the fosmid and their predicted function. (C) Levels of *mes-2* transcript in mixed stage worms measured by qRT-PCR, normalised to levels of *cdc-42* mRNA as internal control and to levels of *mes-2* mRNA in wild-type animals

5.2.5 Extension of lifespan correlates with healthspan in long-lived worms

The healthspan and lifespan of an organism are inherently connected. The goal of ageing research is not necessarily to artificially prolong long life (although this may also be a result) but rather to increase the period of wellbeing and healthy life lived.

C. elegans is a very simple multicellular organism and yet even in this organism one can quite easily distinguish a young individual from an old one just by observation. There are in fact several qualitative and quantitative ways of describing deteriorating health in worms, from general phenotypic observations, through movement and pharyngial pumping speeds, to analysis of fat content, gene expression, and proteome changes (Collins 2008a).

Information about healthspan can be inferred from the shapes formed by the lifespan curves. If the whole population lives longer, the whole curve is shifted in a corresponding fashion to the right; the likelihood is that the whole process of ageing is also slowed down and deleterious effects happen later. Therefore what we are truly looking to find is not (merely) a simple extension of maximal lifespan, but a robust and replicable change in healthspan. For that reason I decided to monitor markers of health span in some of the long-lived strains described earlier in this thesis. Figure 5.13 presents representative mutant worms from an ageing population over time.

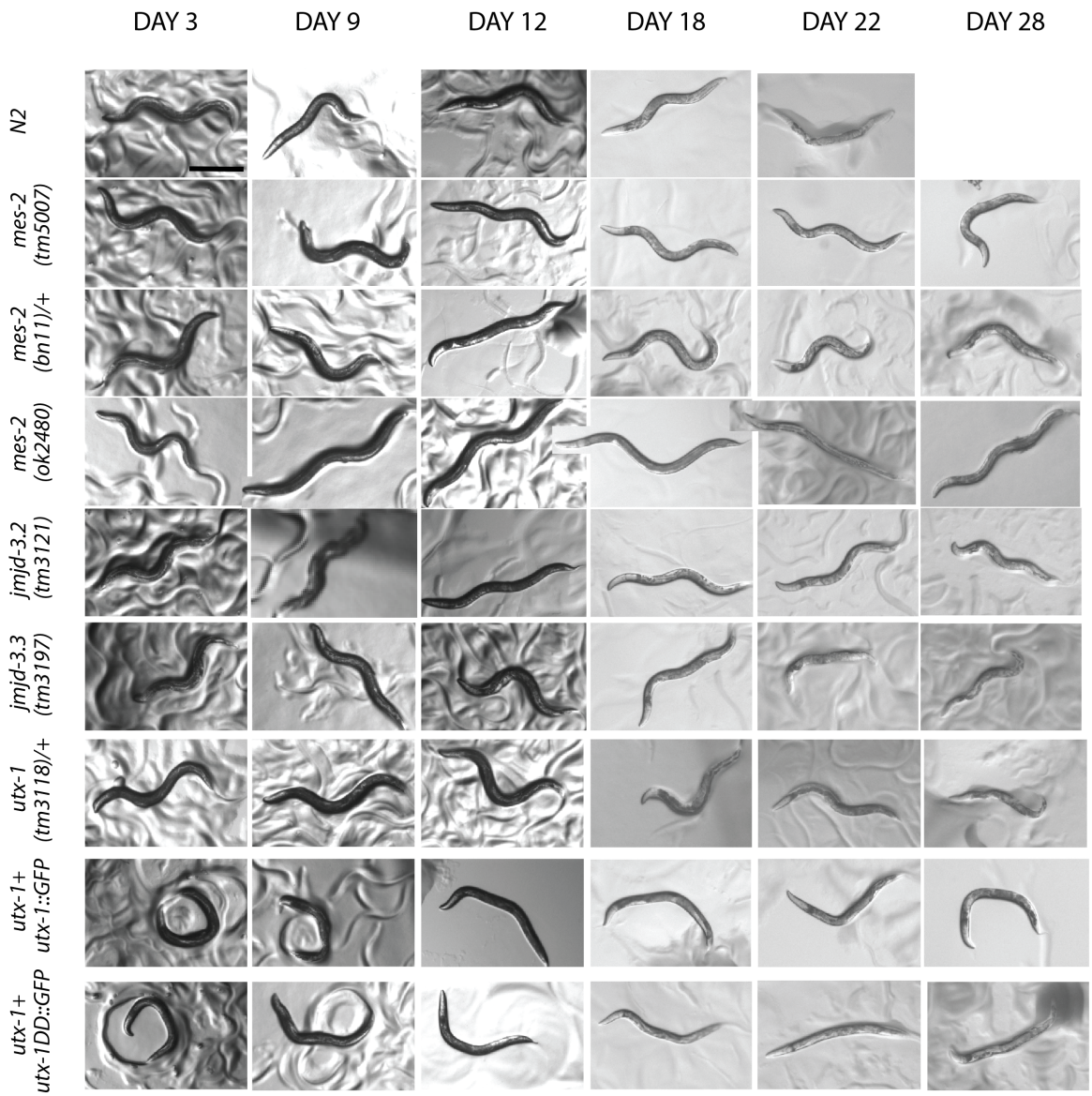


Figure 5.13 Longer lifespan correlates with longer healthspan in analysed mutants. Representative phase contrast images of various long-lived mutants compared to wild type animals at chosen days of adulthood. Note the lack of an image of an N2 worm at day 28 which is due to 100% mortality reached by that time point. Image acquisition: Leica MZIII dissection microscope with attached Hamamatsu camera and HCImage Live (V3.0) imaging software. Scale bar shown in N2 day 3 image applies to all images and represents 500 μm.

The progressive deterioration of the tissues can be readily seen in Figure 5.13 and it is relatively easy to compare the effect of longevity on the appearance of the worm. One striking point is the observation of growth and then progressive shrinking of the worms as they age (e.g. see N2 at day 3, compared to day 12 and finally to day 22). This has previously been described in the literature, and it has even been suggested that the initial size of the worm can be a good predictor of its maximum lifespan (Pincus, Smith-Vikos, and Slack 2011): worms that are larger early in life tend to live longer. Interestingly, the *mes-2(ok2480)* mutant seems to resist this age-dependent shrinking; what is more it seems to continue growing for much longer than other (even long-lived) mutants and reaches a larger size. There are also other easily observable differences, especially at later stages of life: comparing *utx-1* heterozygous mutants with a WT *utx-1* rescue (*utx1(tm3118)+utx-1::GFP*) and *utx–1DD* rescue (*utx-1(tm3118)+utx-1DD::GFP*), it is clear that by day 28 the strain overexpressing the enzymatically active *utx-1* is looking healthier and bigger than the other two strains.

An alternative more quantitative approach to healthspan estimation is a thrashing assay in which a worm is put into a liquid and allowed to move freely (Collins 2008b). The average number of "thrashes" per minute (body bend into one direction and back) as compared to a wild-type worm is an estimate of the health of the worm. Increased thrashing early in adulthood does not necessarily translate to a longer lifespan, however the resistance to a decrease in thrashing rate over chronological time strongly suggests worms with increased movement capabilities and therefore, indirectly, to an increase in healthspan.

In Figure 5.14, I show the changes in thrashing rates in various long-lived mutants with age. Young WT worms have an average thrashing rate of 99.4 thrashes/minute, this rate decreases at day 9 and by day 12, over 50% decrease in average thrashing rate is observed (40.9 thrashes/minute). The age-related decrease in thrashing is visible in all strains, however, all of the long lived strains retain the movement capabilities better than the WT strain. Overall, as Figure 5.14 shows, by this measure all the mutants with even slightly prolonged life have a

greater healthspan than wild-type worms. This is especially evident at day 12 and day 15. At day 15, the average thrashing rate of WT worm was 24.6 thrashes/min. In contrast, thrashing rate of all long-lived mutants at day 15 was above 40 thrashes/min (almost double the rate, one way ANOVA and T-test, $p<0.01$). This experiment has also been repeated without the putative confounding activity of FUDR. Comparable results were obtained, suggesting FUDR has no effect on healthspan extension (Appendix 7).

Unfortunately this technique, despite being quite robust, has several potential problems. Firstly, I could not assess any of the worms overexpressing *utx-1* or *jmjd-3.2* because of the effect of a co-injection marker (*rol-6*), which causes the worms to express a 'roller' phenotype. This marker affects movement of *C. elegans* on both solid media and in the liquid, it does not however cause changes to lifespan (Appendix 8). Since this causes worms to have trouble with thrashing or swimming, it makes the thrashing assay impossible to perform and or quantify in a way that can be related to data from non-roller worms. Additionally confounding was the increased size (especially length) of the *mes-2(ok2480)* mutant which made the thrashing scoring problematic. For example, at day 9, it seems as if thrashes/min are actually lower in that mutant, despite it being in good health and living longer than the wild-type worm. In fact it turns out that its increased length makes the worms curl more than thrash in the liquid, giving rise to an apparently lower thrashing rate (although the amplitude of the movement was actually greater). This suggests that this technique might be better suited to comparing worms later in life, where the potential for differences in amplitude is likely lower and would most effectively be used in combination with other techniques (such as lifespan, phenotype observation, movement tracking on solid media).

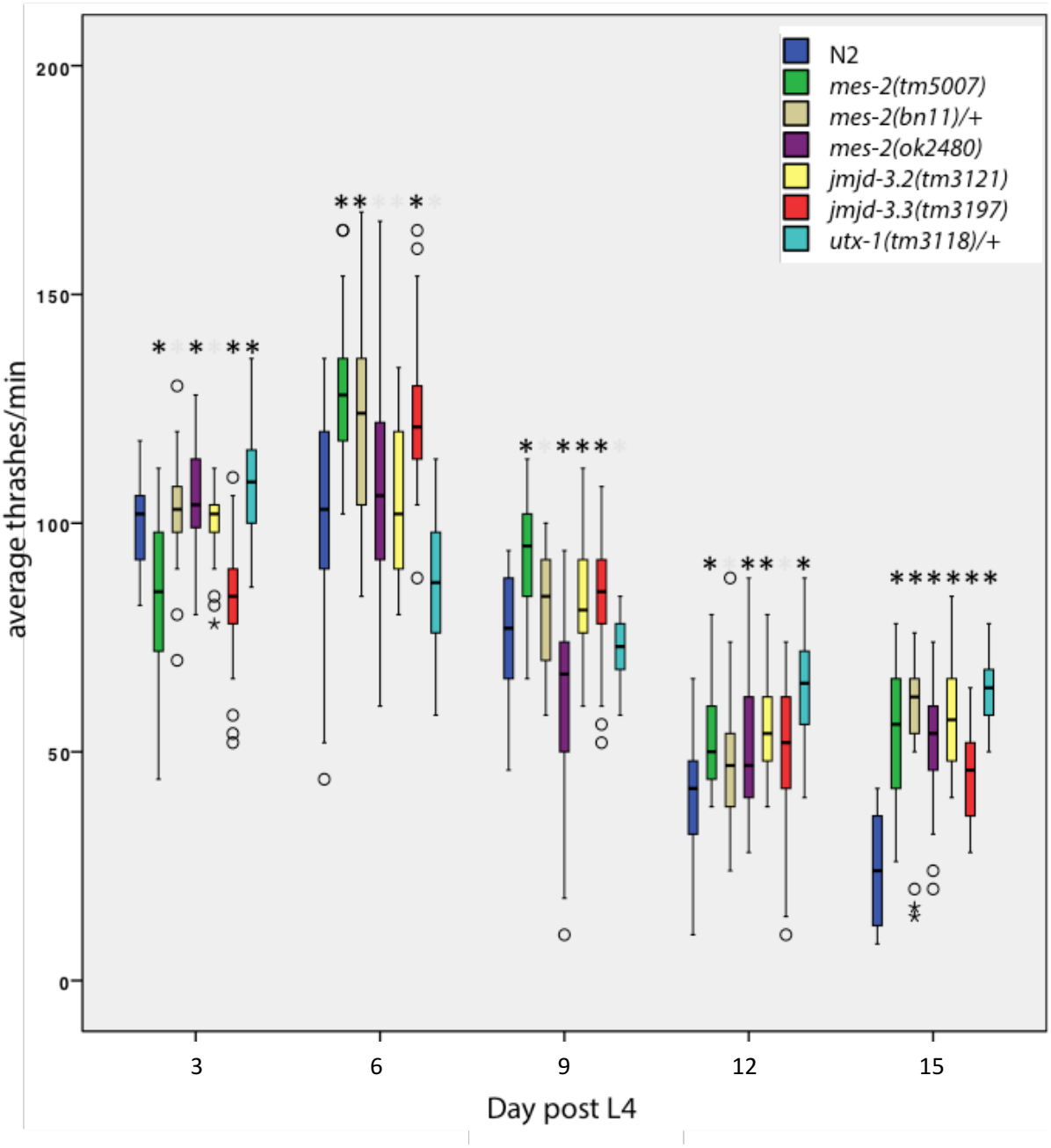


Figure 5.14 Healthspan improvement can be quantified using a thrashing assay.

10 worms per strain were used for every time point, recording thrashing during three 30s periods. '*' above the box-whisker plot signifies a statistically significant difference from a WT strain at the day of the test (One way ANOVA and Student's T- test, $p<0.01$). 'o' above and below the plot and '*' under the plot signifies outliers and extreme outliers respectively. The box represents the interquartile range of data, the central line indicates the mean, whiskers show 95% confidence interval. Data was analysed and plotted using SPSS software.

5.3 DISCUSSION

5.3.1 Members of H3K27me3 demethylase family affect lifespan

Knowing that *jmjd-3.2* is a member of a family of demethylases, I investigated other members of the family as well as the *jmjd-3* triple mutant for possible evidence of overlap in function. Neither *jmjd-3.1* nor *jmjd-3.3* RNAi knock-down resulted in lifespan extension. Interestingly, mutation in both of these genes do not affect global levels of methylation according to previously published data (Vandamme et al. 2012). The triple mutant extended lifespan to the same degree as *jmjd-3.2,* however Western blotting to determine the changes in global methylation levels suggests that there is a relative global increase in H3K27me3 in the multiple mutant when compared to any of the single *jmjd-3* mutants (Vandamme et al. 2012). Additionally there is a marked decrease in both H3K27me2 and me1 in the triple mutant as compared to any of the single mutants. Taken together, the data from lifespan curves and Western blots, these data suggest a degree of redundancy in methyltransferase function of the JMJD-3 proteins but not in the lifespan regulating function. This might be evidence supporting the hypothesis of decoupling of the *jmjd-3.2* role as methyltransferase and as lifespan regulating protein.

Interestingly, JMJD3 in human cells has been shown to be mostly activated under particular stress conditions (Agger et al. 2009), which could be the reason why *jmjd-3.1* and *jmjd-3.3* mutations have little effect on total H3K27 methylation levels. Their function may only become crucial when *C. elegans* is challenged by difficult environmental conditions.

Mutation in the final member of the demethylase family, *utx-1* has been previously described as having a lifespan extending consequence in *C. elegans* (Maures et al. 2011; Jin et al. 2011). The depletion of both *utx-1* (post-developmentally) and *jmjd-3.2* (via mutation) in the same organism resulted in an additive lifespan extension (Figure 5.4), which implies that these two demethylases could work by different mechanisms.

5.3.2 Overexpression of demethylases results in lifespan extension

In this thesis, initially, I have shown that loss-of-function mutations in both *utx-1* and *jmjd-3.2* demethylases extend lifespan (Figure 4.9 and Figure 5.5). Interestingly, both of these demethylases also increase *C. elegans* lifespan when overexpressed under their genomic promoter (Figure 5.7 and Figure 5.10). There are, however, several crucial differences between these two genes. *utx-1* overexpression-driven lifespan extension is dependent on its demethylase activity (Figure 5.6), whilst longevity is increased in strains with an extrachromosomal array expressing either the functional *jmjd-3.2::GFP* or the enzymatically inactive *jmjd-3.2DD::GFP* (Figure 5.10). This suggests that lifespan extension via *jmjd-3.2* overexpression is independent of enzymatic activity, rather it is its presence as a scaffolding protein that confers the phenotype or perhaps its ability to stabilise various complexes. These results correlate with the observation made by De Santa et al. (2009) who showed that in human cells, JMJD3 does not actually alter all of the H3K27me3 loci it is associated with.

Another important difference between overexpression of the two demethylases is the effect of background mutation in the strain. In the case of *jmjd-3.2*, overexpression of the gene in either the wild-type N2 or the *jmjd-3.2(tm3121)* background results in a similar level of lifespan extension. Knowing that *jmjd-3.2(tm3121)* in itself also induces longevity, this suggests that the overexpression and loss-of-function could work through the same pathway. Conversely, overexpression of *utx-1* in a *utx-1(tm3118)* background gives significantly greater lifespan extension than in an N2 background. That suggests that the effects of *utx-1* mutation and overexpression could be additive and therefore occur via at least partially non-overlapping pathways.

Interestingly, overexpression of either of the discussed demethylases result in very different levels of expression of the proteins, as *utx-1* is mostly a ubiquitous protein, while *jmjd-3.2* overexpression under the endogenous promoter is restricted to head neurons as previously described (Figure 5.11). This is mirrored in Western blot quantification of UTX-1 and

JMJD-3.2 in overexpression strains which show that levels of UTX-1::GFP are significantly higher than those of JMJD-3.2::GFP (Figure 5.11, *utx-1::GFP* overexpressing strains marked with '*').

That such a limited expression of *jmjd-3.2* still can drive significant lifespan extension is notable, and begs many questions as to the mechanism it uses to achieve longevity. Is it the expression in the adult neurons that is important or the fact that *jmjd-3.2* is the most abundant H3K27 demethylase in early development? This question may be answered by the use of RNAi against known neuronally-expressed longevity-regulating genes such as *osm-3* (Alcedo and Kenyon 2004) in the strain overexpressing *jmjd-3.2*. If lifespan extension in this experiment is additive, it could imply that it is embryonic rather than neuronal *jmjd-3.2* function that is important for longevity. Overexpression of *jmjd-3.2* under a specific neuronal promoter could also be investigated. If this resulted in lifespan extension, it would strongly indicate that is is the neuronal role of *jmjd-3.2* that establishes longevity.

5.3.3 *mes-2*-containing fosmid expression extends lifespan

Expression of a *mes-2*-containing fosmid from an extrachromosomal array results in lifespan extension despite the fact that levels of *mes-2* transcript seem to be unchanged. Germline is known to be the major site of MES-2 expression, however, (Holdeman, Nehrt, and Strome 1998) also note low levels of MES-2 in the intestine of the worm, which I also confirmed using a strain containing a single integrated copy of *mes-2::GFP* (data not shown). Due to repression of extrachromosomal arrays in the germline (Kelly and Fire 1998), the only potential over-expression site of *mes-2* would thus be intestine. Overexpression of *mes-2* in only 16 nuclei of the worm might therefore be undetected when looked for using whole-worm extracts. However, biological effect on lifespan could still be conferred, despite the changes in relatively small population of cells. It has been suggested that mutations in *mes-2* actually lead to derepression of extrachromosomal arrays (Kelly and Fire 1998), meaning that fosmid

expression in a *mes-2* mutant background should therefore be derepressed, thus could provide one way to gain more insight into this mechanism of lifespan extension.

5.3.4 Extension of lifespan correlates with healthspan in long-lived worms

Organismal lifespan has become an easy proxy for scientists studying the biology of ageing. However what is even more important is that in all of the long-lived worm mutants described in this thesis, prolonged lifespan correlates very well with prolonged healthspan. Using *C. elegans* as a model means that there are however many limitations as to what we can quantify as a measure of health decline caused by ageing. General phenotypic changes such as tissue degradation or gonadal misplacement, though initially obvious, are difficult to quantify. This is why other measures have been developed such as the movement assays (Collins 2008b). In fact, movement and strength have been strongly correlated with healthspan and probability of death in many organisms, including humans (Laukkanen, Heikkinen, and Kauppinen 1995; Rantanen et al. 1999; Shinkai et al. 2000) indicating that this kind of assay might be very informative.

The thrashing assay performed (see Figure 5.14) showed that by day 15, all of the long-living strains outperformed a wild-type strain in thrashing rate. As I have discussed (see section 5.2.5), some problems with the use of particularly long mutants e.g. *ok2480*, meant that earlier in life, differences are not as distinguishable. However, an improvement on this technique could be used where both the thrashing rate and the amplitude of thrashes is taken into consideration. This kind of experiment can be accomplished using a video camera and already developed worm-tracker software (Husson et al. 2005).

Furthermore, developments have been made in techniques that allow measurement not only of a worm's motility but also its strength. 'Nemaflex' is a microfluidic device that includes agar-based micro-pillars, that when displaced by a crawling worm become a measure of the force the animal is able to exert (Laranjeiro, personal communication, *C. elegans* 2015 20th

International meeting). This kind of device, not only gives valuable quantitative data but also can be used for different mutants, such as the *rol-6* mutants that cannot be measured by a conventional thrashing assay.

In summary, in this chapter I have shown that overexpression of the lysine demethylases *utx-1* and *jmjd-3.2* results in lifespan extension. The mechanisms through which these genes upregulate lifespan are however separate, with *jmjd-3.2* independent and *utx-1* dependent on the demethylase enzymatic activity. Further I show that all examined long-lived mutants also exhibit extended healthspan. The next step is to investigate the pathways through which these genes regulate longevity.

CHAPTER 6: MECHANISMS OF LIFESPAN EXTENSION BY H3K27 METHYLATION REGULATORS

6.1 INTRODUCTION

As shown in the previous chapters, change in the wild-type expression of several H3K27 methylation-controlling factors can result in the extension of *C. elegans* lifespan. In this chapter, I will explore the pathways that these factors function through, as well as investigate changes at the level of histone modifications and gene expression.

6.1.1 Tissue-specific modulation of lifespan

Considering the complexity of the ageing phenotype, the fact that changes to just one gene can significantly prolong lifespan is truly remarkable. What is more, these changes do not have to be introduced in the whole organism in order to have an effect but only in certain crucial tissues. It has been confirmed in a number of species that signals from some tissues like neurons or intestine have more effect on lifespan regulation than signals from the rest of the organism.

In *Drosophila melanogaster,* mutations in the *Mgat1* gene (which codes for an enzyme involved in paucimannose N-glycan synthesis important for homeostasis of cell membranes and control of attachment of receptors to the cell surface) result in lifespan decrease as well as various locomotion and brain abnormalities (Sarkar et al. 2010). Interestingly, these experiments showed that neuronal-specific rescue of *Mgat1* not only rescues the wild-type lifespan but also significantly lengthens it, suggesting a lifespan-controlling function of *Mgat1* that is restricted to the CNS. Another experiment from a fly model shows that moderate inhibition of IIS pathways in somatic stem cells extends lifespan by improving the proliferative homeostasis of the gut, in this way connecting gut signalling to lifespan (Biteau et al. 2010). The group responsible for this discovery also indicated a significant correlation

between rates of intestinal proliferation and lifespan for a large number of *D. melanogaster* mutants.

Further experiments on the tissue specificity of lifespan regulation have been conducted in *C. elegans* and mostly concentrate on three tissues: gonad, neurons and gut. Initial experiments suggesting that neuronal signalling can be a significant part of the lifespan regulation network came from the Kenyon lab (Apfeld and Kenyon 1999). They knocked down nine genes leading to various defects in sensory cilia and observed lifespan extension (*daf-19, che-2, che-13, osm-1, osm-5, osm-6, che-3, che-11, daf-10*). As *C. elegans* senses its environment through its ciliated sensory neurons, this suggested that sensing changes in the outside environment (such as abundance of food) are important for establishing longevity. In work following that, Alcedo and Kenyon (2004) ablated gustatory and olfactory neurons and showed that both can have a significant effect on lifespan regulation via different pathways (as ablation of either type of neuron increases lifespan, and ablation of both resulted in additive lifespan extension).

Similar crucial ablation studies were also reported by the Kenyon lab in the worm gonad; these showed that ablation of germline precursors cells (Z2 and Z3) results in significant lifespan extension. However, if the somatic gonad precursor cells (Z1 and Z4) are destroyed early in development together with the germline precursors, this lifespan extension is completely abrogated, suggesting an important role for somatic gonad in lifespan control (Hsin and Kenyon 1999). Interestingly, lifespan control pathways by gonad and neuronal tissues converge, as ablating all germline precursor cells (Z1-Z4) as well as olfactory neurons re-established the longevity phenotype observed following the removal of the Z2 and Z3 cells (Alcedo and Kenyon 2004). The authors suggested that hormonal control from the neurons could be at the basis of this regulation, sending the signal and allowing the somatic gonad to influence lifespan.

Further evidence supporting neuronal control of lifespan was provided by Wolkow et al. (2000) who showed that restoring *daf-2* expression solely in the neurons of *daf-2* mutants restores wild-type lifespan, while muscle- or intestine- driven *daf-2* was not able to rescue this phenotype. In contrast to that, Libina, Berman, and Kenyon (2003) conducted a similar experiment, but in a *daf-2 daf-16* double mutant (with a short, *daf-16*-like lifespan). In an elegant set of experiments they showed that expression of *daf-16* under a neuronal specific promoter (*unc-119*) could only restore 5-20% of the *daf-2* longevity phenotype, while muscle specific promoter (*myo-1*) could not rescue the phenotype at all. Intriguingly, an intestine specific promoter (*ges-1*) was able to restore over 50% of the phenotype, suggesting this tissue is where most of the *daf-16* longevity function resides, despite DAF-16 being expressed in many cell types.

6.1.2 H3K27 methylation and gene expression

H3K27me3 is mostly associated with the repression of transcription in a cell-type specific manner, mostly around the Transcription Start Site (TSS) of the repressed genes. Due to the importance of epigenetic control of gene regulation, changes in global levels of H3K27me3 have become a point of interest, however it has been found that the changes in global levels of H3K27me3 depend on the animal, tissue and ageing model used. For example in aged brain of the SAMP8 (Senescence Accelerated Prone) mouse, global levels of H3K27me3 increase with age (Wang, Tsai, Yew, et al. 2010) in agreement with data from Liu et al. (2013), who looked at methylation changes in ageing muscle stem cells of a mouse. Conversely, HGPS cells (accelerated ageing model) have got an altered epigenetic landscape with a shift towards a general loss of H3K27me3 (Pegoraro et al. 2009). This also seems to be the case for *C. elegans* somatic cells, as shown by Maures et al. (2011).

To fully understand these data it is important to correlate changes in histone marks with their effect on gene expression and therefore protein levels. Early attempts at looking at protein level changes come from Johnson and McCaffrey (1985) who showed, using 2D PAGE gel

analysis, that there are not many relative changes in types and levels of proteins made in young and old worms (only about 1% of 600 proteins). This is in sharp contrast to around 11% of proteins that change during development. This became one important argument against the "programmed ageing" hypothesis. Following this protein work, Fabian and Johnson (1995) also investigated changes in gene expression profiles by probing *C. elegans* cDNA libraries with cDNAs isolated form yound and old nematodes. In this early work, they found only a handful of genes (exactly twelve) with altered expression in aged animals. This could be attributed to the crude techniques used in comparison to the currently available microarray and sequencing technologies.

Thanks to advances in molecular techniques, Lund et al. (2002) identified around 200 genes (1% of the nematode genome) that change with age in adult *C. elegans*, some of which act through insulin signalling. However none of the major regulatory nodes like *daf-2* or *age-1* were identified in this work. These results in worms correlate well with studies in ageing mice muscle tissue that found only 2% of the genes to be significantly up- or downregulated over time. (Lee, Weindruch, and Prolla 2000). Together, these findings suggest that ageing is probably not the result of significant changes in global gene expression. It is more plausible that small, cumulative changes in expression of particular genes, as well as stochastic effects of molecular and cellular damage, have greater effects on the ageing phenotype.

H3K27me3 is generally correlated with transcriptional repression (Pauler et al. 2009). Interestingly, however, Young et al. (2011) identified genes that are highly expressed despite showing high levels of H3K27me3. In their work, they noted that a significant proportion of genes with the H3K27me3 mark could be divided into three groups: 'promoter', 'TSS' and 'broad' (see Figure 6.1). TSS profile genes are described as having a strong and narrow peak of enrichment of H3K27me3 around the TSS, but with little to no methylation associated with the gene body. The opposite profile is the 'broad' category, in which genes are strongly enriched for H3K27me3 in the gene body, but have lower trimethylation upstream of the TSS.

The last type of methylation is the promoter group, which shows a significant, broad peak of H3K27me3 upstream of the TSS, with methylation depleted in the gene body.

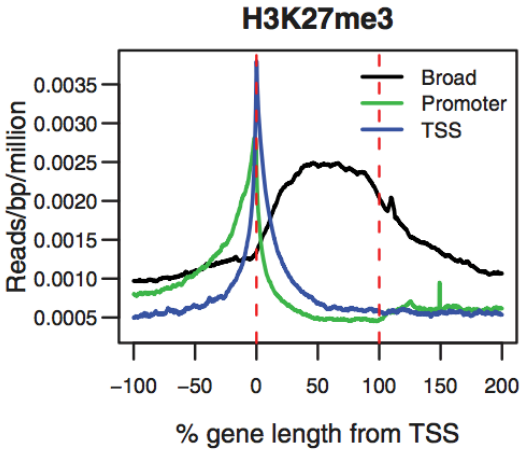


Figure 6.1 Three types of H3K27me3 profiles across the gene – from (Young et al. 2011). Average scale enrichment plot was obtained from ChIP-seq in embryonic stem cells.

These three categories correlate with particular types of gene expression. The 'broad' group of genes show highest transcriptional repression and therefore lowest gene expression. The TSS marked genes also fall into the 'repressed' category, as their mRNA levels were significantly lower compared to an average gene on the array analysed. On the other hand, the 'promoter' set of genes was highly expressed despite having high levels of H3K27me3 at their promoters. Gene ontology analysis performed on the 'promoter' group of genes shows enrichment for cell signalling, especially Ras pathway involvement (Young et al. 2011).

Interestingly, the 'promoter' profile of H3K27me3 was also correlated with H3K4me3, H3K36me3 marks and RNA pol II binding, which are all signs associated with active transcription (which is consistent with high expression levels). The other classes did not show any enrichment for H3K36me3 or RNA pol II (although the TSS class was also strongly enriched for H3K4me3). Young et al. (2011) suggested that genes with H3K27me3 'promoter' type profiles are actually highly expressed while the 'TSS' category of genes show bivalent chromatin (containing both activating and repressing marks) and are therefore poised to respond to regulatory cues.

The work cited in this section implies that ageing might not be a result of global changes, in this case in histone modifications or gene expression. It also suggests a range of potential

roles for H3K27me3. Thus, global changes in methylation are more complex than originally imagined; this suggests that they might not be informative in terms of understanding lifespan regulation.

6.1.3 Aims

In earlier chapters I have shown that *utx-1* and *jmjd-3.2* overexpression result in lifespan extension. My data also suggest that they regulate lifespan by different mechanisms as *jmjd-3.2*-overexpression-driven lifespan extension is not dependent upon demethylase function while *utx-1* overexpression only extends lifespan if the enzymatically-active form of protein is overexpressed. To look into the mechanisms of this lifespan extension, I assessed the potential targets of UTX-1. First, I tested the correlation between global changes in methylation levels and ageing in various long-lived mutants. Next, I aimed to find out whether changes in H3K27me3 at *daf-2* are responsible for changes in gene expression and longevity in both wild-type worms and various overexpression strains. Finally, I investigated whether known longevity pathways in *C. elegans* could modulate the lifespan extension conferred by my previously-described mutants. I also investigated the tissues responsible for lifespan extension by *utx-1* overexpression.

6.2 RESULTS

6.2.1 Changes in global levels of H3K27 methylation in aged tissues

Work aimed at investigating whether global levels of various modifications change among ageing *C. elegans* as in other models has been reported (Wang, Tsai, Yew, et al. 2010; Liu et al. 2013). Maures et al. (2011) used a germline-less mutant *glp-1* mutant to investigate global changes in H3K27me3 in somatic cells. In their publication Maures et al. (2011) shows a significant loss in H3K27me3 levels with age. This finding correlates with the finding that human HGPS cells also lose their repressing mark as they age (Shumaker et al. 2006). There is however a possibility that the decrease is an artefact of the loading of the Western Blot (H3 levels used as loading control also decrease significantly with age).

Observation of global changes in H3K27me3 that can be correlated with the age, prompted me to investigate whether global H3K27 methylation levels could be a good predictor of lifespan. Due to problems with H3K27me3 antibody, in next figure I present data obtained with H3K27me2 antibody, and try to correlate that with what we know about trimethylation of H3K27 and longevity of strains in question. Decrease in H3K27me3 with age as described in literature by Maures et al. (2011) could be correlated with increased levels of H3K27me2 or H3K27me1. Figure 6.2 A and B, show that in wild type strain (N2), at day 10, there is a marked increase in H3K27me2. This observation could be suggestive of low levels H3K27me2 being therefore a mark of longevity.

I also investigated whether changes in the H3K27 methylation status found in ageing N2 worms can be observed in ageing *utx-1*-overexpressing animals (Figure 6.2 A and B). The reason why I chose these mutants for investigation, is a significant lifespan increase of *utx-1+utx-1::GFP* strain in comparison to *utx-1+utx-1DD::GFP* (Figure 5.6 and Figure 5.7). despite their identical genetic backgrounds (the *utx-1(tm3118)* mutation). Figure 6.2 A and B shows that the above mentioned strains, do differ from each other in their global H3K27me2.

At day 1 levels of H3K27me2 in both strains are comparable to that of a WT worm. However, at day 10, there is a marked increase in H3K27me2 in *utx-1+utx-1::GFP* strain but not in the strain overexpressing enzymatically inactive protein. Interestingly strain overexpressing *utx-1* has a significant lifespan extension, which doesn't support the hypothesis of the low levels of H3K27me2 being correlated with longevity.

However, considering the correlation of loss of H3K27me3 and potential rise of H3K27me2 with age in WT worms, I decided to investigate this further in other various long-lived mutants. I assessed levels of H3K27me2 in mixed stage population of *C. elegans* (compared to wild-type strain). The data in Figure 6.2 (C) shows that there is a considerable variation in global levels of H3K27me2 between various long-lived mutants that does not correlate with lifespan. This suggests that the correlation of changes in methylation levels with age that might be true for wild-type worms does not necessarily apply to various mutants. In fact, most of the mutants presented in Figure 6.2 (A) appear to have increased global levels of H2K27me2. Considering the fact that we are looking at global changes in whole worm extract (mixed somatic and gonad tissues), this experiment needs to be repeated, to investigate tissue specific changes with age (or at least differentiating gonads from somatic tissues) to provide clearer results.

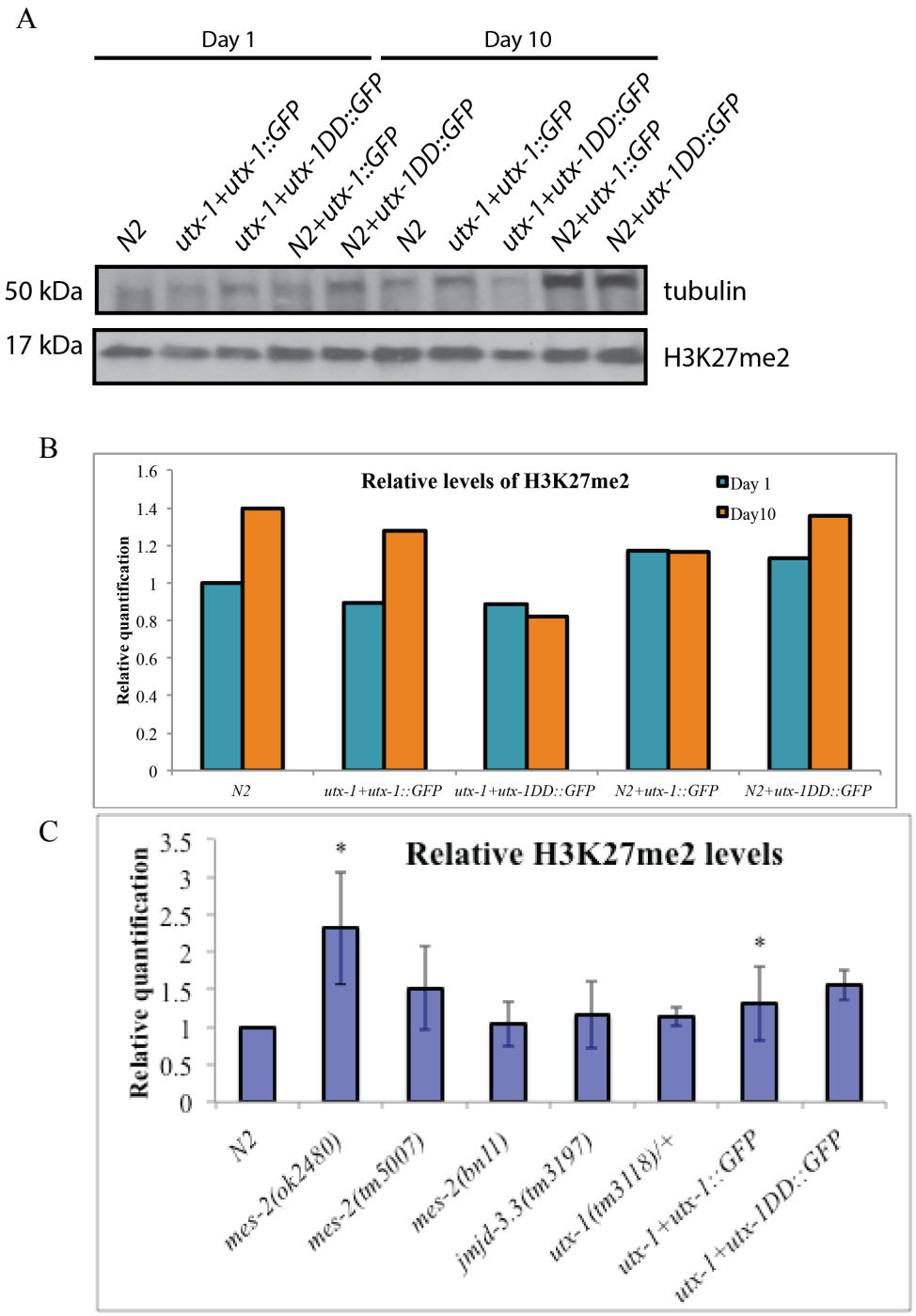


Figure 6.2 Changes in global H3K27 dimethylation in long-lived mutants. (A and B) Western blot and its quantification showing changes in H3K27me2 in long-lived *utx-1* overexpression lines as compared to N2 worms. Quantification of western blots is normalised to tubulin levels, lack of error bars results from quantification of a single Western blot. (C) Image J quantification of western blot experiments (data not shown) probing for H3K27me2, normalised to tubulin. Each strain has at least 3 biological repeats and the bar graph shows an average level of H3K27me2 relative to N2 levels. Error bars represent a standard error of the samples. '*' represents samples that are statistically significantly changed from N2 sample.

6.2.2 Changes in H3K27 methylation status and transcript levels at candidate genes

Due to the fact that global levels of H3K27 methylation might not be a dependable longevity predictors, I decided to investigate whether changes to H3K27me3 at a particular locus (e.g. the *daf-2* gene) can be correlated with ageing, using qPCR following Chromatin Immunoprecipitation with an H3K27me3 antibody, in long-lived and wild-type animals. This will allow me to compare changes in H3K27m3 to variations in gene expression at the loci with age.

In these experiments, I used *cdc-42* as an internal control that has been previously shown to be a good control gene for qRT-PCR, as the transcript levels do not vary significantly with various conditions used (Hoogewijs et al. 2008). I decided to use this gene as a control for both ChIP-qPCR and for qRT-PCR. ChIP primers for this and all of the other genes evaluated were designed around the 5'UTR (see Appendix 2), where the H3K27me3 modification is known to be present. The results of ChIP against H3K27me3 at the 5'UTR of *cdc-42* are presented in Figure 6.3. This bar plot shows that there are no significant changes in H3K27 methylation status of *cdc-42*, between day 1 and day 10, or strain used, showing it is a good ChIP control region.

The gene chosen for investigation is *daf-2*, primarily because it is an important player in lifespan regulation of *C. elegans*, but also because research by Jin et al. (2011) and Maures et al. (2011) indicates the *daf-2* promoter as the major site by which loss of *utx-1* by mutation or RNAi has its effect on lifespan. Demethylase loss causes prolonged H3K27me3 at the *daf-2* promoter leading to *daf-2* repression and therefore prolonged lifespan (Jin et al. 2011). To investigate what kind of changes occur at the level of histone modifications and transcript level in aged animals, I used the same primers as Jin et al. (2011) for both *daf-2* qRT-PCR and ChIP-qPCR. Firstly, I investigated changes in wild-type worms to see if I can reproduce the findings of Jin et al. (2011). Figure 6.4 illustrates changes in *daf-2* transcript levels and

H3K27me3 changes at its 5'UTR. Levels of *daf-2* transcript are elevated at day 5 and day 10 in comparison to day 1, as expected from previous findings of Jin et al. (2011). Interestingly there is a decrease of the transcript levels at day 15 (compared to day 10), this has not been observed by Jin et al. (2011). Levels of H3K27me3 at day 10 are significantly lower than those at day 1 (T test, $p<0.01$). This correlates with the increase in transcript levels late in life in comparison to day 1 adult. Additionally, my experiment shows a decrease in *daf-2* levels at day 15. This could suggest that most of *daf-2*-dependent regulation of lifespan happens between day 5 and 15 of adulthood, i.e. when most wild-type worms start to deteriorate.

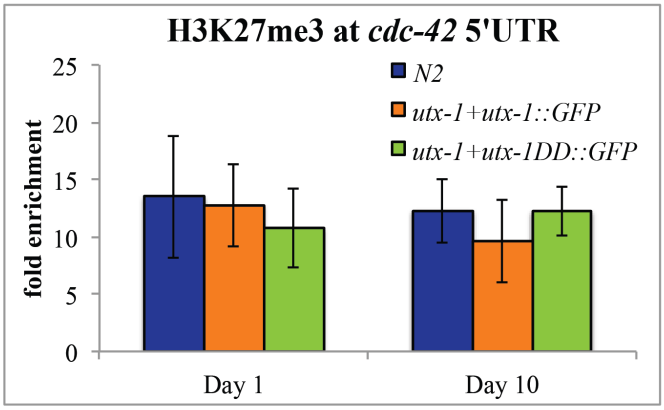


Figure 6.3 Assessment of H3K27me3 changes at 5'UTR of *cdc-42* control gene. ChIP data are presented as fold enrichment over 'no IP' control using the ΔΔCt method. Error bars represent ± standard deviation between the three technical repeats and mathematical error progression.

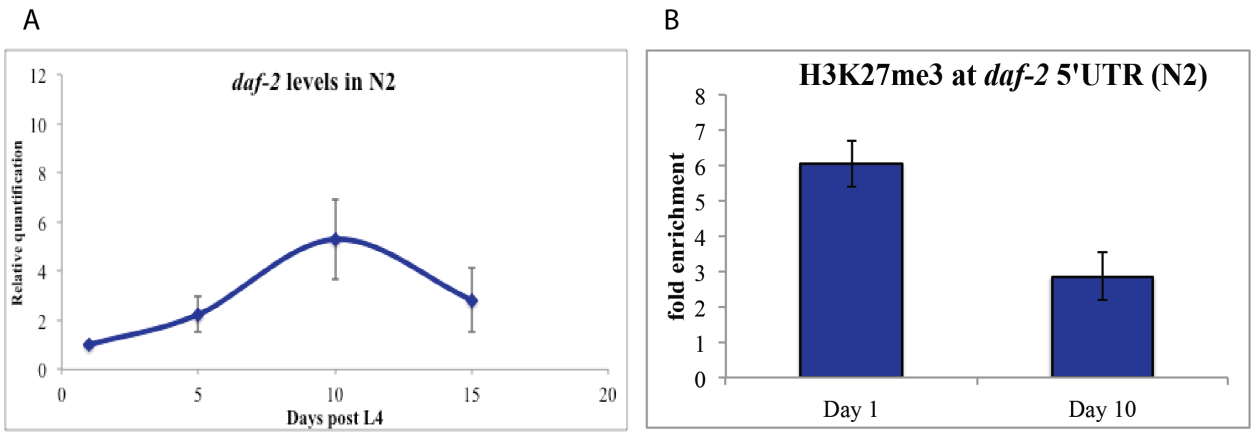


Figure 6.4 Assessment of changes in *daf-2* transcript levels and levels of H3K27me3 at 5'UTR of *daf-2* gene in the N2 strain. (A) qRT-PCR data showing changes in transcript levels of *daf-2* with age. Data have been pooled from at least 2 separate biological repeats and error bars represent ± standard deviation between the replicates. Quantification is relative to levels of transcript in day 1 wild-type animals and normalised to levels of *cdc-42*. (B) ChIP data are presented as fold enrichment over 'no IP' control using the ΔΔCt method. Error bars represent ± standard deviation between the three technical repeats and mathematical error progression.

The results shown in the section 5.2.2 suggest that the effect of *utx-1* overexpression on lifespan operates though a different mechanism to the *utx-1(tm3118)* mutation or depletion of *utx-1* by RNAi (due to differences in lifespan between overexpression of *utx-1* in wild-type and mutant backgrounds). Loss of UTX-1 has been hypothesised by Jin et al. (2011) to increase lifespan via increased chromatin repression of *daf-2* is consistent with my observations. If overexpression of *utx-1* also resulted in changes in chromatin regulation at *daf-2* promoter, the increase in the abundance of UTX-1 would be expected to lead to decreased H3K27me3 at the *daf-2* locus and therefore loss of its repression and lifespan decrease, which does not match the observed phenotype (lifespan increase).

To confirm my hypothesis of separate pathways of lifespan control by *utx-1* (mutant vs overexpression strains), I investigated changes in H3K27me3 at *daf-2* promoter and levels of *daf-2* in *utx-1(tm3118)+utx-1::GFP* and *utx-1(tm3118)+utx-1DD::GFP* mutants. Figure 6.5

shows that overexpression of *utx-1* does indeed lead to lower levels of H3K27me3 at *daf-2* 5'UTR as compared to a wild-type strain control (Figure 6.5 B) and that correlates with high levels of *daf-2* expression in early adulthood (day 1 and day 5, Figure 6.5 A). An intriguing observation is that levels of H3K27me3 at the *daf-2* locus decrease between days 1 and 10 in the *utx-1(tm3118)+utx-1::GFP* strain similarly to WT strain (Figure 6.5 B). This methylation loss, however, does not correlate with *daf-2* transcript levels that seem to remain unchanged when comparing days 1 and 10 of adulthood (as decrease in H3K27me3 should increase transcript levels as seen in WT strain, see Figure 6.4). This suggests that the lifespan extension in strains overexpressing *utx-1* does not correlate with H3K27me3 levels at the *daf-2* locus, indicating that overexpression of UTX-1 prolongs lifespan in a manner that is independent of H3K27 methylation at *daf-2*.

Levels of H3K27me3 at *daf-2* promoter in *utx-1(tm3118)+utx-1DD::GFP* strain could be interpreted as a mimic of *utx-1* RNAi or a *utx-1* mutant, as methylation levels rise between day 1 and day 10 (Figure 6.5 B), which would agree with its lifespan phenotype (lifespan extension similar to that of *utx-1(tm3118)* heterozygous mutant, see Figure 5.6, 35% and 40% mean lifespan extension over WT strain respectively, Log Rank Test between the two strains, p=0.49). This is in agreement to data showed by Jin et al. (2011), where *utx-1* RNAi resulted in retention of H3K37me3 at the *daf-2* locus in day 10 animals. The initial H3K27me3 levels at day 1 are, however, significantly lower (T-test, $p<0.01$) in this strain than in wild-type worms. This might be due to aberrant activity of other demethylases during development and larval stages. Levels of *daf-2* transcript still rise with age in this mutant strain, however the rise is seen later than in wild-type worms. This information unfortunately cannot be correlated with results from Jin et al. (2011) as this group did not investigate the changes in *daf-2* levels in *utx-1* mutant worms.

Further, I also investigated changes in *daf-2* levels in animals overexpressing *jmjd-3.2* as I have suggested they extend lifespan via a different pathway to *utx-1* overexpression. In Figure

6.6 both strains (*jmjd-3.2(tm3121)+jmjd-3.2::GFP* and *jmjd-3.2(tm3121)+ jmjd-3.2DD::GFP*) show no significant changes in *daf-2* levels over time, however, the levels of *daf-2* seem to be significantly higher early in life as compared to a wild-type strain (Figure 6.4(A)). By day 10 levels of *daf-2* mRNA seem comparable between strains overexpressing *jmjd-3.2* and WT worms. This could be suggestive of a loss of transcriptional regulation of *daf-2* in worms overexpressing *jmjd-3.2* however, it will need to be investigated further.

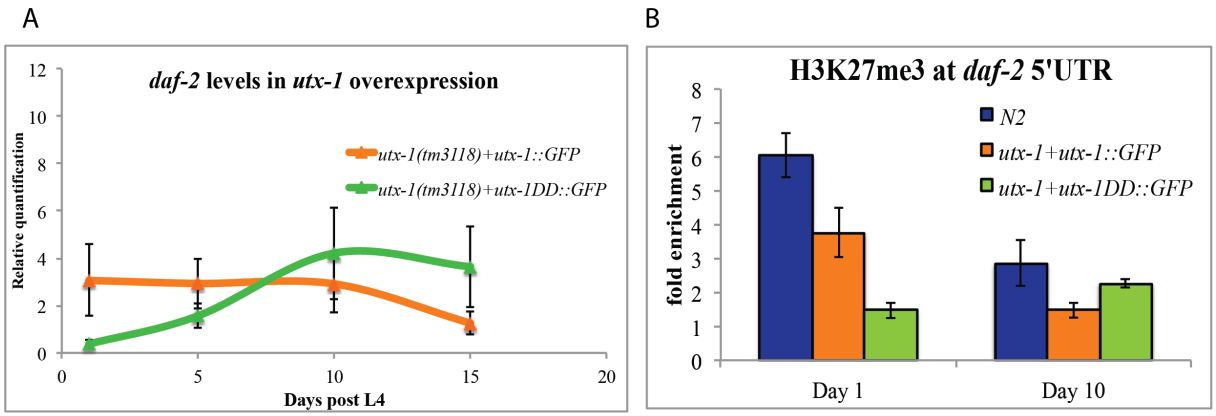


Figure 6.5 Assessment of changes in *daf-2* transcript levels and levels of H3K27me3 at 5'UTR of *daf-2* gene in *utx-1* overexpression strains. (A) qRT-PCR data showing changes in transcript levels of *daf-2* with age. Data have been pooled from at least 2 separate biological repeats and error bars represent ± standard deviation between the replicates. Quantification is relative to levels of transcript in day 1 wild-type animals and normalised to levels of *cdc-42* mRNA. (B) ChIP data are presented as fold enrichment over 'no IP' control using the ΔΔCt method. Error bars represent ± standard deviation between three technical repeats and mathematical error progression.

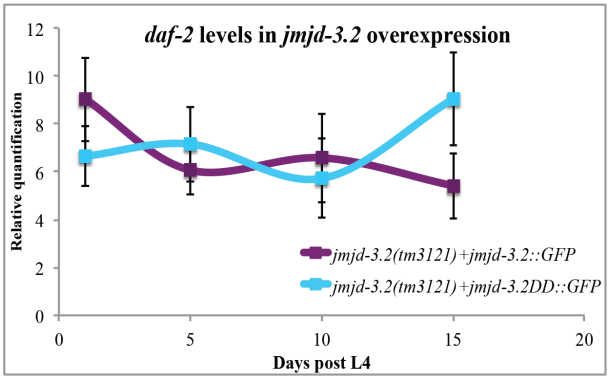


Figure 6.6 Assessment of changes in transcript levels of *daf-2* gene in *jmjd-3.2* overexpression strains. qRT-PCR data showing changes in transcript levels of *daf-2* with age. Data have been pooled from at least 2 separate biological repeats and error bars represent ± standard deviation between the replicates. Quantification is relative to levels of transcript in day1 wild-type animals and normalised to levels of *cdc-42* mRNA.

6.2.3 Regulatory interaction among the demethylases

I also decided to take a closer look at the potential regulatory interactions between the two demethylases under investigation. Figure 6.7 highlights the differences in *utx-1* and *jmjd-3.2* gene expression at day 10 between the strains. It clearly shows that levels of *utx-1* are depleted in the strain overexpressing *jmjd-3.2*, while levels of *jmjd-3.2* are significantly lower in *utx-1* overexpressing strains in comparison to wild-type worms. This regulatory relationship is a novel observation and it is going to be very important to pinpoint whether this relationship is important the demethylase-based lifespan extension.

The data presented in sections 6.2.1 and 6.2.2 show that global changes in methylation are not good predictors of lifespan in longevity mutants. Next, I confirmed that lifespan extension driven by *utx-1* overexpression must be operating through a different pathway to *utx-1* mutation by showing that levels of H3K27me3 decrease with age at *daf-2* locus. This suggests that *daf-2* locus control by UTX-1 via H3K27me3 cannot drive longevity in this strain. Mindful of the fact that longevity control in *utx-1* overexpression strain is dependent on its

enzymatic activity, I decided to find out if other longevity pathways could be targets of the lysine demethylase in lifespan control.

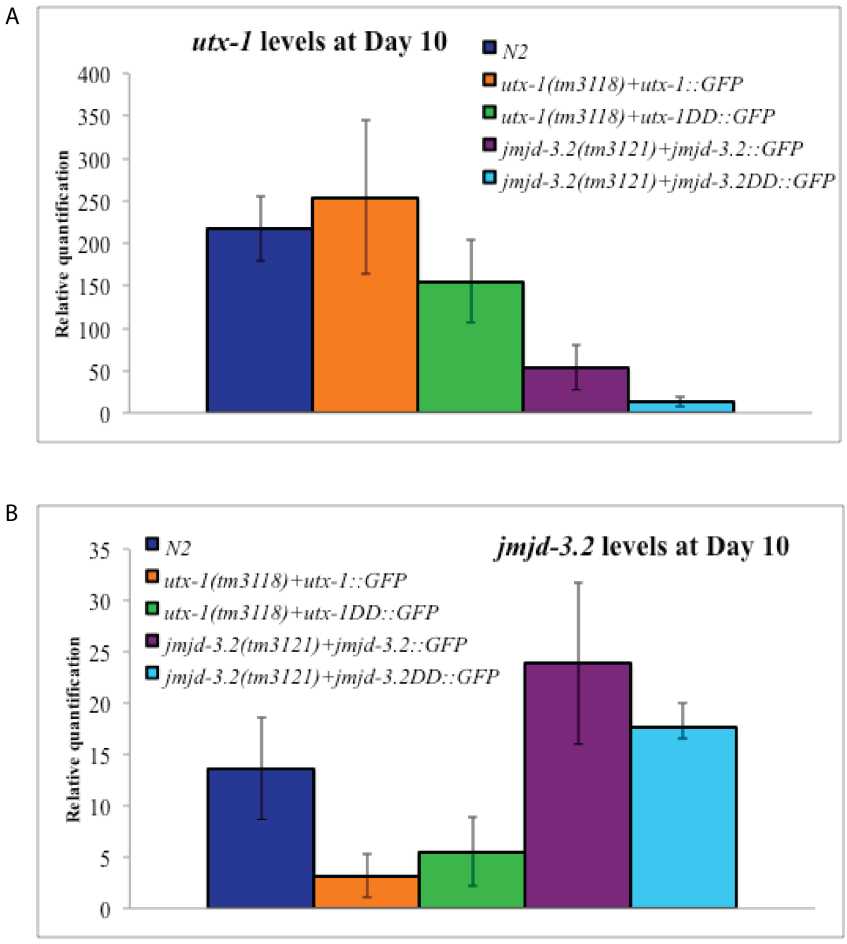


Figure 6.7 Changes in levels of *utx-1* and *jmjd-3.2* genes in various strains. qRT-PCR data showing changes in (A) *utx-1* and (B) *jmjd-3.2* transcript levels between N2 and *utx-1* overexpression and *jmjd-3.2* overexpression strains at day 10. Data have been pooled from at least 2 separate biological repeats and error bars represent standard deviation between the replicates. Quantification is relative to levels of transcript in day 1 wild-type animals and normalised to levels of *cdc-42*.

6.2.4 *daf-16* dependence of the longevity phenotypes in H3K27 methylation regulators

One of the most important pathways regulating ageing in *C. elegans* is the IIS pathway. Non-silent mutation or epigenetic changes to any of the genes involved results in significant lifespan alteration. Mutations in *daf-2, age-1, akt-1,* for example, are known to prolong *C. elegans* lifespan (Kenyon 2010b) while mutations in *daf-16* to shorten it significantly (Larsen, Albert, and Riddle 1995). Control of this pathway was for a long time thought to be concentrated upstream of the FOXO transcription factor DAF-16, eventually converging at DAF-16 and causing it to be either sequestered from or directed to the nucleus via phosphorylation by the AKT complex (Paradis and Ruvkun 1998). *daf-2*-mediated lifespan extension is also known to be *daf-16*-dependent, meaning that *daf-2* mutants subjected to *daf-16* RNAi have a short lived phenotype; this is one of the epistasis experiments that clarified the pathway.

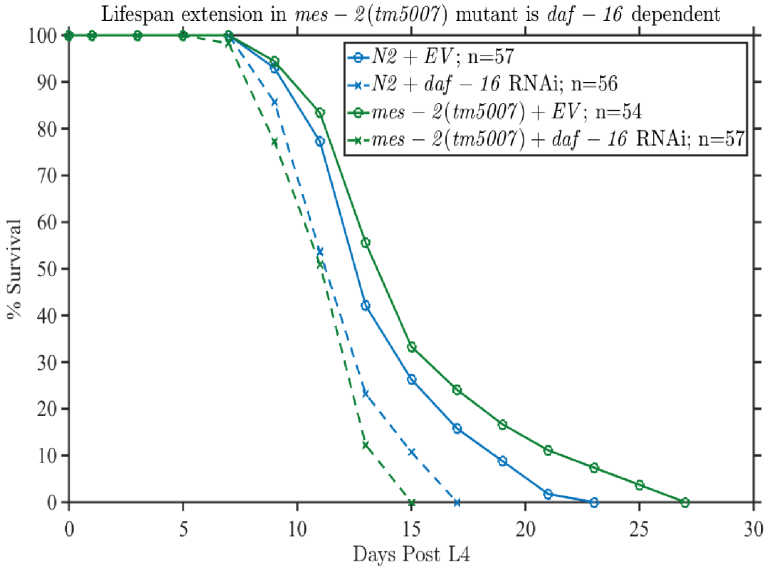
One important question one can ask about novel genes regulating ageing is whether that regulation is *daf-16*-dependent. To answer this question, I subjected the strains described in chapter 5 to *daf-16* RNAi and observed changes in lifespan as before. Figure 6.8 shows the consequences of *daf-16* knock-down in various *utx-1*, *jmjd-3.2* and *mes-2* mutants as well as in strains overexpressing *utx-1*. In all of the tested genetic backgrounds, *daf-16* RNAi resulted in dramatic lifespan shortening, comparable to that seen in wild-type background on *daf-16* RNAi (positive control).

This could suggest that even if many of these genes work initially in different pathways (as previously suggested), their effects seem eventually to converge at DAF-16. Distinguishing the early onset of ageing from general sickness that could cause an increase in mortality is often important for studies on higher organisms. Due to its simplicity, in *C. elegans* there are fewer potential phenotypes that could be worsened compared to a higher vertebrate. However, some information can be inferred from the lifespan curves: *daf-16* RNAi treated worms do not

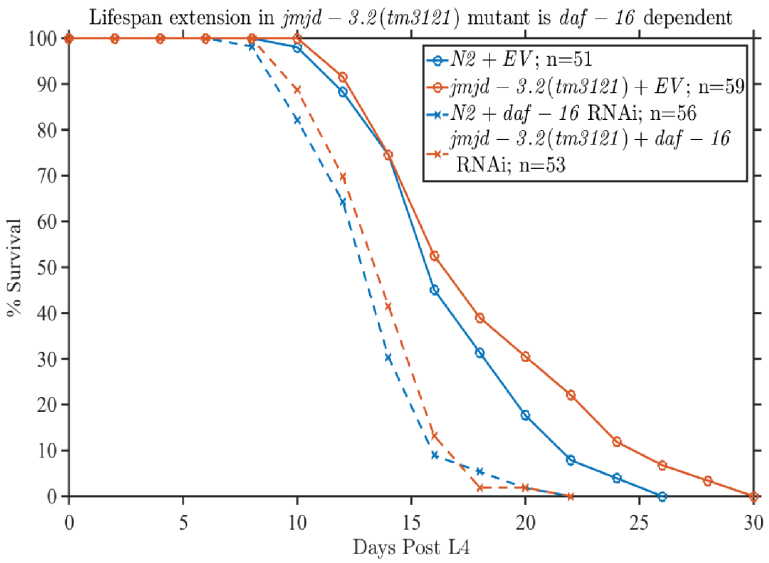
start dying early in adulthood (day 1-8), but, similarly to their wild-type counterparts, most of them finish egg laying and only then does mortality start to increase. Additionally, *daf-16* RNAi does not increase aberrant phenotypes (bagging etc.), which could lead to increased death rates either. Depletion of *daf-16* in all of the mutant strains leads to their lifespan curves matching those for N2 on *daf-16* RNAi in both statistics (Figure 6.8 and Table 6.1) and the curve shape. If exposure to *daf-16* RNAi resulted in general sickness, increase in aberrant phenotypes and early (pre-egg-laying) deaths this would be mirrored in survival curves dropping even more steeper and earlier than in wild-type worms on *daf-16* RNAi. The fact that such incidences were not observed, may suggest that the longevity phenotypes of the strains tested in the above experiment are entirely *daf-16*-dependent

The only two lines that did not show complete *daf-16* dependency are *utx-1(tm3118)+utx-1::GFP* and *N2+utx-1DD::GFP* strains; the lifespan of both was still significantly decreased on *daf-16* RNAi compared to the EV control, although they were slightly longer lived than N2 worms fed on a *daf-16* RNAi (Table 6.1).

A



B



C

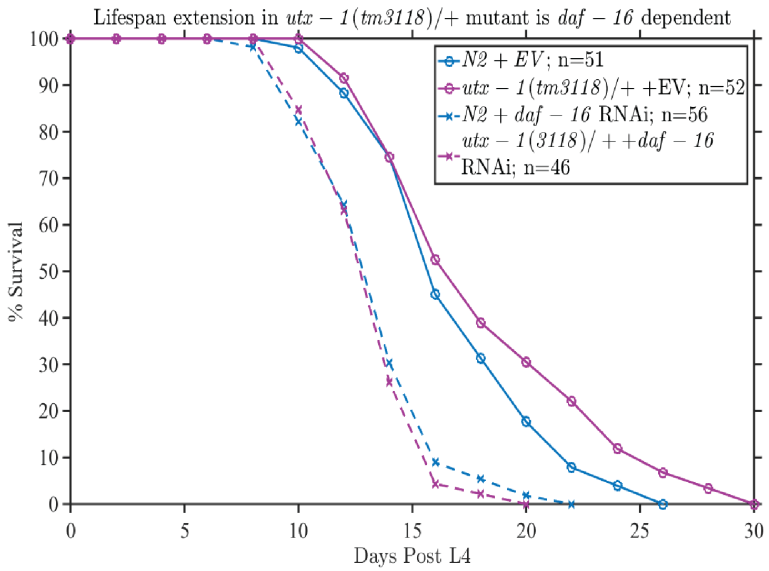
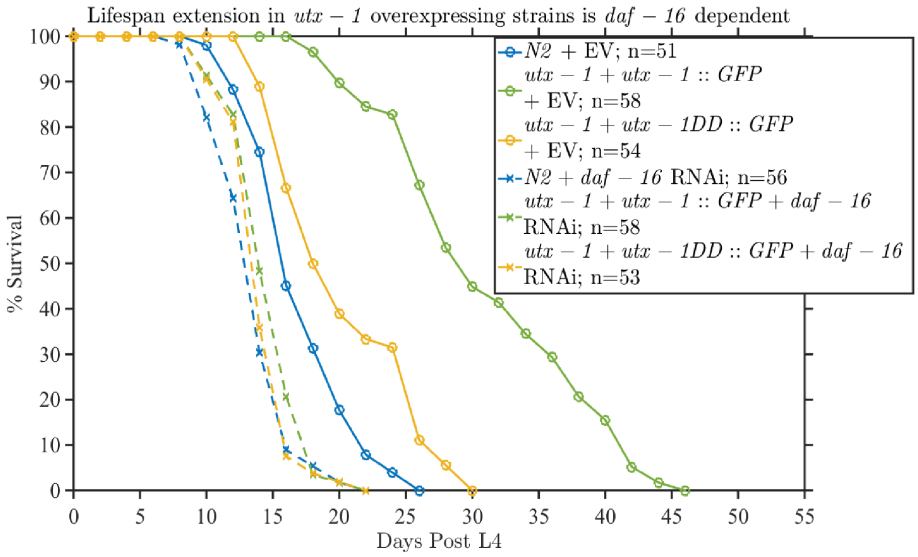


Figure 6.8 (part 1/3) Lifespan extension in *mes-2, utx-1* and *jmjd-3.2* mutants and *utx-1* overexpression strains is dependent on *daf-16*.

D



E

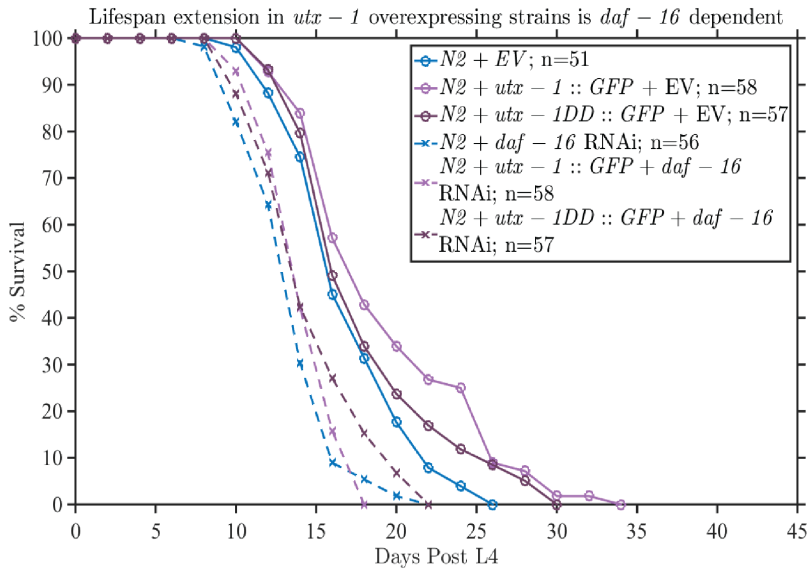


Figure 6.9. (part 2/3) Lifespan extension in *mes-2*, *utx-1* and *jmjd-3.2* mutants and *utx-1* overexpression strains is dependent on *daf-16*.

F

strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	14.30	0.44	15	23
N2 + *daf-16* RNAi	12.46	0.31	13	17
mes-2(tm5007) + EV	15.59	0.60	15	27
mes-2(tm5007) + *daf-16* RNAi	11.77	0.27	13	15

strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	17.33	0.52	16	26
N2 + *daf-16* RNAi	13.82	0.37	14	22
jmjd-3.2(tm3121) + EV	18.64	0.63	18	30
jmjd-3.2(tm3121) + *daf-16* RNAi	14.34	0.35	14	22
utx-1(tm3118)/+ + EV	19.31	0.72	18	32
utx-1(tm3118)/+ + *daf-16* RNAi	13.61	0.33	14	20
utx-1(tm3118) +utx-1::GFP + EV	31.34	0.99	30	46
utx-1(tm3118) +utx-1::GFP + *daf-16* RNAi	14.97	0.33	16	22
utx-1(tm3118) +utx-1DD::GFP + EV	20.52	0.68	18	30
utx-1(tm3118) +utx-1DD::GFP + *daf-16* RNAi	14.56	0.34	16	22
N2 +utx-1::GFP + EV	20.55	0.78	20	36
N2 +utx-1::GFP + *daf-16* RNAi	14.53	0.30	16	18
N2 +utx-1DD::GFP + EV	17.16	0.52	16	28
N2 +utx-1DD::GFP + daf-16 RNAi	15.02	0.44	14	22

Figure 6.9 (part 3/3) Lifespan extension in *mes-2, utx-1* and *jmjd-3.2* mutants and *utx-1* overexpression strains is dependent on *daf-16*. Lifespan extension of (A) *mes2(tm5007)* (B) *jmjd-3.2(tm3121)* (C) *utx-1(tm3118)* (D) *utx-1+utx-1::GFP* and (E) *N2+utx-1::GFP* strains on empty vector control bacteria (when compared to N2 strain) is abolished when these worms are subjected to *daf-16* RNAi, which on its own (control N2+*daf-16* RNAi) also decreases lifespan (see statistics in **Table 6.1**). The experiments on all strains (A-E) were performed at the same time and therefore control strains (N2+EV and N2+*daf-16*) were re-used. (F) Mean lifespan and 50% mortality points are shown for all strains presented in A-E.

Table 6.1 Log-Rank statistics test for *daf-16* dependence ('+' marks an increase in lifespan, '-' a decrease and '0' no apparent change)

strain 1	strain 2	strain 1 (control) vs strain 2 lifespan change	p value (Log Rank Test)	sig.
N2 + EV	N2 + *daf-16* RNAi	-	p<0.01	***
N2 + EV	*mes-2(tm5007)* + EV	+	p<0.01	***
N2 + *daf-16* RNAi	*mes-2(tm5007)* + *daf-16* RNAi			
mes-2(tm5007) + EV	*mes-2(tm5007)* + *daf-16* RNAi	-	p<0.01	***
N2 + EV	*jmjd-3.2(tm3121)* + EV	+	p<0.01	***
N2 + *daf-16* RNAi	*jmjd-3.2(tm3121)* + *daf-16* RNAi	0	p=0.36	
jmjd-3.2(tm3121) + EV	*jmjd-3.2(tm3121)* + *daf-16* RNAi	-	p<0.01	***
N2 + EV	*utx-1(tm3118)/+* + EV	+	p<0.01	***
N2 + *daf-16* RNAi	*utx-1(tm3118)/+* + *daf-16* RNAi	0	p=0.53	
utx-1(tm3118)/+ + EV	*utx-1(tm3118)/+* + *daf-16* RNAi	-	p<0.01	***
N2 + EV	*utx-1(tm3118) +utx-1::GFP* + EV	+	p<0.01	***
N2 + *daf-16* RNAi	*utx-1(tm3118) +utx-1::GFP* + *daf-16* RNAi	+	p=0.04	*
utx-1(tm3118) +utx-1::GFP + EV	*utx-1(tm3118) +utx-1::GFP* + *daf-16* RNAi	-	p<0.01	***
N2 + EV	*utx-1(tm3118) +utx-1DD::GFP* + EV	+	p<0.01	***
N2 + *daf-16* RNAi	*utx-1(tm3118) +utx-1DD::GFP* + *daf-16* RNAi	0	p=0.17	
utx-1(tm3118) +utx-1DD::GFP + EV	*utx-1(tm3118) +utx-1DD::GFP* + *daf-16* RNAi	-	p<0.01	***
utx-1(tm3118) +utx-1::GFP + EV	*utx-1(tm3118) +utx-1DD::GFP* + EV	-	p<0.01	***
utx-1(tm3118) +utx-1::GFP + *daf-16* RNAi	*utx-1(tm3118) +utx-1DD::GFP* + *daf-16* RNAi	0	p=0.38	
N2 + EV	*N2 +utx-1::GFP* + EV	+	p<0.01	***
N2 + *daf-16* RNAi	*N2 +utx-1::GFP* + *daf-16* RNAi	0	p=0.26	
N2 +utx-1::GFP + EV	*N2 +utx-1::GFP* + *daf-16* RNAi	-	p<0.01	***
N2 + EV	*N2 +utx-1DD::GFP* + EV	0	p=0.15	
N2 + *daf-16* RNAi	*N2 +utx-1DD::GFP* + daf-16 RNAi	+	p=0.04	*
N2 +utx-1DD::GFP + EV	*N2 +utx-1DD::GFP* + *daf-16* RNAi	-	p<0.01	***
N2 +utx-1::GFP + EV	*N2 +utx-1DD::GFP* + EV	-	p<0.01	***
N2 +utx-1::GFP + *daf-16* RNAi	*N2 +utx-1::GFP* + *daf-16* RNAi	0	p=0.15	

6.2.5 Germline dependence of the longevity phenotypes in H3K27 methylation regulators

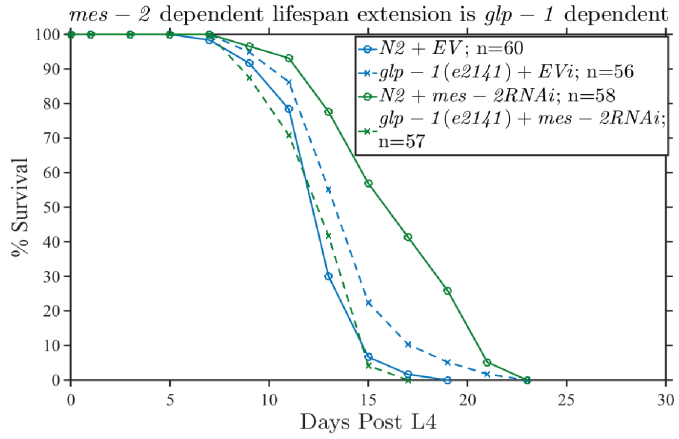
As mentioned before (section 6.1.1), there are particular tissues in *C. elegans* that have proved to be key regulatory sites of ageing, for example the intestine, neurons and germline. The germline particularly, has been the subject of intensive research investigating what causes the germline cells to become essentially 'immortal' such that they can transfer the genetic material in pristine condition to the next generation. For this to be possible, they must be much more resistant to environmental stresses and need to be controlled differently to somatic cells, as there are no significant ageing phenotypes found in germline cells.

The crucial experiments in highlighting the germline's effect on longevity, as mentioned before, involved ablating the Z2 and Z3 germline precursor cells (Hsin and Kenyon 1999). One mutant duplicating the gonad-associated longevity phenotype is *glp-1(e2141)*. It is a temperature sensitive mutant, which is sterile at the restrictive temperature (25°C) but develops and reproduces normally at 15°C (Kodoyianni, Maine, and Kimble 1992). This has proven to be a very useful tool in dissecting the role of the germline cells or germline signalling in various mutant worms. I decided to use this mutant in combination with gene-specific RNAi to investigate germline dependence of lifespan extension for *mes-2, jmjd-3.2* and *utx-1* (Figure 6.9).

Table 6.2 Log-Rank statistics test for *glp-1* dependence.

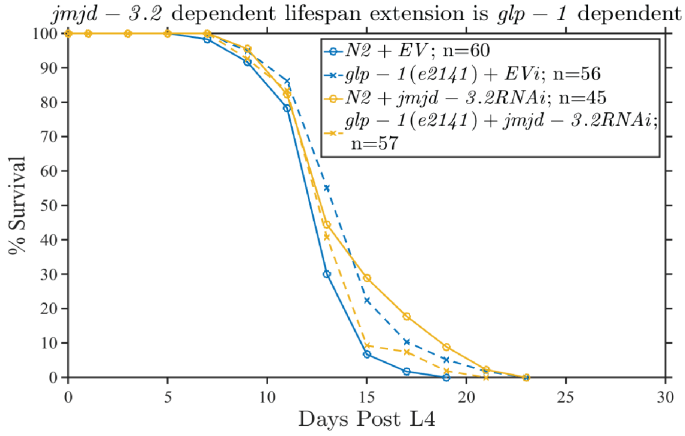
strain 1	strain 2	strain 1 (control) vs strain 2 lifespan change	p value (Log Rank Test)	sig.
N2 + EV	*glp-1* + EV	+	p<0.01	***
N2 + EV	N2+ *mes-2 RNAi*	+	p<0.01	***
N2+ *mes-2* RNAi	*glp-1+ mes-2 RNAi*	-	p<0.01	***
glp-1 + EV	*glp-1+ mes-2 RNAi*	-	p<0.01	***
N2 + EV	N2+ *jmjd-3.2 RNAi*	+	p<0.01	***
N2+ *jmjd-3.2* RNAi	*glp-1+ jmjd-3.2 RNAi*	0	p=0.13	
glp-1 + EV	*glp-1+ jmjd-3.2 RNAi*	0	p=0.09	
N2 + EV	N2+ *utx-1 RNAi*	+	p<0.01	***
N2+ *utx-1* RNAi	*glp-1+ utx-1 RNAi*	+	p=0.05	*
glp-1 + EV	*glp-1+ utx-1 RNAi*	+	p<0.01	***

A



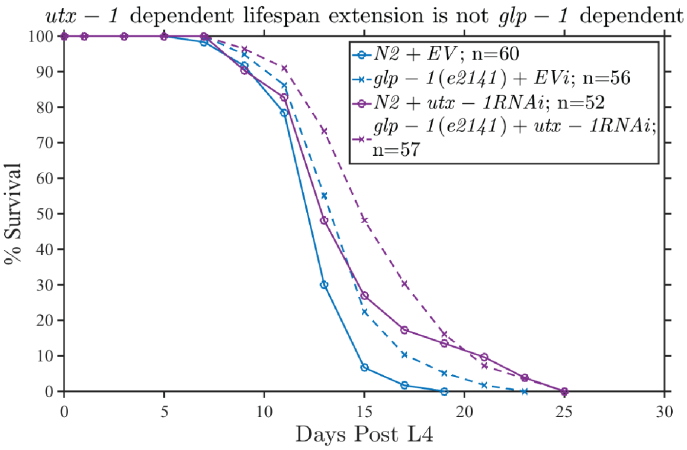
strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	13.13	0.27	15	19
glp-1(e2141) + EV	14.52	0.37	15	23
N2 + *mes-2 RNAi*	16.93	0.47	17	23
glp-1(e2141) + *mes-2* RNAi	13.08	0.32	13	17

B



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	13.13	0.27	15	19
glp-1(e2141) + EV	14.52	0.37	15	23
N2 + *jmjd-3.2 RNAi*	14.60	0.49	15	23
glp-1(e2141) + *jmjd-3.2* RNAi	13.70	0.33	15	21

C



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2 + EV	13.13	0.27	15	19
glp-1(e2141) + EV	14.52	0.37	15	23
N2 + *utx-1 RNAi*	14.85	0.55	15	25
glp-1(e2141) + *utx-1* RNAi	16.32	0.49	15	25

Figure 6.9 *glp-1* dependence of lifespan extension by *mes-2*, *jmjd-3.2* and *utx-1* RNAi.
(A) *mes2* RNAi and *glp-1* mutant extend lifespan on their own but not in combination (B) *jmjd-3.2* RNAi lifespan extension is not additive to *glp-1*-dependent lifespan extension (C) *utx-1* RNAi extends lifespan of *glp-1* mutant. WT and *glp-1* strains were cultivated at *glp-1* permissive temperature (15°C) and shifted to the restrictive temperature (25°C) at L1 stage. Both strains were subjected to RNAi (EV or gene in question) at L4 stage onwards. The experiment was performed at the same time, therefore the control strains for A, B and C (N2+EV, *glp-1*+EV) were re-used. For all Log-rank test values see **Table 6.2**.

It has been previously described that *utx-1*-dependent lifespan extension is *glp-1*-independent as *utx-1* and *glp-1* lifespan extensions are additive (Jin et al. 2011; Maures et al. 2011). I used this result as a positive control in my experiment (Figure 6.9(C)), indeed showing an additive lifespan extension of *glp-1* strain on *utx-1* RNAi. In Figure 6.9 A and B, I am showing changes in lifespan of *glp-1* mutant when subjected to *mes-2* or *jmjd-3.2* RNAi. In WT background, both RNAi extended lifespan, as shown previously (Figure 3.4 (C) and (O)). However, neither *jmjd-3.2* nor *mes-2* knock-down in *glp-1* strain additionally extended lifespan, suggesting that they might be working in the same pathway as *glp-1*-dependent germline signalling.

It is intriguing to see that *jmjd-3.2,* expressed mostly in neurons, may act through germline signalling. An alternative explanation takes into consideration the fact that *jmjd-3.2* is the most widely expressed demethylase during development (Vandamme et al. 2012); it therefore could interact with the *glp-1* pathway in the embryo. The fact that *jmjd-3.2* has got a *glp-1* dependent function in longevity regulation while, *utx-1* has been confirmed to act in a *glp-1* independent fashion, could be further evidence supporting the hypothesis that *utx-1* and *jmjd-3.2* based lifespan extensions work in different ways. Additionally, *mes-2* also shows *glp-1* dependence, which agrees with the fact that it is known to be working predominantly in the germline (Capowski et al. 1991; Holdeman, Nehrt, and Strome 1998).

The data shown in the last two sections (6.2.4 and 6.2.5) support the hypothesis that lifespan extensions dependent on changes in H3K27 methylation regulators are achieved by different pathways. Lifespan extension regulated by *mes-2* and *jmjd-3.2* are both *glp-1* and *daf-16*-dependent, while extension by *utx-1* RNAi is *glp-1*-independent. Further, I showed that overexpression of *utx-1* regulates lifespan extension in a *daf-16*-dependent manner (Figure 6.8), despite the previous finding suggesting that *utx-1* overexpression does not regulate lifespan by modulating H3K27me3 on *daf-2* 5'UTR (Figure 6.5). Knowing that *daf-16* is an

important downstream effector of *daf-2*, it suggests that the canonical IIS signalling pathway is not the only lifespan-related regulation of *daf-16*.

Next, I concentrate on the long-lived *utx-1* overexpression strains and examine if oxidative stress resistance is correlated with longevity as well as whether there are particular tissues that govern the lifespan extension

6.2.6 *utx-1* overexpression-mediated oxidative stress resistance

Prolonged lifespan in most laboratory model organisms often correlates with increased resistance to environmental stresses. For a long time it has been thought that the two are connected. This was tested in *C. elegans* by Kim and Sun (2007), who performed a screen for longevity, based on resistance to ROS stress. Interestingly, the experiment has not resulted in identification of many longevity genes suggesting that the relationship between ROS resistance and longevity might be one sided (i.e. long-lived mutants are often resistant to stress, but the stress resistant mutants are not necessarily long-lived). There are many potential stresses that a worm can be subjected to: temperature, UV radiation, oxidative stress, to name the most common. I decided to investigate oxidative stress by subjecting worms to paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), which catalyses the formation of ROS in living cells by interfering with the electron transport. The effect of paraquat, as well as other stress inducers, has been previously tested in *utx1* knock-down worms by Jin et al. (2011), who suggested that *utx-1* RNAi enhanced resistance to all of the tested stress conditions (UV, ROS, high temperature). Considering that *utx-1* overexpression might be acting through a parallel pathway to *utx-1* depletion, I decided to check the stress resistance of *utx-1* overexpression lines.

Results are summarised in Table 6.3 and Figure 6.10 (based on the work of Abigail Guillermo, a rotation student in our lab, under my supervision). I show that as compared to a WT strain, *C. elgans* exposed to *utx-1* RNAi survives on average 10 hours longer after being

subjected to 10 mM of paraquat on the NGM plates (which is over 10% of maximum survival of a WT worm). A long lived strain overexpressing *utx-1* also survives significantly longer than the control (Log Rank Test, $p<0.01$). Conversely, worm strain overexpressing enzymatically dead form of *utx-1* has got both shorter maximum survival and shorter median survival (hours of exposure when 50% worms are still alive).

Our results confirm that knock-down of *utx-1* results in an increased resistance to oxidative stress. Furthermore, the *utx-1(tm3118)+utx-1::GFP* strain also has increased resistance to paraquat. Interestingly, this strain has a significantly longer lifespan than that of *utx-1(tm3118)/+* (Figure 5.7). For the purpose of this experiment we used *utx-1* RNAi as a positive control showing that depletion of *utx-1* increases stress resistance. We decided to use *utx-1* RNAi as a proxy to mimic the effect of *utx-1* heterozygous mutation. Significantly longer lifespan of *utx-1(tm3118)+utx-1::GFP* strain does not therefore translate to significantly higher stress resistance (Log Rank Test, N2+*utx-1* RNAi vs *utx-1(tm3118)+utx-1::GFP*, $p=0.39$), suggesting that it is the loss of UTX-1 that promotes stress resistance, whilst its overexpression has no additional protective effects. Furthermore, overexpression of the *utx-1* DD version of the protein seems increase the sensitivity to oxidative stress. This is a curious observation, suggesting that perhaps the enzymatically dead protein might have some dominant negative effects in stressful environments.

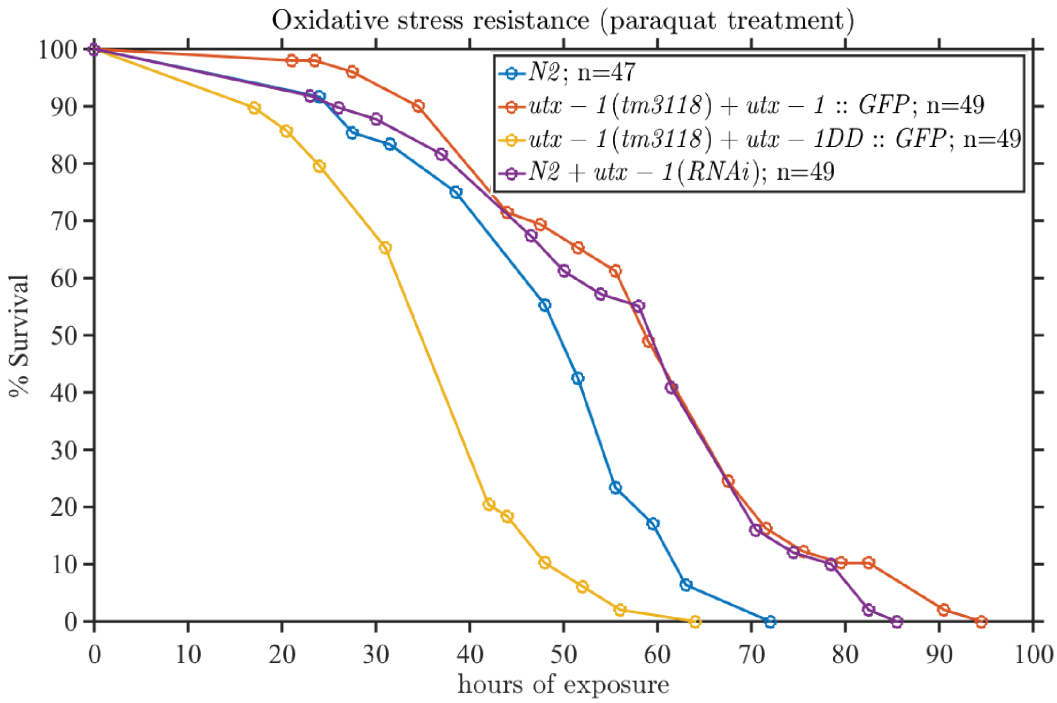


Figure 6.10 Oxidative stress resistance of the *utx-1* overexpression strains. All strains have been subjected to 10 mM of paraquat (final concentration in agar) upon reaching adulthood and remain subjected to it throughout the experiment. Resistance of both *utx-1* knock-down and *utx-1(tm3118)+utx-1::GFP* to paraquat is significantly enhanced over the wild-type strain (Log Rank Test, $p<0.01$). The *utx-1(tm3118)+utx-1DD::GFP* strain is however more sensitive to oxidative stress (Log Rank Test, $p<0.01$). Experiment performed by Abigail Guillermo under my supervision.

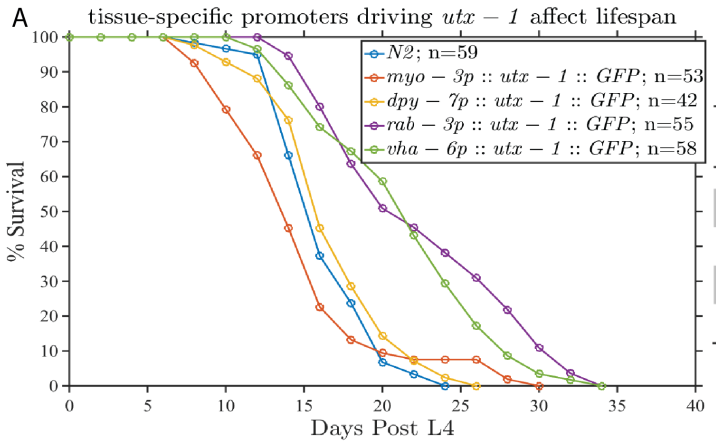
Table 6.3 Comparison of the effect of the changes in *utx-1* on paraquat resistance and longevity.

strain	paraquat resistance	lifespan extension
N2	-	-
N2 + *utx-1* RNAi	yes	yes
utx-1(tm3118)+ utx-1::GFP	yes	yes
utx-1(tm3118)+ utx-1DD::GFP	no	yes

6.2.7 Tissue-specific *utx-1* expression results in lifespan extension

In collaboration with the Salcini lab we created 4 worm strains with *utx-1* driven by tissue-specific promoters and assessed their lifespan. Expression of *utx-1* in neurons (*rab-3* promoter) or intestinal cells (*vha-6* promoter) result in statistically significant lifespan extension (Figure 6.11). In contrast, muscle (*myo-3*) and hypodermal (*dpy-7*) expression of *utx-1* did not affect lifespan. Both neuronal and intestine driven *utx-1* extended lifespan to a similar degree, which is also comparable to the extension of lifespan seen in the *N2+utx-1::GFP* line (Figure 5.9), where *utx-1* is controlled by its endogenous promoter.

This suggests that lifespan extension in *utx-1*-overexpressing strains is governed by at least two tissues: neurons and intestine. This in turn sheds light on previous results presented in this chapter. The fact that expression of the *utx-1,* in terms of lifespan control, is only important and therefore regulated in these two tissues might suggest that the data representing the changes in H3K27me3 and transcript levels at *daf-2* locus might be confounded by presence of other tissues due to analysis of whole animal extracts. Therefore, a technique that isolates neurons or intestine may lead to enrichment in meaningful signal.



strain	Mean lifespan		Age in days at % mortality	
	day	std. error	50%	100%
N2	16.54	0.40	16	24
N2 + myo-3P::utx-1::GFP	15.06	0.69	14	30
N2 + dpy-7-3P::utx-1::GFP	17.05	0.58	18	26
N2 + rab-3P::utx-1::GFP	22.80	0.80	22	34
N2 + vha-6P::utx-1::GFP	21.72	0.71	22	34

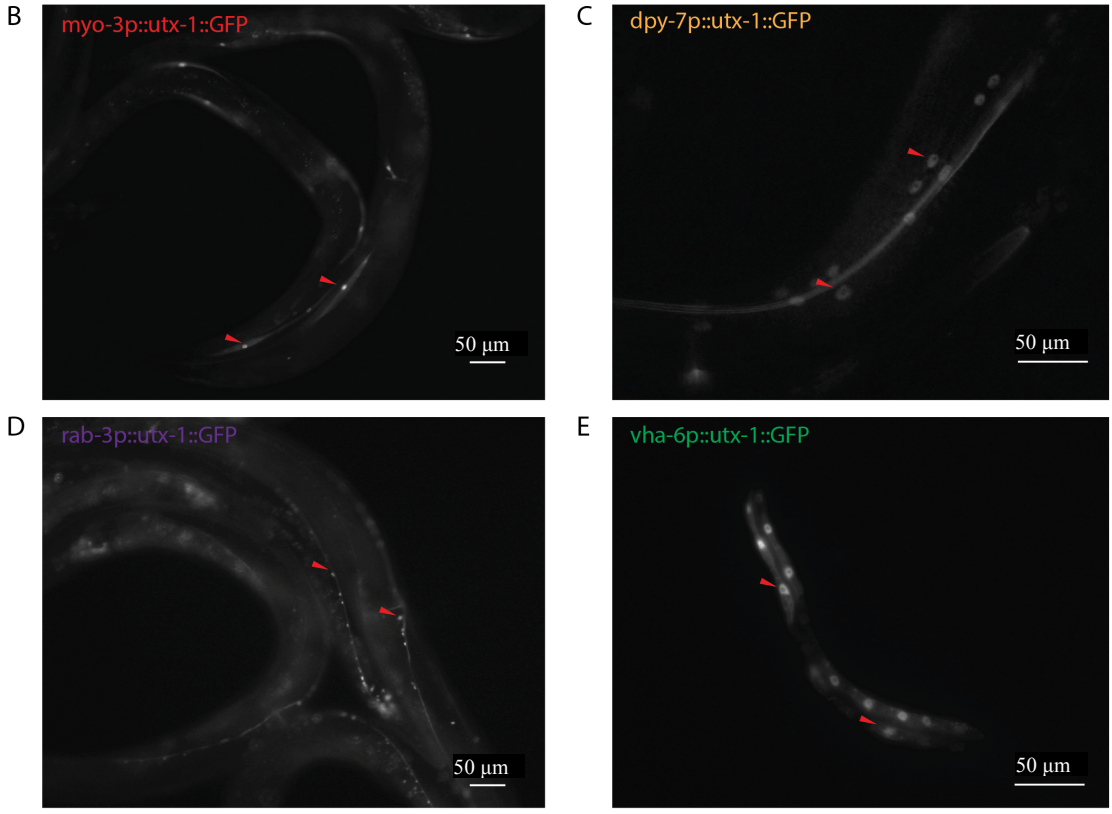


Figure 6.11 Expression of *utx-1::GFP* driven by neuronal or intestine specific promoter but not muscle or hypodermis leads to lifespan extension. (A) Lifespan curves of worm lines expressing tissue specific promoters driving *utx-1::GFP* in an N2 background. *rab-3* and *vha-6* driven *utx-1::GFP* causes statistically significant lifespan extension (Log Rank Test, $p<0.01$ as compared to N2) (B-D) expression of UTX-1::GFP from tissue-specific promoters, red arrows point to representative GFP positive nuclei. (B) muscle-expressed *myo-3* promoter (C) hypodermis-expressed *dpy-7* promoter (D) pan-neuronal-expressed *rab-3* promoter (D) intestine-expressed *vha-6* promoter.

6.3 DISCUSSION

6.3.1 H3K27 methylation and gene expression changes in aged *C. elegans*

Maures et al. (2011) propose that there is a significant loss of H3K27 trimethylation with age in somatic tissues. This therefore rather significant loss of repression should thus correlate with a global increase of gene expression and significant upregulation of transcription of many genes. Such upregulation has, however, not been confirmed in work by Johnson and McCaffrey (1985), Fabian and Johnson (1995) or Lund et al. (2002). This begs the question of the functionality of H3K27me3 late in life. If there is no correlation between the repressive mark and upregulation of transcript levels, this suggests that the mark could either lose stringent correlation with repression or might act together with changes in other histone marks. This could affect chromatin domains and nuclear structure in aged cells.

Replication of the ChIP data shown (Figure 6.4 and Figure 6.5), as well as use of antibodies against both H3K27me2 and H3K27me1, will be very important to draw final, meaningful conclusions as to the mechanisms involved here. The changes at H3K27 also need to be correlated with changes in other histone modifications to show the full spectrum of possible control.

Apart from global changes in H3K27 methylation, I also presented data from ChIP-qPCR and qRT-PCR at the *daf-2* locus investigating whether changes at this particular key regulatory hub could explain the longevity phenotypes observed in the *utx-1* and *jmjd-3.2* overexpression strains. Firstly, my data in wild-type animals agrees with previously published results (Jin et al. 2011) suggesting an increase in *daf-2* levels with age, associated with decreased levels of H3K27me3 at its promoter. Further, I show that the prolonged lifespan of the *utx-1(tm3118)+utx-1::GFP* strain does not in fact correlate with H3K27me3 levels at the *daf-2* locus. This indicates that overexpression of UTX-1 prolongs life in a manner independent of H3K27 methylation at *daf-2*.

From my other experiments I concluded that longevity driven by *utx-1* overexpression is (at least in major part) dependent on *daf-16*, as *daf-16* RNAi ameliorated most of the lifespan extension of this strain. This means that UTX-1 overexpression could be regulating lifespan by demethylating other proteins like transcription factors, working either at *daf-2* or other parts of the IIS pathway upstream of *daf-16* transcription regulation.

Furthermore, the finding that *daf-2* levels, which are relatively unchanged between days 1 and 10 (Figure 6.5), do not match the observed decrease in the H3K27me3 mark in the *utx-1(tm3118)+utx-1::GFP* strain suggests the existence of another overriding control for *daf-2* levels. This control would have to be independent of H3K27me3, but could still involve UTX-1 as a lysine demethylase. Alternatively, it could be argued that a decrease in H3K27me3 at the 5'UTR of the gene needs to be considered as a part of a whole pattern of various histone modifications over the gene promoter and gene body. This, as suggested by Young et al. (2011), might be crucial to differentiate a repressive mark from an activating or bivalent one.

In worms overexpressing *jmjd-3.2*, the potential lack of changes in *daf-2* levels with age might not be representative of the effect that *jmjd-3.2* overexpression could have on *daf-2* gene regulation. That is mostly due to the fact that in the adult worm, *jmjd-3.2* is only present in several head neurons and therefore the bulk of *daf-2* regulation would probably happen in those cells. Neuronal cells are known to be involved in regulating lifespan (for example, *utx-1* expressed solely in neurons can extend lifespan - Figure 6.11), and therefore due to the nature of the experiments in which expression is averaged across tissues of the whole worm, the real effects can be hidden in the background 'noise'. On the contrary, *jmjd-3.2* overexpression prolongs lifespan independently of its demethylase activity (Figure 5.10). This suggests that long lifespan in these strains is also independent of changes in H3K27me3 at *daf-2* or elsewhere in the genome. However, this assumption needs to be confirmed by performing ChIP-qPCR for H3K27me3 in *jmjd-3.2* overexpression strains.

Overall, changes in H3K37me3 at the *daf-2* locus correlate well with UTX-1 levels: in the *utx-1(tm2118)+utx-1::GFP* strain, trimethylation is lost with age which can be linked to the increased presence of this demethylase, while in the *utx-1(tm2118)+utx-1DD::GFP* strain H3K27me3 is gained at *daf-2* because of the presence of *utx-1* mutation in the strain background (Figure 6.5). Furthermore, if these are indeed effects of *utx-1* overexpression or *utx-1* mutation, it suggests that UTX-1 has a stronger effect late in life. This also correlates with the observation that in wild-type worms, levels of *utx-1* rise with age (Jin et al. (2011) and Appendix 10). These data could imply that with age, chromatin becomes more susceptible to the effects of UTX-1, for example by changes in other histone modifications that would increase the efficiency of UTX-1 recruitment. To fully understand these changes, we need to perform ChIP-seq to investigate the changing profiles of H3K27me3 and other related histone modifications like H3K4me3 or H3K27ac.

Additionally, in section 6.2.3, I suggest a regulatory interaction between *jmjd-3.2* and *utx-1*, as in *utx-1* overexpression strains, *jmjd-3.2* levels are significantly lower, while in *jmjd-3.2* overexpression strains *utx-1* level are unusually low. This interaction seems especially curious as the demethylases not only function in different tissues but also at different times during development (*jmjd-3.2* is the most abundant demethylase in the family during early development, being later substituted by *utx-1* (Vandamme et al. 2012)). I also provide evidence supportive of my hypothesis that *utx-1* overexpression prolongs life via a different pathway than depletion or mutation of the gene, but is, interestingly, still demethylase-dependent. Conversely *jmjd-3.2* is only present in head neurons, and overexpression likely acts through the same pathway as *jmjd-3.2* mutation and is not dependent on the presence of demethylase activity.

6.3.2 *daf-16* dependence

To examine whether the long-lived strains investigated in this thesis exert their control of lifespan via IIS signalling, I used RNAi against *daf-16* in order to deplete this gene product.

Remarkably, despite the variety in potential pathways through which H3K27 methylation regulators could affect lifespan, all of these converge at DAF-16 as *daf-16* RNAi resulted in significant decrease in lifespan in all of the examined strains (back to levels of WT worms on *daf-16* RNAi). Intriguingly, the lifespan of some of the strains overexpressing *utx-1* subjected to *daf-16* RNAi, despite being significantly shorter than on the EV control, were still longer than the *N2+daf-16* RNAi control. This might imply that although most of the regulation of lifespan is mediated by the IIS pathway, there could also be some unrelated effects of UTX-1, perhaps controlling the methylation of other independent proteins.

6.3.3 *glp-1* dependence

Expression of extrachromosomal arrays is usually silenced in the germline (Kelly and Fire 1998), and therefore, to investigate the dependence on germline signalling, one needs to study either gonadal laser-ablated animals, or a *glp-1* temperature-sensitive strain that is sterile at the restrictive temperature (25°C). It needs to be noted that *C. elegans* lifespan is also temperature dependent and worms live shorter at 25°C compared to 20°C. This is the reason why all of the control lines (wild type) have been moved up to 25°C, at the same time as *glp-1* mutants (at L1 stage).

My results point to the conclusion that both *jmjd-3.2*- and *mes-2*-dependent lifespan extensions are also germline-dependent (Figure 6.9), while confirming the germline-independent mode of action of *utx-1* (Jin et al. 2011). JMJD-3.2 is expressed mostly in neuronal tissue in the adults (Vandamme et al. 2012), and although it might seem initially contradictory that it acts in a *glp-1*-dependent manner, it has been shown that neuronal inputs to germline signalling can affect lifespan (Alcedo and Kenyon 2004), therefore confirming the validity of a germline-dependent *jmjd-3.2* longevity mechanism.

MES-2 is expressed primarily in the gonad; its involvement in germline signalling is therefore not so surprising. However, notably, the results could also be interpreted in a slightly different

manner. Not only does *mes-2* RNAi fail to extend lifespan in the *glp-1* mutant, it actually decreases it (Table 6.2 and Figure 6.9; Log Rank Test *N2+mes-2 RNAi* vs *glp-1+mes-2 RNAi* $p<0.01$). That could suggest that it is *glp-1*-derived lifespan extension that is dependent on the presence of MES-2. Interestingly, in one recent publication (Ni et al. 2012), it was suggested that *mes-2*-dependent lifespan extension is *glp-1-in*dependent. Due to the disparities between these results, I used qRT-PCR to quantify *mes-2* transcript levels in *glp-1* worms fed on *mes-2* RNAi to confirm knock-down of the *mes-2* transcript (Appendix 11). Loss of *mes-2* in *mes-2* RNAi fed worms is shown, however, loss of *mes-2* in the *glp-1* EV control sample is also evident. Considering the fact that *mes-2* is largely expressed in the germline and *glp-1* mutants at 25°C are sterile due to lack of gonad, the decreased levels in *mes-2* transcript are likely to be a consequence of a lack of gonad. This suggests that *glp-1*-induced sterility may mimic a *mes-2* loss-of-function mutation.

Taken together, *glp-1*-dependent lifespan extension is *mes-2*-dependent while loss of GLP-1 causes significant loss of MES-2 as a consequence of germline lack. These two observations seem to contradict each other. It is possible that *mes-2* is not only expressed in germline cells but also in somatic gonad cells or in other tissues in low abundance. For example, Holdeman, Nehrt, and Strome (1998) report low levels of MES-2 antibody binding in intestine. This would suggest that *glp-1* lifespan extension may require trace levels of MES-2 in somatic gonad and/or intestine that are not detectable by qRT-PCR.

Considering that in chapter 4 I suggested that *utx-1* depletion and *utx-1* overexpression may regulate lifespan via separate pathways, the question remains whether lifespan extension dependent on *utx-1* overexpression is also *glp-1*-independent. This would need to be addressed by crossing the worms with the *utx-1* extrachromosomal array into the *glp-1* mutant and assessing the lifespan of this new strain.

6.3.4 Stress resistance

Worms depleted for *utx-1* mRNA have been shown by Jin et al. (2011) to have increased resistance to heat, UV and oxidative stress. I decided to investigate whether *utx-1* overexpression strains also show resistance to environmental stress. In the investigation we included *utx-1* knock-down worms as a positive control to replicate previous findings. Our results suggest that overexpression of enzymatically active *utx-1,* but not the demethylase-dead form, results in resistance to oxidative stress (Figure 6.10). Worms overexpressing *utx-1* had a comparable response to *utx-1* knock-down worms to the presence of paraquat in the media, despite the fact that the former strain live significantly longer under non-stressful conditions. By contrast, overexpression of *utx-1DD::GFP* resulted in decreased stress resistance compared to wild-type worms. This could be because of a potential dominant negative effect of the DD version of the protein, which could act by an increased protein accumulation affecting cellular processes (for example, affecting proteosomal degradation). This needs to be investigated by observation of *utx-1::GFP* and *utx-1DD::GFP* accumulation after exposure to paraquat. Further, additional experiments studying *utx-1* overexpression in the wild-type background will prove very useful in determining whether *utx-1* overexpression has any protective effects, and whether the demethylase-dead version of the protein is in fact detrimental to the stress response. Use of tissue-specific overexpression of *utx-1* could also provide valuable information about the potential source of ROS protection. Further alternative stress inducing stimuli can be used like high temperature or UV to find out whether the potential dominant negative effect of the DD *utx-1* is specific to ROS response.

To find out if there are significant inherent differences in stress-related gene expression between enzymatically active *utx-1* overexpression and the *utx-1DD* overexpression line, we plan to investigate levels of expression in the following genes previously linked to resistance to oxidative stress: *gst-4, nlp-7, ent-1* and *cup-4* (Park, Tedesco, and Johnson 2009). In their research, Park et al. (2009) claim that *gst-4* and *nlp-7* are upregulated as a response to

oxidative stress while *ent-1* and *cup-4* become downregulated. We suggest that there may be differences in expression of those genes between the WT and DD *utx-1* overexpression strains after exposure to oxidative stress.

6.3.5 Tissue-specific *utx-1*-dependent lifespan extension

There is a significant body of work on tissue-driven lifespan control in *C. elegans*, most often identifying neurons, intestine and gonads as the most influential tissues. Signals from multiple tissues might be responsible for the final lifespan of the organism; it has been shown that some longevity control signals are integrated from various tissues (for example neurons are thought to be excreting a hormones that can be received in somatic gonad and allows lifespan extension (Alcedo and Kenyon 2004)). To reveal tissues via which *utx-1* overexpression-driven lifespan extension is controlled, we expressed *utx-1* under tissue-specific promoters and found that both neuronal and intestinal expression of *utx-1,* but not hypodermal or muscular, can result in significant lifespan extension (Figure 6.11).

Taking into account the tissue specificity of *utx-1*-driven longevity as well as the observation that *daf-2* has been shown to exert its lifespan-regulatory function via neurons (Wolkow et al. 2000) and *daf-16* acts mostly by intestine (Libina, Berman, and Kenyon 2003), it could suggest that *utx-1* overexpression modulates longevity via control of genes in the IIS pathway. As the lifespan extension of both neuronal- and intestine-driven *utx-1* strains are comparable (Figure 6.11), a question emerges as to whether these represent two separate pathways of lifespan control or the same pathway in different tissues. To answer this, we are planning to engineer a strain that overexpresses *utx-1* in both neurons and intestine. If this results in an additive lifespan extension it would imply that both neuronal and intestinal pathways of lifespan control are separate and that expression of *utx-1* in both intestine and neurons is necessary for full lifespan extension. Additionally, if lifespan of this strain is longer than that of the *N2+utx-1::GFP* strain (where *utx-1* is under the control of its endogenous promoter),

this might suggest that the endogenous levels of *utx-1* are not contributing to lifespan control in one or both of the tissues.

In this chapter, I have suggested that global levels of H3K27 methylation might be a poor proxy for determining longevity. Further, my data supports the hypothesis that overexpression of *utx-1* controls longevity though a different pathway to mutation in *utx-1*. Additionally I have suggested the molecular pathways through which long-lived mutants described in this thesis regulate lifespan. Finally I have shown that expression of *utx-1* in only neurons or intestine cells is sufficient for the *utx-1*-dependent lifespan extension shown. Because only two of the four examined tissues are important for *utx-1*-dependent lifespan control, we should re-examine changes to H3K27me3 and transcript levels at the *daf-2* locus in a tissue-specific manner, as the results shown in this chapter might be confounded by the presence of other tissues in the worm extracts. This would involve utilising tissue-specific qRT-PCR and ChIP techniques to address these questions for example by using FACS to sort GFP-positive nuclei.

CHAPTER 7: DISCUSSION

7.1 AGEING AND SOCIETY IN THE 21ST CENTURY

Increased access to information, instead of educating society seems to increase distrust toward scientists, whether on the matter of vaccines, the greenhouse effect, or a cure for cancer. New developments in the ageing field are no different, as every publication is instantly reported on and often mis-represented in the press, just like the "anti-oxidant" craze several years back. Additionally, there is also a great deal of false advertisement for "anti-ageing" products where scientific terms are hijacked to make the customer more likely to buy the product (e.g. DNage cream, resveratrol in dietary supplements, and so forth). With all these "anti-ageing" merchandise on the shop shelves, one would think that ageing research would benefit from the exposure and therefore would be one of the best-funded areas of research. The truth is actually quite the opposite. Furthermore, research into ageing is sometimes described as immoral and 'playing god', as people may regard it as the scientist's attempt to interfere with a natural process. This, to me, is a very hypocritical argument for several reasons. Firstly, since developing simple medical procedures, discovering antibiotics and increasing hygiene, the average length of human life has been steadily increasing. Secondly, research into curing cancer, Alzheimer's or diabetes (other natural processes!), if successful, will also achieve exactly the same thing – they will extend human life. Finally, the current drive in our society to look (and feel) young by using various beauty products, dietary supplements or even invasive operations is also trying to achieve a form of "rejuvenation".

Some of the controversy behind ageing research may be spurred by scientists who make bold statements about curing ageing, which might sound presumptuous or even simply bogus to an average person. For example, Aubrey de Grey, a theoretical scientist known for his claims that the first person to live to 1000 years old has already been born and that immortality is in fact within scientific reach, has been given both funding from the private sector and media

coverage. However, in the scientific community it is felt by many that his claims are precipitous.

Most scientists that study biology of ageing are reluctant to be this bold in their claims or even goals. In fact, the current most common objective in ageing research is not necessarily extension of *lifespan* but more likely an extension of *healthspan* – the period lived free of age-related pathologies. However, the rate of ageing is difficult to quantify, and therefore scientists usually use extension of lifespan as the marker for successful ageing interventions, even though extended life is not the goal itself.

7.2 AGEING RESEARCH

A very important point to make when talking about ageing research is to emphasise that a change in just a single gene in many model organisms can significantly extend lifespan. We do not need many troublesome interventions from several fronts to achieve a result; instead, when one gene is tweaked, the biology does the rest – and the animal lives a much longer and healthier life than the control. This is best-described using Cynthia Kenyon's words: what we are trying to utilise is the fact that "animals have a latent potential to live much longer than they normally do" (Kenyon 2010b).

Ageing is a very difficult biological problem to understand, and in fact we are far from fully doing so. The biggest challenge in ageing research is to distinguish cause from effect. For this reason we only have just started to find real causes of frailty and ageing pathologies.

This is one reason that there are still multiple theories to try to explain why molecular changes in ageing tissues occur (see Chapter 1). Even very recently, a new one was proposed: it describes ageing as a "quasi-program", a "shadow" of developmental growth (Blagosklonny 2013). Similarly to some previous hypotheses, it suggests, that ageing is *not* programmed and cannot be selected for, rather, it is a consequence of a tightly-regulated developmental process. It states that many TOR pathway genes work to promote ageing via antagonistic

pleiotropy (in brief, it is an idea that proteins essential early in development drive ageing later in life). The "quasi-programmed hyperfunction" theory can, in fact, explain several observations in *C. elegans* ageing, such as the destructive effect of yolk accumulation late in life (Gems and de la Guardia 2013). However, just like other ageing theories it fails to explain the full extent of phenotypes seen with age (for example the fact that *C. elegans* males also age despite not experiencing yolk accumulation, (Shen and Hodgkin 1988)). In fact, I find it difficult to distinguish the core assumptions of this theory from the general points of the antagonistic pleiotropy theory proposed by Williams over a half a century ago (Williams 1957).

The emerging picture of ageing is truly a complicated one. The pathways controlling lifespan that have been initially elucidated as separate entities are now merging into a convoluted and highly complex set of co-dependent networks. We are only starting to realise how many of the factors involved in longevity control may be working in several processes and even in multiple different ways, depending on their co-regulators, cellular environment and cues from other pathways. Because of this, any changes made to a single protein can resonate in various parts of the cell. This is why research on model organisms is so vital. Biology does not happen in a vacuum; cellular environments are full of enzymes and there is co-dependency between cells, tissues and organs, leading to complicated phenotypes that cannot be fully seen when only using cell culture experiments (which, of course, also have their place in ageing research).

C. elegans is proving to be an extremely and increasingly useful tool for ageing research. The ease of manipulation, the ability to obtain large populations of worms and quick generation turnover were some of the reasons why it was the first organism chosen for the initial RNAi screens for longevity (Hamilton et al. 2005; Hansen et al. 2005). These continue to be powerful recommendations for its use in complex research.

7.3 RNAi SCREEN FOR LONGEVITY PHENOTYPES

Ten years after the first RNAi screens were conducted, the search for factors affecting longevity in *C. elegans* is still ongoing. In the RNAi screen presented in this thesis, I attempted to find novel longevity regulators amongst the "chromatinome" of *C. elegans* (i.e. amongst genes affecting chromatin).

Due to the semi high-throughput nature of the method, any RNAi screen has some flaws and will contain a number positive and false negative results. In my screen, I used the accumulation of autofluorescent pigment (lipofuscin) as a biomarker of ageing, and scored the phenotypes of worms subjected to RNAi on two separate days to decrease the potential for false hits. This, however, did not eliminate all of the potential for error. Critical assessment of my RNAi screen must include the fact that, first of all, I used a biomarker that is not fully established in the field and, what is more, is not easily quantifiable with the tools I used (as discussed earlier). However, as mentioned in section 3.3.1, several internal controls were introduced to ensure consistency in the scoring system. Furthermore, false negative results could be the genes that, despite having a lifespan prolonging effect, did not affect lipofuscin while false positive results could affect lipofuscin accumulation but not ageing (for example by upregulating autophagy or general protein turnover). These hits would have been ruled out using my secondary RNAi screen, investigating lifespan extension.

Despite these potential drawbacks, the screen revealed 4 confirmed, strong positives; genes that, when knocked-down or mutated, increased the lifespan of *C. elegans*. Furthermore, comparing my screen to previously published and peer-reviewed longevity screens, I have shown that my rate of identification (1.2%) lies in between the identification rates of both genome wide screens (0.1-0.5%; (Hamilton et al. 2005; Hansen et al. 2005)) and the screen utilising a library of essential genes (Curran and Ruvkun 2007). This suggests that my approach was successful and comparable to published data.

We chose to investigate chromatin-associated genes for longevity as they have been shown to be major regulators of gene expression and are known to be involved in all biological processes (Berger et al. 2009; Carrer and Wellen 2015; Gonzalez-Aguilera, Palladino, and Askjaer 2014; Martino et al. 2014; Vercelli 2016). This gave me confidence that some of the examined factors would have a role in regulating longevity as well. It has to be noted that the promiscuous roles of chromatin modifiers might result in the identification of factors difficult to study due to their potential effects at many targets, the effects of which may cancel each other out. Further, that any of these factors had a role associated with alteration of chromatin environment (like histone methylation) might not necessarily mean that this is the role that they had in regulation of longevity. The fact that many methyl- and acetyl- transferases are able to modify not only histones but also other proteins is a possibility we have to keep in mind (for example methylation of many transcription factors like E2F1, RB, STAT3 has been reported (Zhang, Wen, and Shi 2012) as well as acetylation of general transcription factors like TFIIE and TFIIE (Imhof et al. 1997) and acetylation of ISWI by GCN5 (Ferreira et al. 2007)). Additionally, enzymes may also have a role as scaffolding proteins or stabilising complexes. When investigating chromatin modifiers it is easy to assume that the histone modifications seen at a particular locus are associated with a direct functional effect of the enzymatic presence. This approach however, might be flawed; what we need to take into consideration is the fact that modifications could be merely a by-product of recruitment of the factors for different purposes. This is the reason for which we investigated the importance of the demethylase activity in lifespan extension, as well as began an investigation into the targets of the lysine demethylases in longevity regulation.

7.4 CHROMATIN MODIFIERS AFFECTING LONGEVITY

The four enzymes that I identified from my longevity screen are *isw-1*, *cbp-1, mes-2* and *jmjd-3.2*. All of these genes, when either knocked-down by RNAi or mutated extend lifespan. Considering that the initial RNAi library was chosen to include the genes regulating

chromatin, it should have come as no surprise that three out of the four genes are thought to work closely together. However, the fact that all of them are known to regulate modification of the same lysine is, perhaps, a little bit more intriguing. CBP-1 is a histone acetyltransferase (Victor et al. 2002), MES-2 is a methyltransferase (part of the Polycomb complex, (Bender et al. 2004)) and JMJD-3.2 is one of the family of demethylases (Vandamme et al. 2012), all proposed to target H3K27, suggesting this could be a key regulatory residue.

Interesting information can be inferred from previous publications that involve homologues of these modifiers in other model organisms. First of all, CBP-mediated lysine acetylation (which is an active chromatin mark) has been shown to work antagonistically to the repression by Polycomb complex (Tie et al. 2009). Furthermore, in a following publication (Tie et al. 2012), they showed that Utx (a homologue of JMJD-3.2, also investigated in this thesis) is physically associated with CBP *in vivo* in order to modulate the antagonism of the repressive marks associated with the Polycomb complex.

Additionally, in *Drosophila*, CBP (Shi et al. 2009), Utx (Zhang et al. 2013) and Jmjd3 (Sola et al. 2011) have all been found to be able to bind and modify p53 affecting its activity (for example, CBP can acetylase p53 in the nucleus to stabilise it, while in the cytoplasm it works to promote its turnover). This line of research has not yet been explored in *C. elegans*, however considering the flexible roles of *cep-1* (the p53 homologue) in lifespan regulation in *C. elegans* (Arum and Johnson 2007; Ventura et al. 2009; Baruah et al. 2014), the possible interactions of JMJD-3.2, CBP-1 and UTX-1 with CEP-1 could provide some interesting insights into lifespan regulation by these enzymes.

An interesting observation I additionally made during the investigation of these chromatin modifiers is that three out of four of them can have phenotypic effects when heterozygous. Both *cbp-1* and *utx-1* effects on longevity have been investigated in heterozygous strains due to the lethality of the homozygous mutation. Mutations in both genes result in significant

lifespan extension despite still containing a single functional copy of the gene, which suggests that haploinsufficiency is at the core of the observation of increased lifespan in these *C. elegans* strains. In fact, exact levels of some of the proteins do seem to be important as *cbp-1* RNAi in the *rrf-3* strain (RNAi hypersensitive) has been shown to result in shortening of the lifespan (Zhang et al. 2009). This finding might seem contrary to the one shown in my thesis (Figure 3.4 (B)) where *cbp-1* RNAi increases lifespan. This could be due to the importance of the dose of CBP-1 protein i.e. loss of some of it increases lifespan, however, loss of significant proportion (in *rrf-3* strain) has profound consequences and results in lifespan shortening.

One of the *mes-2* alleles investigated (*bn11*), can also extend lifespan as a heterozygote (see Appendix 9). What is more, its effect in the heterozygous state is just as significant as the effect of the homozygous (null) mutation. This can be explained by a potential dominant negative role of the *bn11* mutation, for example in stabilising non-functional complexes. For example, if *bn11* mutation causes disruption to methyltransferase domain of MES-2, but does not affect the domains responsible for binding MES-3 and MES-6, then in the heterozygote, a proportion of the PRC2 complex, will become sequestered by a enzymatically inactive MES-2. This could therefore mimic a *mes-2* RNAi phenotype by dominant negative role of mutant *mes-2*.

The effect of heterozygous mutations are not very common in *C. elegans*, so seeing these effects in three of the genes in this work may be a significant observation, suggesting either that dose-dependence is a general feature important in regulation of longevity or that it is specific to the factors that affect chromatin.

7.5 CHANGES IN CHROMATIN ORGANISATION

As shown in my experiments using ChIP and qRT-PCR at target genes, mutations or overexpression of the histone demethylases can significantly alter both histone modification and transcript levels of the genes in question.

However, there is another level of potential control of chromatin, that has not been yet mentioned, and one that must be included for the full picture of age-related cellular changes to emerge. This is the 3D folding and spatial organisation of the nucleus both during and out of the cell cycle. Chromosomes do not just occupy random positions in the nuclei, they have their preferred locations relative to both the nuclear periphery and interior of the nucleus (Bickmore 2013). Gene-dense regions of the chromosomes have been shown to associate with the interior of the nucleus (Boyle et al. 2001; Cremer et al. 2001; Küpper et al. 2007) while gene-poor regions like pericentric regions have been shown to be connected to the nuclear periphery (Gilchrist et al. 2004; Guelen et al. 2008). This 3D organisation of the nucleus can become reorganised in quiescent or senescent cells, with movement of the gene-poor regions away from the periphery observed in these states (Bridger et al. 2000; Mehta et al. 2010). The nuclear periphery is organised by attachment of the heterochromatin to nuclear lamins, therefore various laminopathies also affect the special organisation of the cell nucleus (Dechat et al. 2008; Chen et al. 2014). Importantly, accelerated ageing in HGPS cells have been connected to a defective lamin-A gene product (progerin) and it has also been suggested that the presence of progerin alters some of the heterochromatic interactions with the nuclear periphery and affects the 3D structure of the chromatin (Kubben et al. 2012).

The effect of the changes to the histone modifiers described in this thesis would therefore need to be investigated in the light of their potential effects on the spatial organisation of the nucleus, using FISH probes, or 3C and 4C technologies.

7.6 CHROMATIN IN BALANCE

The most interesting point about the enzymes investigated and their effect on longevity is the fact that despite their opposing functions, manipulation results in the same phenotype. In summation, the lifespan extension could be achieved by:

- loss of *isw-1*
- loss of *cbp-1*
- loss of *utx-1*
- loss of *jmjd-3.2*
- loss of *mes-2*
- overexpression of enzymatically active *utx-1*
- overexpression of *jmjd-3.2* (both enzymatically active and demethylase dead)
- expression of *mes-2*-containing fosmid

These results imply that it is not the type of the change in the chromatin that is important, but perhaps rather change itself. It could suggest that change to any of those enzymes can result in changes in spatial organisation of the chromatin and therefore have downstream effects on particular genes, not by direct effect of mutation or overexpression of the histone modifiers but rather by altering the chromatin neighbourhood and perhaps affecting the localisation of loci in euchromatin or heterochromatin.

These kinds of changes could result in an alteration of the 'balance' of forces determining the state of the cell nucleus. This balance is the sum of the action of various modification enzymes, including the ones presented in this thesis. Notably, many have been shown to act at lysine 27 of histone H3, thus implicating chromatin changes in this control of balance. However, it has to be noted that many enzymes can have multiple functions and multiple targets. For example, the lysine modifiers discussed above might act upon various transcription factors, and therefore indirectly affect chromatin balance.

One new hypothesis that might provide a framework to understand this is the idea of "chromesis", a chromatin-specific recapitulation of the idea of hormesis. This is the observation that a complex system will resist change and find a new equilibrium dependent upon changing factors (Rattan 2008). Chromesis implies that changes to chromatin structure could alter the delicate balance of the nucleus and therefore induce stress response pathways and as an indirect consequence, prolong lifespan. This hypothesis seems to explain why changes in opposing chromatin-affecting enzymes all result in lifespan extension.

7.7 FUTURE WORK

This thesis opens many avenues for further investigation into the effect of H3K27 modifiers on longevity. Firstly, it will be important to elucidate the connection between CBP-1, UTX-1, JMJD-3.2 and CEP-1 (the *C. elegans* homologue of p53). Intriguing studies in *D. melanogaster* determined that CBP regulates p53; CBP can also bind to Utx (Tie et al. 2009; Tie et al. 2012). These data serve as a great starting platform to investigate the relationship between these proteins and their potential effect on longevity in *C. elegans*. This would require the generation of double mutants, and perhaps the utilisation of co-purification studies to investigate the complexes in which each of the proteins might play a role.

Secondly, considering that both loss-of-function and overexpression of the demethylases leads to extended lifespan, it would be interesting to find out whether overexpression of *mes-2* also results in longer life. This was initially attempted by expression of a *mes-2*-containing fosmid, however levels of the transcript proved unchanged potentially due to repression of extrachromosomal arrays in the gonad which is the major site of MES-2 action. The fact that the strains expressing the fosmid still lived longer than the wild-type control can have two explanations. Either one of the other genes on the fosmid can also regulate lifespan, or *mes-2* is overexpressed in intestine and results in lifespan extension (expression in the intestine cells has been reported by(Holdeman, Nehrt, and Strome 1998)). The latter would be difficult to

assess using whole worm extracts as there are only 16 intestine cells in the worm and therefore the overexpression might not be apparent in qRT-PCR data. This problem might be remedied by crossing the fosmid-containing strain into *mes-2(tm5007)* background, as one of the reported effects of *mes-2* mutation is derepression of extrachromosomal arrays in the gonad (Holdeman, Nehrt, and Strome 1998).

Additionally, the overexpression strains might be used to answer a number of other queries that arose from the data collected. For example, overexpression of either or both *jmjd-3.1* and *jmjd-3.3* could provide more information about the effect of *jmjd-3* family on *C. elegans* lifespan. Considering that the loss of each separately does not affect lifespan and loss of both, does not increase lifespan extension of the *jmjd-3.2* mutant (see Figure 5.2 and Figure 5.3), it will be interesting to see whether increased gene dosage could affect the worm's longevity. Further, to understand the source of longevity regulation by overexpression of *jmjd-3.2* (which is expressed ubiquitously early in development but only in adult neurons) two experiments could be conducted. First, if expression of *jmjd-3.2* under a neuronal promoter leads to lifespan extension, this could point to the adult localisation of the JMJD-3.2 being crucial as it would suggest that only the neuronally expressed JMJD-3.2 is responsible for the effect on lifespan. Next, if knock-down of *osm-3* (a neuronal-expressed gene that in depletion causes lifespan extension) in *jmjd-3.2* overexpression strains leads to an additive lifespan extension, this would point to the embryonic JMJD-3.2 being important for longevity regulation (as *jmjd-3.2* would therefore work though separate pathway to the neuronally-controlled longevity and hence result in additive lifespan extension). Another use of the overexpression strains would be to further confirm the existence of separate mechanisms of *utx-1* and *jmjd-3.2* overexpression-dependent lifespan extension. To do this, we could overexpress *utx-1* in the *jmjd-3.2(tm3121)* background. The opposite, however, would be difficult to achieve because of the embryonic lethality of the *utx-1(tm3118)* strain.

I have shown that lifespan extension can be achieved by expressing *utx-1* under either intestinal or neuronal promoters in wild-type background, and to fully understand the contribution of each tissue to the establishment of longevity, we will express *utx-1* in both intestine and neurons. Additive lifespans would suggest separate mechanisms and a role for *utx-1* in longevity regulation independently in both tissues. Further, depletion of *utx-1* by RNAi in adult worms results in longer lifespan as compared to depletion through the larval stages (Maures et al. 2011). It would be interesting to understand whether there is a temporal regulation of the *utx-1* overexpression. This could potentially be achieved using a heat shock promoter and subjecting the worms to increased temperature at various developmental stages. However worm heat shock promoters are known to result in a significant about of leaky expression and therefore it might not provide meaningful data.

Mindful of the fact that *utx-1* overexpression-dependent lifespan extension was shown to be specifically dependent on its enzymatic activity, we have investigated *daf-2* 5'UTR H3K27me3 as a potential target of its demethylase activity. I have shown that H3K27me3 on *daf-2* 5'UTR decreases at day 10 as compared to day 1 of adulthood (concomitant with overexpression of the demethylase), which suggests that *daf-2* regulation by H3K27me3 cannot be the source of this lifespan extension. Considering that the increased longevity seen is both *daf-16* and *glp-1* dependent (as shown earlier: Figure 6.8 and Figure 6.9), it is going to be important to identify potential targets of the lysine demethylase in those pathways. Additionally, *mes-2* potential targets and the importance of its methyltransferase activity in lifespan extension could be investigated using drugs like 3-deazaneplanocin A (which inhibits the methyltransferase activity of the PRC2 complex (Chiba et al. 2012)).

Finally, at a chromatin-wide level, we could use FISH to establish whether the 3D structures of nuclei change with age, and how this is affected by overexpression of the demethylases I am studying. This approach can be complemented using 3C and 4C, which would investigate the changes in the proximity of particular DNA sites across the genome. Additionally use of

ChIP-seq will allow us to determine the changes in the profile of the H3K27me3 as well as other chromatin marks across the promoters. Knowledge of the changes in all epigenetic marks could be essential to fully understand the dynamics of ageing chromatin.

These approaches, combined with RNA-seq to investigate changes in gene expression between young and old, wild-type and long-lived strains, will be key to correlate shifts in chromatin modifications with their effects on transcription in aged cells. Finally, all of the above approaches should also be additionally extended by examining epigenetics in cell (or tissue) specific manner. Initial approach could be to separate the germline from somatic tissues as these would cause the biggest variance in results, due to the inherent difference in tissue role (disposability of soma and 'immortality' of germline). This can be done either mechanistically by gonad dissection, genetically using germless mutants (like *glp-1*) or using GFP tagged cells and sorting using FACS. Some work has been done to try to use GFP markers to assess the changes in ageing tissues however further research needs to be done to optimise current approaches.

APPENDIX

APPENDIX 1. List of genes in the 'chromatinome' library donated by Dr Gino Poulin. The library is a re-grid from the *C. elegans* RNAi library created by Ahringer lab (Kamath et al. 2003). List of genes was curated by WormBase annotation of chromatin related function.

gene name	**sequence name**
mes-2	*R06A4.7*
wdr5.1	*C14B1.4*
rbbp5	*F21H12.1*
ash-2	*Y17G7B.2*
rab-5	*F26H9.6*
smo-1	*K12C11.2*
lin-59	*T12F5.4*
C18E3.5	*C18E3.5*
C10H11.8	*C10H11.8*
B0261.1	*B0261.1*
tom-1	*M01A10.2a*
F55F8.3	*F55F8.3*
tag-345	*F55F8.5*
F56H1.3	*F56H1.3*
C06A5.3	*C06A5.3a*
T23H2.3	*T23H2.3*
K06A5.8	*K06A5.8a*
unc-37	*W02D3.9*
H06O01.2	*H06O01.2*
F15C11.2	*F15C11.2a*
ekl-1	*F22D6.6*
tag-298	*C01H6.7*
lin-61	*R06C7.7*
pbrm-1	*C26C6.1a*
wdr-23	*D2030.9a*
gna-2	*T23G11.2*
taf-5	*F30F8.8*
mys-4	*C34B7.4*
rsd-6	*F16D3.2*
sec-12	*K02B12.3*
D1081.8	*D1081.8*
prp-4	*C36B1.5*
gei-11	*F32H2.1a*

thoc-3	*F32H2.4*
rad-54	*W06D4.6*
gei-17	*W10D5.3a*
uev-3	*F26H9.7*
rba-1	*K07A1.11*
lin-53	*K07A1.12*
sur-6	*F26E4.1*
npp-17	*F10G8.3*
ned-8	*F45H11.2*
mus-101	*F37D6.1*
prp-17	*F49D11.1*
ubc-12	*R09B3.4*
hda-3	*R06C1.1*
uba-2	*W02A11.4*
Y18D10A.9	*Y18D10A.9*
smu-1	*CC4.3*
Y87G2A.11	*Y87G2A.11*
W04A8.1	*W04A8.1*
taf-1	*W04A8.7*
F47G4.4	*F47G4.4*
mys-2	*K03D10.3*
ekl-4	*Y105E8A.17*
uev-1	*F39B2.2*
Y54E5B.2	*Y54E5B.2*
ubc-16	*Y54E5B.4*
Y39G10AR.18	*Y39G10AR.18a*
Y39G10AR.9	*Y39G10AR.9*
mbtr-1	*Y48G1A.6*
Y71F9AL.17	*Y71F9AL.17*
ubc-3	*Y71G12B.15*
Y71G12B.1	*Y71G12B.1a*
prp-21	*W07E6.4*
W07E6.2	*W07E6.2*
T02H6.1	*T02H6.1*
F52C6.4	*F52C6.4*
F52C6.12	*F52C6.12*
F33H12.1	*F33H12.1*
set-11	*F34D6.4*
T05A8.5	*T05A8.5*
F14D2.11	*F14D2.11*
C16C8.4	*C16C8.4*
C16C8.13	*C16C8.13*
C16C8.14	*C16C8.14*
C16C8.5	*C16C8.5*

C16C8.11	*C16C8.11*
F40E12.2	*F40E12.2*
F54D10.7	*F54D10.7*
C16A11.3	*C16A11.3*
C16A11.4	*C16A11.4a*
H20J04.2	*H20J04.2*
ZK430.7	*ZK430.7*
tsn-1	*F10G7.2*
F33G12.2	*F33G12.2*
fzy-1	*ZK177.6*
F59E12.1	*F59E12.1*
rbbp5	*F21H12.1*
cpf-1	*F28C6.3*
F46C5.9	*F46C5.9*
set-17	*T21B10.5*
D2013.3	*D2013.3*
wdfy-2	*D2013.2*
scp-1	*D2013.8*
Y53C12B.1	*Y53C12B.1*
ufd-3	*C05C10.6a*
mec-15	*T01E8.4*
pro-1	*R166.4*
ect-2	*T19E10.1a*
rad-23	*ZK20.3*
gpb-1	*F13D12.7*
F43G6.11	*F43G6.11a*
F43G6.6	*F43G6.6*
ZK930.1	*ZK930.1*
btf-1	*F15D4.1*
bub-3	*Y54G9A.6*
hda-6	*Y51H1A.5*
Y48B6A.1	*Y48B6A.1*
nurf-1	*F26H11.2c*
ubc-15	*Y110A2AR.2*
ubc-20	*F40G9.3*
ubl-1	*H06I04.4a*
wdr5.1	*C14B1.4*
brc-1	*C36A4.8*
F56F3.4	*F56F3.4*
F35G12.4	*F35G12.4a*
set-2	*C26E6.9a*
T10F2.4	*T10F2.4*
K10D2.1	*K10D2.1*
prmt-5	*C34E10.5*

F52C9.1	*F52C9.1*
Y32H12A.8	*Y32H12A.8*
uev-2	*F56D2.4*
C05D2.10	*C05D2.10a*
C28H8.9	*C28H8.9a*
ubq-1	*F25B5.4a*
F47D12.9	*F47D12.9*
C56G2.15	*C56G2.15*
C16A3.1	*C16A3.1a*
C56G2.1	*C56G2.1a*
T26A5.5	*T26A5.5a*
set-1	*T26A5.7a*
F37A4.4	*F37A4.4*
isw-1	*F37A4.8*
brd-1	*K04C2.4*
ubc-18	*R01H2.6*
rfl-1	*F11H8.1*
B0280.9	*B0280.9*
lpd-7	*R13A5.12*
C18H2.4	*C18H2.4*
flt-1	*ZK783.4*
C18H2.1	*C18H2.1*
C18H2.3	*C18H2.3*
C18H2.5	*C18H2.5*
tag-250	*C29E4.5a*
gei-8	*C14B9.6a*
met-2	*R05D3.11*
cec-1	*ZK1236.2*
zfp-1	*F54F2.2a*
F54F2.9	*F54F2.9*
ubc-7	*F58A4.10*
cbp-1	*R10E11.1a*
cor-1	*R01H10.3*
M03C11.8	*M03C11.8*
hpl-2	*K01G5.2a*
athp-1	*C44B9.4*
Y41C4A.11	*Y41C4A.11*
arx-3	*Y79H2A.6*
ssl-1	*Y111B2A.22*
ubq-2	*ZK1010.1*
npp-18	*Y43F4B.4*
set-25	*Y43F4B.3*
lis-1	*T03F6.5*
set-16	*T12D8.1*

dyf-2	*ZK520.3*
Y53G8AR.2	*Y53G8AR.2a*
Y55B1BR.3	*Y55B1BR.3*
F53H1.4	*F53H1.4a*
npp-20	*Y77E11A.13a*
ubc-1	*C35B1.1*
set-9	*F15E6.1*
F29B9.2	*F29B9.2a*
swd-2.2	*C33H5.7*
swd-2.1	*C33H5.6*
mes-6	*C09G4.5*
lin-49	*F42A9.2*
rad-26	*C27B7.4*
daf-10	*F23B2.4*
F38E11.5	*F38E11.5*
pis-1	*T13F2.3a*
uba-1	*C47E12.5a*
rack-1	*K04D7.1*
miz-1	*R13.4*
rbr-2	*ZK593.4*
let-70	*M7.1*
psa-4	*F01G4.1*
F54E12.2	*F54E12.2*
klp-12	*T01G1.1*
sec-31	*T01G1.3*
lex-1	*F11A10.1a*
F28D1.1	*F28D1.1*
VT23B5.2	*VT23B5.2*
qui-1	*Y45F10B.10*
set-26	*Y51H4A.12*
lsy-13	*T06A10.4*
Y116A8C.13	*Y116A8C.13*
siah-1	*Y37E11AR.2*
eri-5	*Y38F2AR.1*
set-23	*Y41D4B.12a*
T10B5.4	*T10B5.4*
F59A7.8	*F59A7.8*
lin-40	*T27C4.4a*
K09D9.11	*K09D9.11*
C49G7.1	*C49G7.1*
B0238.10	*B0238.10*
T05C3.2	*T05C3.2*
let-418	*F26F12.7*
gad-1	*T05H4.14*

mys-1	*VC5.4*
rps-30	*C26F1.4*
spr-1	*D1014.8*
T23B12.1	*T23B12.1*
C10B5.1	*C10B5.1*
atgr-18	*F41E6.13*
H24G06.1	*H24G06.1*
R07B5.8	*R07B5.8a*
hpo-22	*C08B6.7*
gna-1	*B0024.12*
set-22	*Y32F6A.1*
hdac-11	*C35A5.9*
wdr-5.3	*ZC302.2*
tag-135	*D1054.15*
F17C11.10	*F17C11.10*
C06H2.3	*C06H2.3*
elpc-3	*ZK863.3*
R11D1.1	*R11D1.1a*
mes-4	*Y2H9A.1*
sel-10	*F55B12.3a*
swan-2	*F53C11.7*
swan-1	*F53C11.8*
hda-1	*C53A5.3*
set-5	*C47E8.8*
cdt-2	*T01C3.1*
C01G10.1	*C01G10.1*
aipl-1	*K08F9.2*
F26D2.10	*F26D2.10*
T08G3.7	*T08G3.7*
F36D3.5	*F36D3.5*
ZK228.4	*ZK228.4a*
ubc-19	*Y69H2.6*
C25F9.5	*C25F9.5*
Y113G7B.14	*Y113G7B.14*
elp-1	*F38A6.2*
Y39H10A.6	*Y39H10A.6*
che-2	*F38G1.1*
F13C5.2	*F13C5.2*
F39H12.2	*F39H12.2*
ZC53.6	*ZC53.6*
unc-78	*C04F6.4*
C52B9.8	*C52B9.8*
atg-16.1	*F02E8.5*
ifta-1	*C54G7.4*

set-19	*W01C8.3*
set-20	*W01C8.4a*
ubc-17	*B0403.2*
set-12	*K09F5.5*
chd-3	*T14G8.1*
hpl-1	*K08H2.6*
K09A11.5	*K09A11.5*
C29F7.6	*C29F7.6*
set-8	*F02D10.7*
sym-4	*R03E1.1*
wdr-5.2	*K04G11.4*
F23D12.5	*F23D12.5*
rbc-1	*F54E4.1*
csb-1	*F53H4.1*
F53H4.6	*F53H4.6*
osm-1	*T27B1.1*
hda-4	*C10E2.3*
K07H8.3	*K07H8.3*
ing-3	*Y51H1A.4*
pfs-2	*R06A4.9*
pod-1	*Y76A2B.1*
mes-2	*R06A4.7*
ubc-13	*Y54G2A.31*
ubc-14	*Y87G2A.9*
eif-3.I	*Y74C10AR.1*
gpb-2	*F52A8.2*
set-32	*C41G7.4*
Y74C9A.4	*Y74C9A.4a*
Y23H5B.5	*Y23H5B.5*
cyn-15	*Y87G2A.6*
bet-1	*Y119C1B.8a*
jmjd-2	*Y48B6A.11*
sel-2	*F10F2.1*
ubc-8	*Y94H6A.6*
D2023.4	*D2023.4*
elpc-2	*Y111B2A.17*
cash-1	*K07C5.8*
psa-1	*Y113G7B.23*
F55G7.2	*F55G7.2*
B0303.4	*B0303.4*
C29H12.5	*C29H12.5*
F13H8.2	*F13H8.2*
set-4	*C32D5.5*
lin-23	*K10B2.1*

atg-16.2	*K06A1.5*
egl-27	*C04A2.3a*
ada-2	*F32A5.1a*
ubc-6	*D1022.1a*
T09A5.8	*T09A5.8*
rev-1	*ZK675.2*
hda-2	*C08B11.2*
ubc-24	*F49E12.4*
psr-1	*F29B9.4a*
ubc-9	*F29B9.6*
set-15	*R11E3.4*
hdac-6	*F41H10.6a*
T12E12.2	*T12E12.2*
dnj-11	*F38A5.13*
dyci-1	*C17H12.1*
C17H12.13	*C17H12.13a*
T05A12.4	*T05A12.4a*
F32E10.2	*F32E10.2*
F32E10.5	*F32E10.5*
F32E10.6	*F32E10.6*
utx-1	*D2021.1*
tag-279	*F18E9.5a*
ubc-23	*C28G1.1*
ubc-21	*C06E2.3*
ubc-22	*C06E2.7*
F36G3.2	*F36G3.2*
F21G4.4	*F21G4.4*
D1053.2	*D1053.2*
F54F7.7	*F54F7.7*
set-6	*C49F5.2*
W06D11.4	*W06D11.4*
F17A2.3	*F17A2.3*

APPENDIX 2. List of primers used in this thesis.

Primer name	F/R	sequence	Tm	% GC	Primer type
utx-1_g1	F	CATTGAAAGAAGCTTATGAGCAGC	56	42	Genotyping *utx-1 (tm3118)*
utx-1_g1	R	GTCCACGAATACAAGAAATAGT	52	36	Genotyping *utx-1 (tm3118)*
utx-1res_g2	R	AGAGGCACATCAAGTGGAAC	56	50	Genotyping *utx-1 (tm3118)*
utx-1_rt2	F	TTCGGGTTAGGTCTTGTCTACTTG	58	46	qRT-PCR
	R	TCATCATTATTCTCGTGGCTCAG	56	43	qRT-PCR
daf-2_rt1	F	GTGGCGTGAGAATGAAGTGAG	57	52	qRT-PCR
	R	GGAATTTCGTAGAATCCGTTG	53	43	qRT-PCR
daf-16_rt1	F	TCGTCGTCTCGTGTTTCTCCA	59	52	qRT-PCR
	R	TTCCATAGGCACCCGGTAGTG	60	57	qRT-PCR
jmjd-3.2_rt2	F	ATGCCTGGGCTGTCTTCGATTT	61	50	qRT-PCR
	R	AATGCGGAAGGATTGCGTAGGA	60	50	qRT-PCR
jmjd-3.1_rt2	F	ACGCCTGATGTTCTCATTGGCA	61	50	qRT-PCR
	R	TATGCTGCACAGGCACAGGTTTC	62	52	qRT-PCR
jmjd-3.3_rt2	F	CGACCGAACGTATCCACTCACCTC	62	58	qRT-PCR
	R	CGCCCGCTTCTTTGCCATTT	61	55	qRT-PCR
cdc-42_rt1	F	CTGCTGGACAGGAAGATTACG	56	52	qRT-PCR
	R	CTCGACATTCTCGAATGAAG	52	45	qRT-PCR
mes-2_rt1	F	GCGTGATTCTCAGCGAAATA	54	45	qRT-PCR
	R	TGCACATGTACGAGCACACT	58	50	qRT-PCR
daf-2_5'UTR	F	CTCTTCTTCATCTCCCTCCATCACCA	61	50	ChIP-qPCR
	R	ATCCGACGATCAGATGTGTGCTCT	62	50	ChIP-qPCR
daf-16d_5 'UTR	F	CGCGCTTTTTTCGTCTAATTTGCCC	61	48	ChIP-qPCR
	R	TCCGGAATCAGGCCTTGCCG	63	65	ChIP-qPCR
jmjd-3.2_ 5'UTR	F	GTGTGTGTGTTTTTTGTTAATTTCGAGG	58	36	ChIP-qPCR
	R	GCCACCGCTATCCATCTTTTG	57	53	ChIP-qPCR
utx-1_ 5'UTR	F	GACACAGTATTGACCAGTTCACAGTG	59	46	ChIP-qPCR
	R	GAGAATTGTAGGAAACATAACTTGGAG	56	37	ChIP-qPCR
cdc-42_ 5'UTR	F	CTTCCGACCAGTTACCGATGAATC	59	50	ChIP-qPCR
	R	CGGTAAACGCCTTAATAAACGATG	56	42	ChIP-qPCR

APPENDIX 3. List of putative candidates from the RNAi screen for longevity phenotypes.

('- -' score on one of the days, or '-' score on both days)

gene name	known function	protein domains	homologues
F15C11.2	*Ubql-1*; ubiquitin-like, polyubiquitin binding protein	HS chaperone-binding,	*Ubqn* in flies *dsk2* in yeast
D1081.8		Myb TF, SANTVMyb domain	*cdc5l* in humans
rba-1	RBAp48 related; 96% similarity to *lin-53*;	WD-40 repeat, histone-binding protein RBBP4	*RbBP4* in humans *Caf1* in flies *hat2* in yeast
prp-17	Yeast PRP (splicing factor) related	WD-40 repeat	*cdc40* in humans
ubc-12	Uniquitin-conjugating enzyme		*ube2f* in humans *ubc12* in yeast
W04A8.1	Weakly similar to human microcephalin	BRCT domains	
Y39G10AR.9	ATPase-associated factor	WD-40 repeat	*paaf1* in humans
F52C6.12	Ubiquitin conjugating enzyme		*ubc5* in yeast
D2013.3		WD-40 repeat	
ufd-3	Ubiquitin fusion degradation	WD-40 repeat PFU domain, PUL	*Plap* in flies
rad-23	Required for normal axon branching and function in Nucleotide Excision Repair	Ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains, HS chaperonin-binding, XPC-binding domain	*rad23* in yeast, flies and humans
gpb-1	Beta subunit of heterotrimeric G protein	WD-40 repeat, G-protein beta subunit	*gnb1* in humans *Gbeta13F* in flies
F43G6.6 (jmjd-1.1)	HDMT	PHD and JmjC	*Kdm7a* in vertebrates
met-2	HMT required for H3K9me3 and H3K36me3.	SET domain, Methyl-CpG DNA binding	*Spen* in flies
cec-1	uncharacterised	chromodomain	*rtl1* in mice
T26A5.5 (jhdm-1)	Demethylase specific for H3K36?	JmjC	*Kdm2b* in humans
ubc-18	E2 ubiquitin-conjugating enzyme		*ube2l3* in humans *ubc5* in yeast
rfl-1	E1-activating enzyme and ubiquitin-like protein conjugation pathway		*Uba3* in flies
cor-1	Actin binding	Coronin, WD-40 repeat	*coro6* in humans *crn1* in yeast
set-16	Set1/MLL family of HMT, H3K4me in vitro	FY-rich, SET domain, Zinc finger (PHD)	*Mll2* in humans
let-70	E2 ubq conjugating enzyme, essential gene, abundantly expressed at all stages of development		*Eff* in flies *ubc5* in yeast
elpc-3	Elongator complex protein component, predicted N-acetyltransferase activity		*elp3* in yeast
C01G10.1	uncharacterised		
elp-1	Echinoderm microtubule-associated protein	WD repeat	*eml1* in humans
ash-2	COMPASS complex, H3K4me3 methyltransferase	SET domain	*ash2* in mammals
cyn-15	Cyclophilin homologue		*cpr3* in yeast
psa-1	*swsn-1*; SWI3 orthology, SWISNF complex, required for normal levels of fertility; strong genetic interaction with lin-35.		*smatcc1* in humans
set-11	H3K9 methyltransferase	SET domain	*ehmt1/2* in humans

APPENDIX 4. List of RNAi clones that increased lipofuscin accumulation in *C. elegans*

(A) strong effect – lipofuscin accumulation increased on both days of screening ('+ +' score on both days)

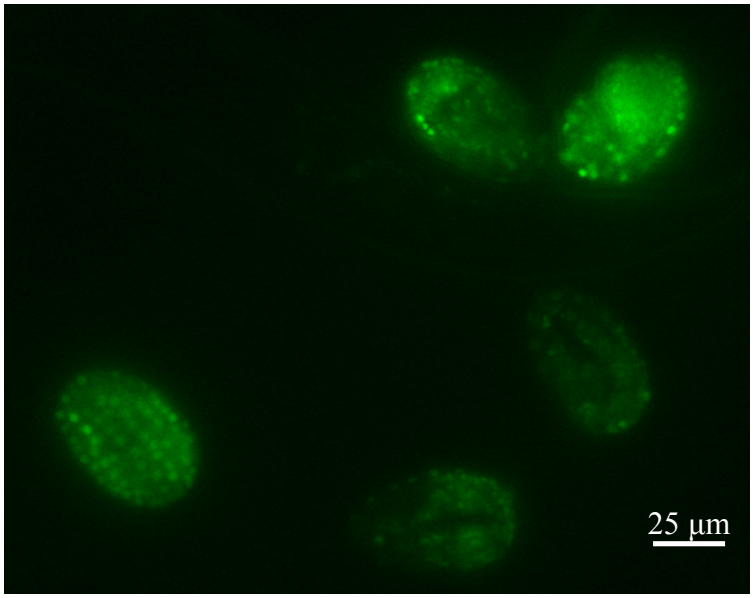
gene name	known function	homologues
C16C8.4	uncharacterised	*ubd* and *uba52* in humans
ZK430.7	uncharacterised	*dcaf13* in humans
ubq-1	uncharacterised	*ubc* in humans
M03C11.8	predicted to have DNA and ATP binding activities	*smarcad1* in humans
set-15	predicted methyltransferase activity	-

(B) weak effects – lipofuscin increase on one of the days of screening ('++' score on one of the days of screening)

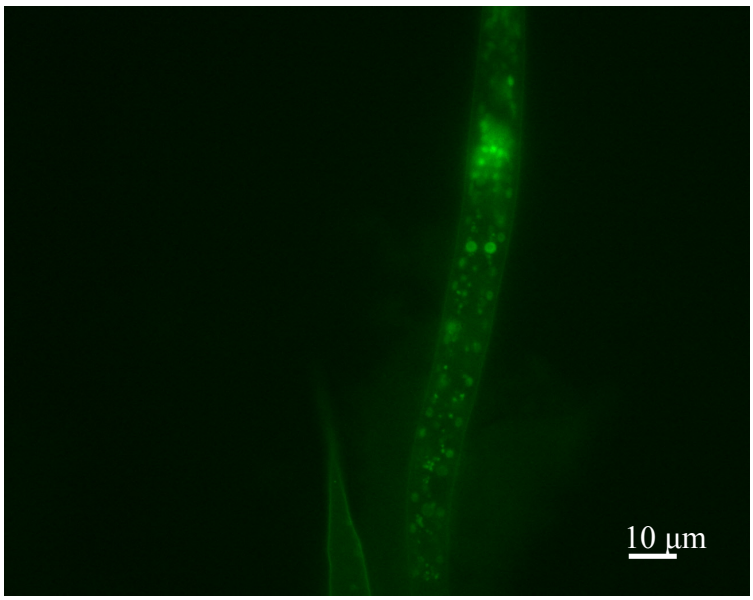
gene name	known function	homologues
tsn-1	component of the RISC complex	*snd1* in humans *Tudor* in flies
pro-1	required for ribosome biogenesis and germline development	*CG12805* in flies
ZK930.1	vps-15 (vacuolar sorting factor)	*pik3r4* in humans
bub-3	mitotic and meiotic spindle assembly checkpoint	*gle2* in yeast
Y48B6A.1	Ribosome biogenesis	*erb1* in yeast
wdr-5.1	part of H3K4 trimethylation complex	*Set1* in yeast
K10D2.1	uncharacterised	*Hira* in humans *hir1* in yeast
C05D2.10	Ribosome biogenesis	*wdr37* in humans
C56G2.1 (akap-1)	uncharacterised	*akap1* in humans
set-9	required for short lifespan, predicted methyltransferase	-
hpo-22 (wdr-20)	Ribosome biogenesis	*Dmwd* and *wdr20* in humans
C25F9.5	uncharacterised	-
F39H12.2	uncharacterised	*wdr-54* in humans
F55G7.2 (dot-1.4)	H3K79 methyltransferase	*dot1l* in humans *dot1* in yeast
rev-1	involved in DNA repair	*Rev1* in humans
ubc-24	ubiquitin conjugating enzyme	*ubc1* in yeast
psr-1	critical for cell corpse engulfment, mediation of phagocytosis	*psr* in flies

APPENDIX 5. Expression of *jmjd-3.2::GFP* in development. Image capture: Zeiss AxioSKOP2 microscope with Zeiss AxioCamMR digital camera, FITC filter, 63x Zeiss objective, exposure of 150 ms.

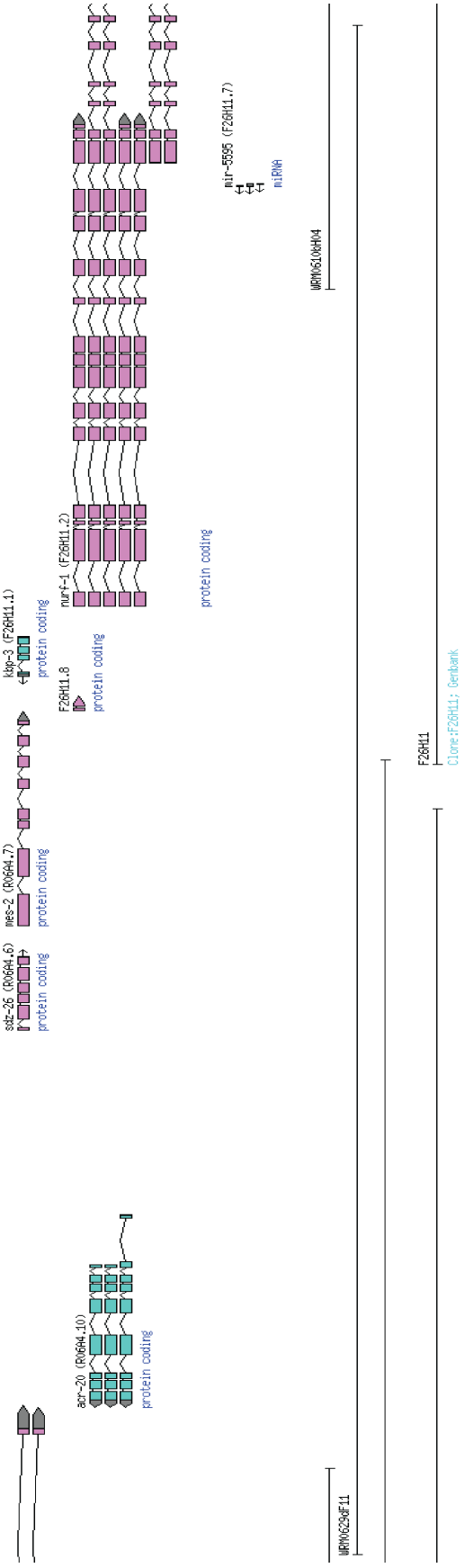
(A) as the embryo develops, levels of JMJD-3.2::GFP decrease (clockwise from bottom left)



(B) in early larval stages (L1 shown) JMJD-3.2::GFP is still visible in most cells

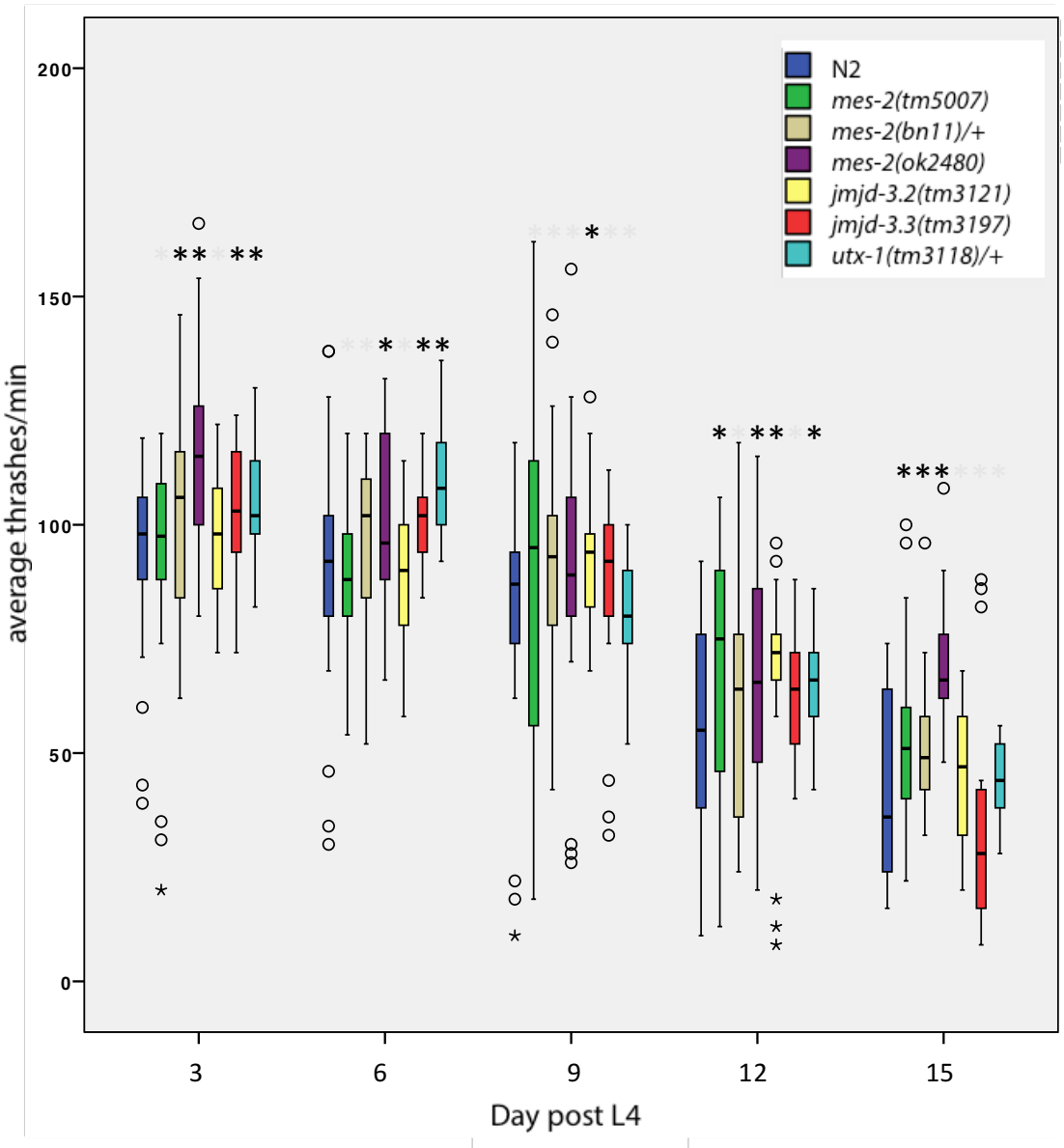


APPENDIX 6. WRM0629dF11 fosmid map. (in orange, marked position of *mes-2*)



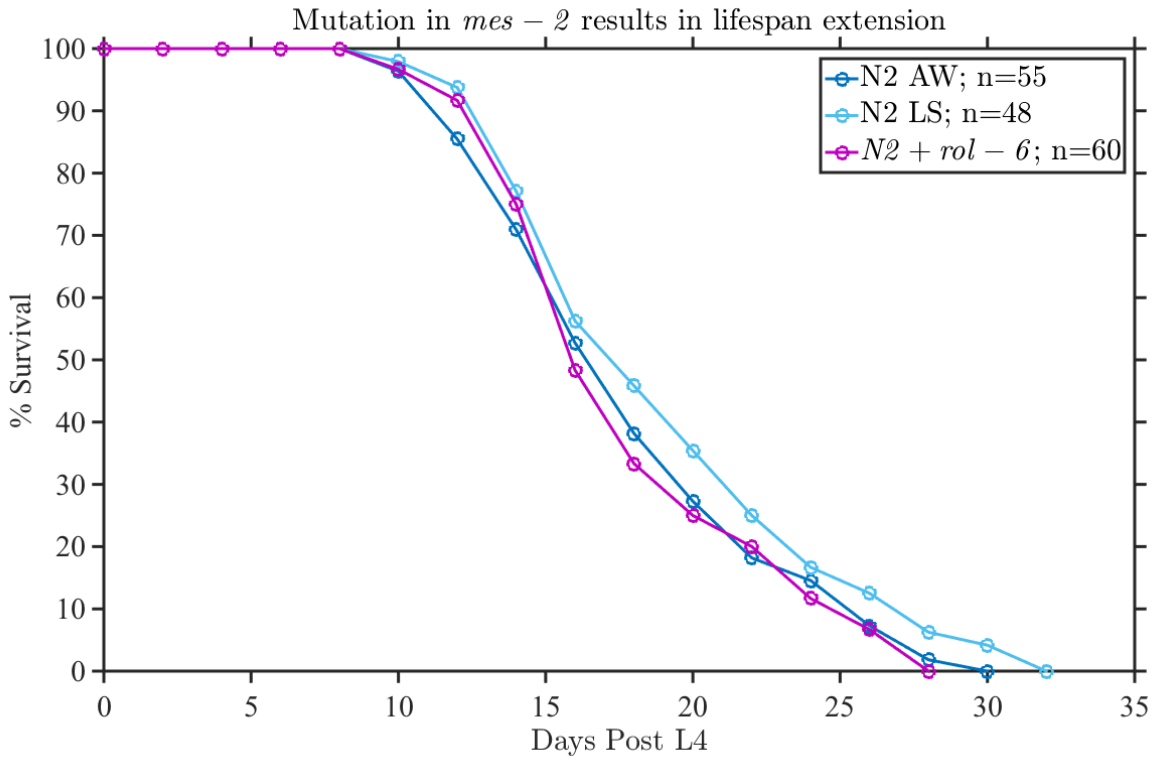
APPENDIX 7. Thrashing assay performed on aged worms not subjected to FUDR. 10 worms per strain were used for every time point, recording thrashing during three 30s periods. There is a marked increase in healthspan in *mes-2* mutants as well as *jmjd-3.2* and *utx-1(tm3118)/+* at day 12. Further, all *mes-2* mutants show significantly increased thrashing rates at day 15.

'*' above the box-whisker plot signifies a statistically significant difference from a WT strain (One way ANOVA and Student's T- test, $p<0.01$). 'o' above and below the plot and '*' under the plot signifies outliers and extreme outliers respectively. The box represents the interquartile range of data, whiskers show 95% confidence interval. Data was analysed and plotted using SPSS software.

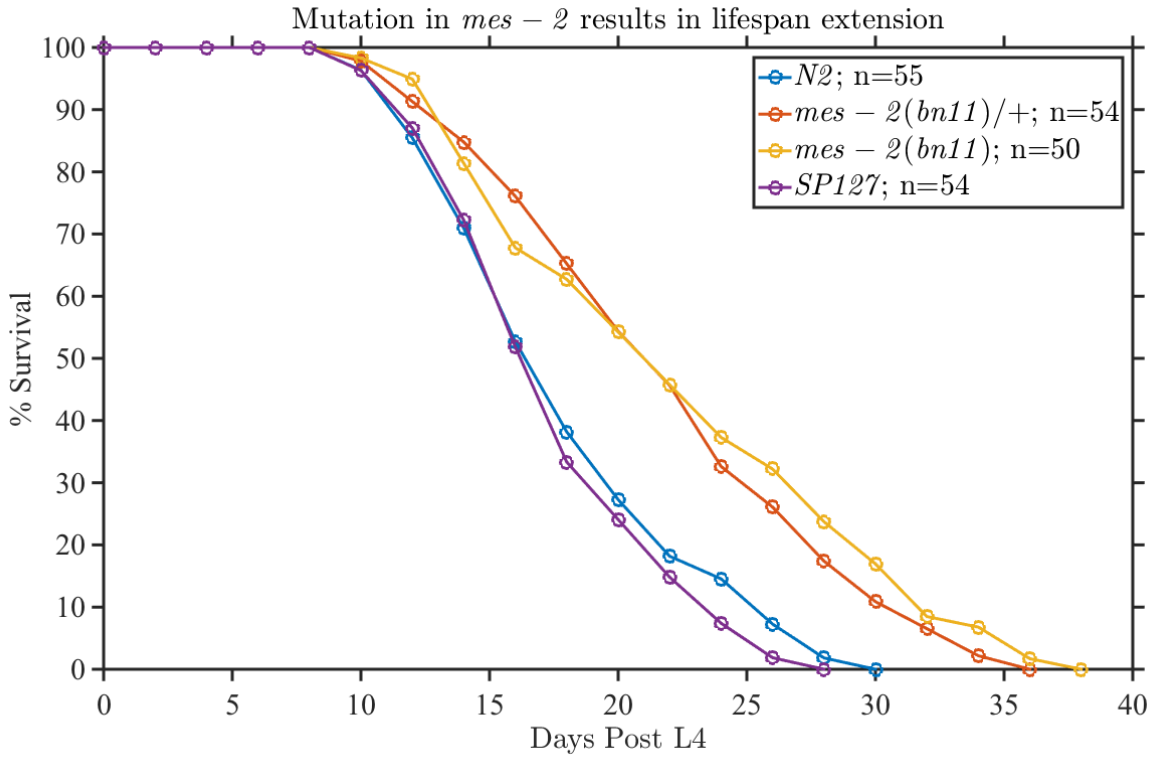


APPENDIX 8. Control lifespan experiments.

Presence of *rol-6* on an extrachromosomal array does not have a statistically significant effect on lifespan. Wild-type worms from the Salcini lab (N2 LS) have lifespan comparable to the N2 worms from the Woollard lab (N2 AW). (Log Rank Test; N2 AW vs N2 LS, p=0.22; N2 AW vs *N2+rol-6*, p=0.64; N2 LS vs *N2+rol-6*, p=0.11).

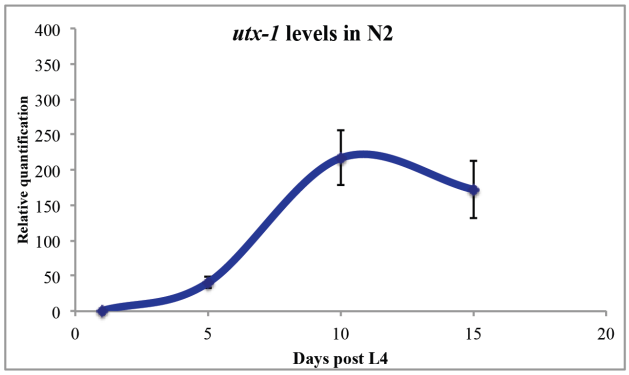


APPENDIX 9. Heterozygous *mes-2(bn11)* mutant extends lifespan to the same extent as the homozygous *bn11* mutation (no statistically significant difference, Log Rank test, p=0.48). Balancer chromosome present in the heterozygous animals has no effect on longevity as shown by SP127 strain that contains the balancer chromosome in the wild-type genetic background.

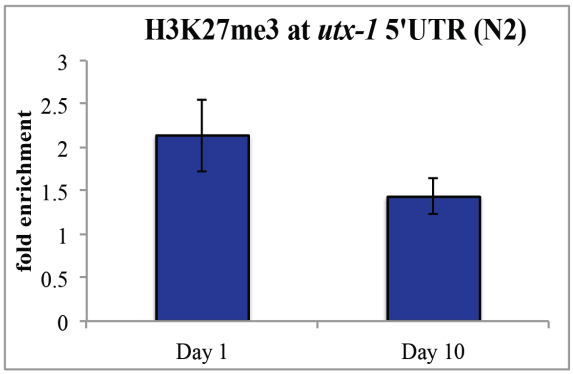


APPENDIX 10. An assessment of changes in transcript levels and levels of H3K27me3 at 5'UTR of *utx-1* gene in N2 strain. (A) qrt-PCR data showing changes in transcript levels of *utx-1* with age. Data has been pulled from at least 2 separate biological repeats and error bars represent standard deviation between the replicates. Quantification is relative to levels of transcript in day 1 wild-type animals and normalised to levels of *cdc-42*. (B) ChIP data are presented as fold enrichment over 'NO IP' control using the ΔΔCt method. Error bars are representative of standard deviation between the three technical repeats and mathematical error progression

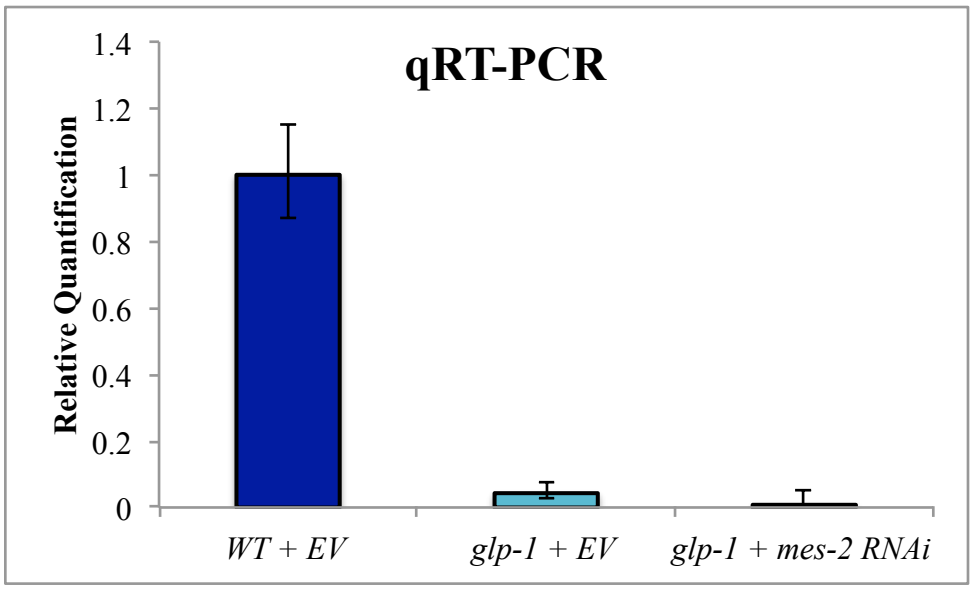
A



B



APPENDIX 11. Levels of *mes-2* mRNA in *glp-1* mutant and in *glp-1* mutant with *mes-2* knock-down. Data has been pulled from at least 2 separate biological repeats and error bars represent standard deviation between the replicates. Quantification is relative to levels of transcript in wild-type animals (EV control RNAi) and normalised to levels of *cdc-42*.



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