

Investigating the role of lysine demethylase UTX-1 in lifespan regulation of *C. elegans*

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ABSTRACT

Investigating the role of lysine demethylase UTX-1 in lifespan regulation of *C. elegans*

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That ageing can be epigenetically regulated has garnered considerable interest due to the implication that ageing is incredibly plastic and therefore, potentially reversible. Overexpression and downregulation of *utx-1* (H3K27me2/3-specific lysine demethylase UTX-1) was found to promote lifespan extension in *C. elegans*. Because *utx-1* is ubiquitously expressed, it was hypothesised that lifespan is differentially affected, depending on the tissue where *utx-1* expression is manipulated. Hypodermis-, intestine- and neuron-specific knockdown of *utx-1* (but not muscle) was found to promote lifespan extension in worms, whereas, overexpression of *utx-1* only in intestine and neurons (but not muscle and hypodermis) promoted longevity. The catalytic activity of UTX-1 was found to be necessary for lifespan extension due to *utx-1* overexpression and RNA-Seq studies showed that *utx-1* overexpression is associated with upregulation of defense and immune response genes – a possible protective mechanism that indirectly promotes lifespan extension in worms. Finally, *hsp-1* (heat-shock protein hsp-70) was discovered to be absolutely required for lifespan extension due to *utx-1* overexpression, suggesting that the heat-shock response is crucial for lifespan regulation mediated by *utx-1* overexpression. Overall, the work in this thesis, which also includes analysis of *cbp-1* (H3K27 acetyl transferase), suggests that lifespan regulation due to H3K27 modification is incredibly complex and that longevity is mediated differentially as a consequence of different doses of modifier, and in specific tissues, likely due to transcriptional changes mediated by the demethylase function of UTX-1.

Thesis Supervisor: Prof. Alison Woollard

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LIST OF ABBREVIATIONS

ACDH-1	Acyl CoA Dehydrogenase-1
AD	Alzheimer's Disease
ADR	Axenic Dietary Restriction
AGE	Advanced Glycation End-products
AKT	Protein Kinase B
ALH-6	Aldehyde Dehydrogenase-6
AMP	Adenosine mono-phosphate
AMPK	AMP-activated Protein Kinase
ANOVA	Analysis of Variance
ATP	Adenosine tri-phosphate
BGI	Beijing Genomics Institute
cAMP	cyclic AMP
CBP	CREB-binding Protein
cDNA	Complementary DNA
CGC	Caenorhabditis Genetics Center
COX	Cytochrome c Oxidase
CR	Caloric Restriction
CRISPR	Clustered, regularly interspaced, short palindromic repeats
CREB	cAMP Response Element Binding
DAF	Abnormal Dauer Formation
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic Acid
DR	Dietary Restriction
EDTA	Ethylenediaminetetraacetic Acid
ENCODE	Encyclopaedia of DNA Elements
ETC	Electron Transport Chain
EV	Empty Vector

FACS	Fluorescence-associated Cell Sorting
FOXO	Forkhead box O
FUDR	5-Fluoro-2'-deoxyuridine
GABA	gamma-Aminobutyric Acid
GFP	Green Fluorescent Protein
GH	Growth Hormone
GSC	Germline Stem Cell
H3K27ac	Histone-3 Lysine-27 acetylation
H3K27me1/2/3	Histone-3 Lysine-27 mono-/di-/tri-methylation
H3K4me3	Histone-3 Lysine-4 tri-methylation
HAT	Histone Acetyltransferase
HD	Huntington's Disease
HGPS	Hutchinson-Guildford Progeroid Syndrome
HLH	Helix Loop Helix
HNF	Hepatocyte Nuclear Factor
IGF	Insulin-like Growth Factor
IIS	Insulin/Insulin-like Growth Factor-1 Signalling
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS	Insulin Receptor Substrate
JAK-STAT	Janus kinase-Signal Transducer and Activator
JMJD	JuMonJi (transcription factor) Domain
KH_2PO_4	Potassium Dihydrogen Phosphate
LG	Linkage Group
MEK1/MAP	Mitogen-activated Protein
MES	Maternal-effect Sterile
$MgSO_4$	Magnesium Sulfate
Na_2HPO_4	Disodium Hydrogen Phosphate
NAC	N-acetyl Cysteine

NAD	Nicotinamide Adenine Dinucleotide
NaOCl	Sodium Hypochlorite
NaOH	Sodium Hydroxide
ncRNA	non-coding RNA
NGM	Nematode Growth Media
NIA	National Institute of Ageing
NLS	Nuclear Localisation Signal
NO	Nitrogen Oxide
ORF	Open Reading Frame
OXPHOS	Oxidative Phosphorylation
PARP	Poly (ADP-ribose) Polymerase
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PIP3	Phosphatidylinositol (3,4,5)-tri-phosphate
PRC	Polycomb Repressive Complex
qRT-PCT	quantitative Reverse Transcription PCR
RFP	Red Fluorescence Protein
RIN	RNA Integrity Number
RISC	RNA-induced Silencing Complex
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
SA	Senescence-associated
SA-β-Gal	SA-β-galactosidase
SASP	Senescence-associated Secretory Phenotype
SATB-1	Special AT-rich Binding Protein-1
SIR	silent information regulator; sirtuin
SWI/SNF	SWItch/Sucrose non-fermentable
TBE	Tris/Borate/EDTA

TCA	Tricarboxylic Acid Cycle
TCF/LEF	T-cell factor/Lymphoid Enhancer Factor
TF	Transcription Factor
TFEB	Transcription Factor EB
TOR	Target of Rapamycin
UPR	Unfolded Protein Response
UTR	Untranslated Region
UTX	Ubiquitously transcribed tetratricopeptide repeats, X chromosome
UTY	Ubiquitously transcribed tetratricopeptide repeats, Y chromosome
UV	Ultraviolet
WHO	World Health Organisation
WS	Werner Syndrome

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CHAPTER 1: INTRODUCTION

1.1. Ageing in the modern society

Advances in modern medicine have allowed us to live longer than ever before, with life expectancy rising significantly in the last century. Because of people living longer, we are becoming an increasingly ageing population. Ageing is broadly defined as "progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age" (Kirkwood & Austad, 2000). Currently, the proportion of the population aged 65 years old and above is at 10% and is predicted to double by 2050 (WHO report on health and ageing, 2015). This dramatic rise in the ageing population poses a huge socioeconomic problem due to increasing disease burden, particularly age-related diseases. In fact, age is the biggest risk factor for age-related diseases such as dementia, cardiovascular disease, diabetes and even cancer (Niccoli & Partridge, 2012). Ageing can also have a detrimental effect on mental health due to stigmatisation, fear of death and bereavement and the idea of "losing one's self". While ageing is seen as an inevitable outcome of growing old, the mechanistic basis has never been fully understood. Studying the biology of ageing could help identify mechanisms that go awry with increasing age.

Ageing is a multifactorial process that leads to progressive physiological decline and it's not far-fetched to think that deteriorating processes in age-related diseases overlap and are not mutually exclusive. Therefore, understanding how we age could reveal actionable mechanisms that can be targeted (for instance, through pharmacological interventions), in order to tackle age-related diseases as whole rather than as discrete maladies.

Furthermore, ageing studies could lead to interventions that improve overall health, resulting in life less spent in morbidity.

1.2. Why do we age?

Before addressing how ageing occurs, there needs to be a clearer consensus on what exactly defines ageing. Understanding how ageing evolves might help in identifying what kind of process it is. Darwinian evolution states that "all species of organisms arise and develop through the natural selection of small, inherited variations that increase an individual's ability to compete, survive and reproduce." Given that ageing is detrimental to fitness and fecundity, the question that needs to be asked is - is ageing subjected to natural selection? Earlier theories explaining why ageing occurs suggests that ageing is an inevitable consequence of wear and tear and does not evolve. However, rates of ageing are highly varied across the phylogeny, suggesting that ageing is subject to natural selection (Kirkwood & Austad, 2000). In extreme cases, ageing does not seem to occur at all, such as in cnidarian *Hydra vulgaris*. In these animals, mortality remained close to zero in a four-year period with no detrimental effects to reproductive capacity, indicating that perhaps ageing is not inevitable (Martínez, 1998; Schaible et al., 2015). On the other hand, some would argue that ageing is a neutral trait because organisms die from extrinsic hazards before the onset of ageing, suggesting that ageing is inconsequential to reproductive success. However, life history traits (such as when an organism is born, reproduces, senesces and dies) evolve in response to its unique ecological circumstances. In this way, an organism's intrinsic mortality is intricately linked to extrinsic hazards (eg. predation). For example, brown rats and bats are similarly sized mammals, suggesting that their intrinsic mortalities would be similar. And yet, the average lifespan of bats is 15 times longer (30 years) compared to rats (2 years). One of the prevailing reasons for this is that bats are subject to less predation (compared to rats) due to their ability to fly,

thus evolving to have a long lifespan. Therein lies the ageing paradox: it is a process subject to natural selection and is not inevitable but is also deleterious to individuals. If that is the case, how can ageing evolve?

In the 1860s, Alfred Russell Wallace argued that ageing evolves to let individuals (who have reproduced) cease to consume resources to increase fitness of their successors (Weismann, 1889). This is further echoed by August Weismann who suggested that ageing removes worn out individuals from the population to make room for the young (Kirkwood et al., 2005; Weismann, 1889). The arguments are circular because both theories assume that ageing (ie. old individuals being worn out) already exists to explain why ageing occurs in the first place. Furthermore, Weismann's idea suggests that resources would be made available to unrelated individuals that would not necessarily inherit the "ageing gene", which constitutes some form of evolutionary cheating. This form of reasoning invokes a group selection argument, which has been widely discredited because it goes against the principles of Darwinian evolution. Lastly, the assumption that an "ageing gene" where the sole function is to cause death is counterintuitive to how evolution progresses. This leads to the general conclusion that ageing is selected against and must be evolving as a side-effect of something else. Indeed, a position statement written by Olshansky, Hayflick and Carnes (2002) states that "ageing is a product of evolutionary neglect, not evolutionary intent" (Olshansky, Hayflick, & Carnes, 2002).

A breakthrough in understanding the ageing paradox was given by Medawar (1952), where he proposed the "mutation-accumulation theory." This posits that ageing evolves as a side-effect of mutation pressure because of the decreasing ability of natural selection to counter its effects at later stages (Medawar, 1952). The mutation-accumulation theory best explains why Huntington's disease (HD), which manifests late in life, is not

eliminated by natural selection as noted by JBS Haldane in 1941. Because the onset of HD is relatively late in life (average onset is 35.5 years), the selective pressure to remove the disease is weak and not removed by natural selection. Additionally, George Williams (1957) proposed that mutations in genes that are beneficial to fitness in early life when natural selection is strong might occur at the cost of detrimental increased rate in ageing. The theory suggests a trade-off effect between fitness in youth and the rate of ageing, which could explain why ageing is adaptive to evolution. This is more commonly known as "antagonistic pleiotropy" or the "trade-off theory" (G. C. Williams, 1957). An example that best exemplifies antagonistic pleiotropy is the tumour suppressor gene p53. Mice carrying a heterozygous mutation associated with activated p53 showed increased resistance to tumour development, but displayed accelerated senescence phenotypes (Tyner et al., 2002).

Echoing the principles of antagonistic pleiotropy, Blagosklonny (2006) proposed the quasi-programmed hyperfunction theory. This states that the non-adaptive continuation of developmental processes during adulthood results in hyperfunction (an excess of a previously benign activity), which causes ageing phenotypes (Blagosklonny, 2006). This is exemplified in a study on *C. elegans* conducted by Ezcurra *et al.* (2018). Yolk protein production (called vitellogenesis) is a required biological program for reproduction and egg-laying in *C. elegans*. Despite cessation of egg-laying, activation (or 'run-on') of vitellogenesis persists (Ezcurra et al., 2018). This persistent activation was found to be maintained by conversion of intestinal mass into lipoproteins (a component of yolk proteins) through intestinal macroautophagy, leading to intestinal atrophy and yolk accumulation – two known senescent pathologies (Ezcurra et al., 2018). The study showed that the run-on of yolk production led to age-related phenotypes, supporting that hyperfunction of biological processes promotes ageing.

While antagonistic pleiotropy is perhaps the most widely accepted theory on the evolution of ageing, concrete molecular evidence of this remains sparse and is only starting to be elucidated. In 1977, Kirkwood argued that rather than looking for specific gene mutations, it might be more informative to look at trade-offs from a physiological perspective. He developed the disposable soma theory which states that an organism must allocate available energy resources between somatic maintenance and reproduction to ensure the greatest chance of success for the offspring (Kirkwood, 1977; Shanley & Kirkwood, 2000). The theory implies that natural selection favours strategies where energy allocated to somatic maintenance is less than adequate for indefinite survival to prioritise reproduction, and this sub-optimal allocation to somatic maintenance is what drives ageing. Based on that logic, it also indicates that over-allocation of resources to somatic maintenance may result in increased lifespan, but at the cost of compromised reproductive capacity of an organism. The disposable soma theory fails to reconcile that many organisms subjected to dietary restriction live longer than when fed *ad libitum* (Changhan Lee, 2016; Guarente & Kenyon, 2000). If ageing is caused by less-than-ideal energy allowance to somatic maintenance, then how is it that abundance of energy (ie. *ad libitum* feeding) leads to a faster rate of ageing compared to scarce conditions? This is addressed in a later iteration of the disposable soma theory (called the Shanley-Kirkwood model) where they propose that energy resource allocation is tailored to provide an optimal life history (Shanley & Kirkwood, 2000). Nevertheless, the theory still fails to explain how certain mutations can cause improved longevity without negatively impacting brood size. For instance, in nematode *Caenorhabditis elegans* (*C. elegans*), worms transgenically overexpressing *sir-2.1* (encoding a histone deacetylase) are long-lived compared with wild-type but have normal brood size (Tissenbaum & Guarente, 2001) (more on this in section 1.3.4.4). Furthermore, several mutants associated with defects in the sensory cilia such as *daf-6*, *che-2* and *osm-1* have extended

lifespan and comparable brood size to wild-type animals (Apfeld & Kenyon, 1999), therefore showing that there is no obvious trade-off between longevity and reproduction. Although no obvious trade-off between longevity and reproduction is seen in the examples given above, it is undeniable that there are long-lived mutants that are sterile or have reduced brood sizes (eg. *glp-1* and *daf-2* mutants) (Arantes-Oliveira, 2002; Kenyon, Chang, Gensch, Rudner, & Tabtiang, 1993). Collectively, this suggests that the trade-off between longevity and reproduction to promote lifespan extension is not a universal phenomenon. To date, it remains challenging to find an all-encompassing theory to explain why we age. Considering the life history traits of an organism in the context of its environment might be more informative in explaining how adaptive mechanisms evolve to affect the rate of ageing.

1.3. How do we age?

The diversity of lifespan across the tree of life suggests that apart from environmental stimuli, ageing is also somewhat genetically programmed. Furthermore, twin studies have shown that 25% of variance in adult human lifespan is accounted for by genetic differences (Steves, Spector, & Jackson, 2012). The discovery by Hayflick and Moorhead (1961) that human fibroblasts have a finite replicative capacity (known as the "Hayflick limit") first demonstrated that ageing is regulated at the cellular level (Hayflick & Moorhead, 1961), sparking future studies into the molecular mechanisms of ageing. Ageing research has received a lot of attention in the last 40 years, leading to a large number of mechanistic theories that drive ageing. It is important to note that there is not one molecular mechanism that fully explains how we age and that mechanisms are likely to overlap with each other. Some of these are highlighted below:

1.3.1. Cellular Senescence

Cellular senescence is a multifaceted process that arrests proliferation of mitotic cells that are susceptible to neoplastic transformation. Senescent cells are characterised by an enlarged morphology, increased senescence-associated ß-Galactosidase (SA-ß-Gal) activity, upregulated DNA damage response and a senescence-associated secretory phenotype (SASP) (Campisi & di Fagagna, 2007). Senescence is triggered by dysfunctional telomeres, DNA damage, overexpression of certain oncogenes such as components of the RAS-RAF-MEK pathway and other stressors (Campisi & di Fagagna, 2007). Senescence is often seen as a protective response when damage is irreparable and threatens to overwhelm the DNA repair machinery. This is mediated by two distinct DNA-damage response mechanisms: p53- and p16-mediated damage response. Damage- and telomere-initiated senescence relies on engaging the tumour suppressor p53. p53 upregulates expression of p21, which is the main transducer of p53 damage response to promote cell cycle arrest and senescence. Physiological mitogens cannot reverse senescence induced by p53. However, inhibition of p53 in senescent human fibroblasts has been shown to reinstate cell cycle progression, suggesting that p53-induced senescence is reversible (Gire & Wynford-Thomas, 1998). Furthermore, inhibition of p21 in senescent fibroblasts bypasses telomere-dependent senescence, corroborating that p53-induced senescence is reversible (Brown, Wei, & Sedivy, 1997). On the other hand, upregulation of the cell cycle inhibitor p16 is a more permanent way of inducing senescence. p16-induced senescence is triggered by oncogene overexpression, chromatin disruption and other stressors (Campisi, 2005). p16 positively regulates the anti-oncogenic Retinoblastoma (pRb) pathway, which inhibits cell cycle progression through heterochromatic repression of cell cycle regulators such as genes targeted by the E2F transcription factor. Once E2F target genes are repressed, senescence becomes irreversible even after p53 inactivation, p16 silencing or pRb inhibition (Campisi, 2005).

Senescent cells are predominantly found in aged tissues and are also present in age-related pathologies such as atherosclerotic lesions, skin ulcers, arthritic joints and precancerous lesions in the liver and prostate (Campisi, 2005). While senescence protects from neoplastic transformation, senescent cells themselves are also found to accelerate ageing by promoting cancer. Studies by Krtolica *et al.* (2001) showed that secreted factors of senescent human fibroblast cells stimulated hyperproliferation and tumourigenesis of preneoplastic epithelial cells and neoplastic epithelial cells respectively (Krtolica, Parrinello, Lockett, Desprez, & Campisi, 2001). Factors secreted by senescent fibroblasts reveal enrichment of pro-inflammatory cytokines, growth factors and proteinases, suggesting that senescent cells promote an environment conducive for neoplastic formation (Coppe, Desprez, Krtolica, & Campisi, 2010).

1.3.2. DNA damage

1.3.2.1. The DNA damage theory of ageing

The DNA damage theory of ageing states that accumulation of unrepaired DNA damage and mutations cause ageing. Mutations are changes in the nucleotide sequence that could lead to production of aberrant proteins, and damage broadly refers to physical and chemical alterations to the double helix structure (Kirkwood, 2005). In Werner syndrome (WS), mutations in the *WRN* gene, encoding a protein with RecQ helicase and endonuclease activity important for DNA repair, cause accelerated ageing (L. Chen & Oshima, 2002). Cells collected from patients with WS have been shown to have more chromosomal abnormalities, DNA double strand breaks and increased frequency of deleterious mutations. Other accelerated ageing syndromes such as Hutchinson-Guildford progeroid syndrome (HGPS), Cockayne syndrome and Ataxia-telangiectasia

syndrome are also caused by mutations in genes involved in maintaining DNA homeostasis (Freitas & de Magalhães, 2011).

1.3.2.2. Telomere Attrition

Telomeres are nucleoprotein structures that cap the ends of eukaryotic chromosomes and protect coding DNA from degradation. DNA polymerases are unable to fully replicate chromosome ends at the lagging strands ("end replication problem"), leading to telomere shortening after each cell division. Telomere attrition is associated with cellular senescence and is considered a hallmark of ageing (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). This has been demonstrated in human fibroblasts where telomere shortening was observed for every passage of human fibroblasts until it reaches senescence, suggesting that telomeres shorten with age (in this case, population doublings) (Harley, Futcher, & Greider, 1990). Telomerases counteract telomere shortening by synthesising telomeric repeats on 3' overhangs of chromosomes and are present in highly proliferative germ cells and some stem cell niches (Bekaert, De Meyer, & Van Oostveldt, 2005). Telomerases are not expressed in somatic cells and the absence is considered a protective measure, since upregulated telomerase activity is associated with abnormal proliferation in many cancers (Blasco, 2005). The link between ageing and telomere length has been demonstrated in mice, where longer telomere lengths correlated with longer lifespan (Armanios et al., 2009; López-Otín et al., 2013; Tomás-Loba et al., 2008). Oxidative stress also induces telomere shortening and cellular senescence, suggesting that telomere shortening might not just act as the 'mitotic clock' of the cell, but also as a marker for stress response (Zglinicki, 2002).

1.3.3. Mitochondria

1.3.3.1. Mitochondrial theory of ageing

Mitochondria are important cellular organelles responsible for energy production in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) in the respiratory chain. Besides energy metabolism, the mitochondria also has roles in signalling, differentiation, cell death and cell growth/cycle (Milena Pinto, 2015). The mitochondrial theory of ageing states that accumulation of mitochondrial DNA (mtDNA) mutations leads to impaired ATP production and subsequent decline of tissue bioenergenesis (Kirkwood, 2005). This is supported by studies showing that cytochrome c oxidase (COX)-deficient mitochondria are observed at a higher frequency in ageing cells found in human cardiomyocytes, gut (colonic crypt stem cells) and neurons (Cottrell et al., 2000; Müller-Höcker, 1989; Taylor et al., 2003). COX is part of the respiratory chain (complex IV) in the mitochondria and expression originates from mtDNA. It is also conventionally used as a biomarker of mtDNA defects (M. A. Johnson, Bindoff, & Turnbull, 1993).

Mitochondrial changes associated with ageing include a decreased capacity to produce ATP and altered morphology, and older tissues have been observed to have less mitochondria and decreased mitochondrial protein levels. Turnover of mtDNA is dependent on biogenesis, dynamics and selective autophagic removal of organelles (mitophagy), and failure at each stage could contribute to ageing. For instance, stimulation of AMPK, a key regulatory factor in mtDNA maintenance, is decreased in the skeletal muscle of old rodents upon pharmacological and physical stimulation and is associated with decreased mitochondrial biogenesis (Reznick et al., 2007). This suggests

that ageing is associated with a decreased capacity to maintain mitochondrial homeostasis.

Additionally, mitochondria undergo fusion/fission dynamics, most likely to avoid heteroplasmy (and therefore maintain mtDNA stability). It is also an adaptive response to stress. For instance, studies in mouse embryonic fibroblasts (MEFs) show that mitochondrial elongation (fusion) is necessary for maintenance of mitochondrial mass (by escaping mitophagy) and more efficient ATP production when subjected to starvation stress (Gomes, Di Benedetto, & Scorrano, 2011). Irreparable mitochondria are targeted for mitophagy (mitochondrial autophagy) and autophagy function characteristically decreases with age and is upregulated in known pro-longevity pathways such as insulin-signalling inhibition (Hars et al., 2007), calorie restriction and sirtuin activation (Gelino & Hansen, 2012). Therefore, it is foreseeable that reduced capacity for mitophagy could contribute to ageing phenotypes.

1.3.3.2. Oxidative Stress

The mitochondria are major sources of reactive oxygen species (ROS) due to production of superoxide anions and hydroxyl radicals in the respiratory chain. In 1970, Harman proposed the "free radical theory of ageing", saying that somatic mtDNA mutations impair OXPHOS resulting in an increase in ROS. ROS damages proteins, lipids and DNA (both nuclear and mitochondrial), therefore causing ageing (Harman, 1956). This theory has since been put into question after findings that patients with impaired OXPHOS did not show accelerated accumulation of mtDNA mutations (Tengan, Gabbai, Shanske, Zeviani, & Moraes, 1997). Furthermore, animals treated with antioxidants to quench ROS showed reduced oxidative damage but did not delay ageing, suggesting that ROS does not necessarily cause ageing (Comfort, Youhotsky-Gore, & Pathmanathan, 1971;

Hagen et al., 2002). ROS are not just detrimental byproducts of mitochondrial metabolism, but are also signalling molecules involved in cell proliferation, cell death and endogenous defenses. Given the role of ROS as cell messengers and stressors, it is possible that age-related mitochondrial deregulation might be caused by an imbalance in ROS levels. This is implied in the "gradual ROS response hypothesis" (hormesis) where ROS is proposed to induce the protective response pathway (Hekimi, Lapointe, & Wen, 2011). Increasing cellular damage with age might induce the stress response even more, leading to increasing levels of ROS and toxicity starts as soon as a certain threshold is reached. The protective effect of ROS is supported in a *C. elegans* study where worms bearing mutations in complex I (*nuo-6*) and complex III (*isp-1*) of the respiratory chain displayed elevated levels of ROS superoxide and elongated lifespan. Antioxidant treatment using N-acetylcysteine (NAC) and vitamin C abolished lifespan extension, suggesting that elevated ROS superoxide was important for longevity (Wen Yang, 2010).

1.3.4. Metabolism

Metabolic diseases such as type-2 diabetes and obesity (commonly referred to as "diabesity") are highly associated with old age. As of 2015, estimates suggest that 415 million people in the world are afflicted with diabetes, a number that is far higher than the 366 million projection (by 2030) made in 2000 (Zimmet, 2017). The dramatic rise of diabetes (and indeed obesity) incidences was so much so that it is now one of the largest epidemics in human history. Furthermore, the continuous rise of life expectancy in the past 50 years is projected to halt due to diabesity (Olshansky et al., 2009). Ageing and metabolic disease is highly interconnected, with diabesity being known to accelerate ageing by inflicting widespread cellular and organ damage. This necessitates the study of mechanisms of metabolic disease to understand how ageing occurs.

1.3.4.1. Metabolic syndrome of ageing

Insulin and leptin are two key hormones that regulate metabolism. After food intake, insulin is released from pancreatic ß-cells to promote glucose uptake and inhibit hepatic glucose output and lipolysis in adipose tissue. Thereafter, insulin signal transduction is mediated by the PI3K/Akt/mTOR pathway. Leptin is produced mainly in the adipose tissue and targets hypothalamic neurons to regulate food intake and appetite.

These pathways become deregulated with age leading to symptoms associated with metabolic syndrome such as insulin resistance. Metabolic syndromes also accelerate ageing by inflicting cellular and organ damage, leading to a vicious cycle of detrimental effects. For example, hyperglycaemia (a symptom of metabolic disorder) enhances formation of advanced glycation end-products (AGE), which causes tissue damage and organ dysfunction (Helen Vlassara, 2014). Obesity is one of the major causative factors of diabetes and it has been previously shown that increased oxidative stress in accumulated fat contributes to the pathogenicity of metabolic syndromes (Furukawa et al., 2017). Studies on genetically obese mice (Ay mouse strain) showed higher levels ROS compared to wild-type mice fed on the same diet, suggesting that obesity is associated with higher oxidative stress. Furthermore, adipose tissue of Ay mice showed an enhanced senescence phenotype due to increased staining of SA-ß-Gal compared to wild-type, and upregulated levels of p53. Given that upregulation of p53 in mice has also been previously associated with accelerated senescence (Tyner et al., 2002), this suggests that obesity promotes ageing possibly through upregulation of p53. Additionally, adipose-specific knockdown of p53 restored insulin sensitivity in Ay mice, suggesting that p53 is also a mediator of insulin resistance. Pro-inflammatory cytokines such as tumour necrosis factor-α (Tnf-α) were upregulated in Ay mice as well (Minamino et al., 2009). Pro-inflammatory cytokines reportedly exacerbate insulin resistance in people

with metabolic disorders (Hotamisligil, Shargill, & Spiegelman, 1993). Collectively, this suggests a possible mechanistic link between obesity, diabetes and ageing (Ahima, 2009).

1.3.4.2. Dietary Restriction

Dietary restriction (DR) refers to the reduction of diet components (such as macronutrients) without incurring malnutrition (cf. Caloric Restriction (CR) where diet is such that total energy consumed is reduced compared to *ad libitum* feeding). DR in multiple model organisms ranging from rhesus monkeys to the simple worm significantly extend lifespan. Two studies on the impact of DR in rhesus monkeys on survival, conducted at the National Institute of Ageing (NIA) and University of Wisconsin (UW) gave confounding results. The NIA found that DR did not impact survival of rhesus monkeys (Mattison et al., 2012), while UW observed a positive effect (Colman et al., 2009). The results were mainly attributed to a difference in experimental design, particularly on the food source used. NIA employed a naturally sourced diet to ensure that micronutrients and minerals were provided (but could be subjected to seasonal change), while UW used a semipurified diet to ensure consistency throughout the experiment. Both food sources had a 60% carbohydrate content but the sucrose component in the NIA diet was significantly lower (<7%) than that of UW (45%). Nevertheless, latest updates on both experiments report significant healthspan benefits such as lowered incidences of cardiovascular disease and cancer in monkeys subjected to DR (Mattison et al., 2017).

In mice, caloric restriction improves metabolic profiles such as increased insulin sensitivity, reduced adiposity and more responsive metabolic shifts between carbohydrate to lipid oxidation in between feeding and resting phase respectively (Riera

& Dillin, 2015). Caloric restriction causes a reduced production of growth hormones and subsequently IGF-1, leading to a general reduction in activity of components in the insulin/IGF-1 signalling (IIS) cascade. Ames dwarf mice are unable to produce growth hormones and have very low levels of circulating IGF-1. They are also characteristically smaller than wild-type littermates and exhibit metabolic alterations such as mild hypoglycemia and increased subcutaneous adiposity. Interestingly, Ames mice are significantly longer lived than wild-type, suggesting that reduction IIS promotes longevity (more on this later) (Brown-Borg, Borg, Meliska, & Bartke, 1996). However, Ames dwarf mice subjected to CR are even longer lived than *ad libitum* fed counterparts, implying that there may be mechanisms distinct from IIS downregulation that regulates CR-mediated longevity (Mattison et al., 2000). Paradoxically, reduced levels of GH and IGF-1 is associated with ageing, indicative of GH and IGF-1 having pleiotropic effects on development and ageing. Like observations in Ames mice, knockout of the insulin receptor substrate-1 (IRS-1) homologue in flies (*chico)* reduces insulin signalling activity and is associated with increased lifespan compared to wild-type flies. *chico* flies are also phenotypically smaller than wild-type animals, corroborating the role of insulin-signalling in growth and development (Clancy et al., 2001). Additional lifespan extension was also observed under certain protocols of DR in *chico* flies, suggesting that DR-mediated longevity is only partially due to downregulation of insulin signalling (Clancy, 2002).

1.3.4.3. Nutrient sensing

The mammalian target of Rapamycin (mTOR) is a serine/threonine kinase that functions as an intracellular energy sensor and is activated by growth hormone stimulation or amino acids to promote growth and metabolism (Finkel, 2015). Genetic and pharmacological downregulation of mTOR reportedly promotes longevity in mice (S. C. Johnson et al.,

2013; Wu et al., 2013), flies (Kapahi et al., 2004) and worms (Robida-Stubbs et al., 2012). Inhibition of mTOR in yeast (Kaeberlein et al., 2005), worms (M. Hansen et al., 2007) and flies (Kapahi et al., 2004) does not have an additive effect on animals subjected to DR, suggesting that mTOR might (at least partially) regulate DR-mediated lifespan extension. However, exact mechanisms for this are difficult to isolate due to pleiotropic functions of mTOR. For instance, mTOR is involved in mediating mitochondria function, protein synthesis, insulin signalling and autophagy. Mechanistic elucidation of how mTOR regulates lifespan and DR-mediated lifespan is further complicated by context-specific effects of mTOR. For example, mTOR positively regulates mitochondrial respiration in mammalian cells (Ramanathan & Schreiber, 2009; Schieke et al., 2006), but negatively regulates oxygen consumption in unicellular yeast (Bonawitz, Chatenay-Lapointe, Pan, & Shadel, 2007). Furthermore, tissue-specific studies in mice show that inhibition of mTOR activity in skeletal muscle leads to muscle dystrophy (Bentzinger et al., 2008), while inhibition in adipose tissue leads to enhanced mitochondrial respiration and increased resistance to diet-induced obesity (Polak et al., 2008). Non-cell autonomous functions of mTOR has also been reported where enhanced self-renewal and preservation of intestinal stem cells (ISCs) due to CR was mediated by downregulation of mTOR in neighbouring niche cells (Yilmaz et al., 2012). The study suggests a novel role of mTOR downregulation and CR in promoting stem cell function, which is known to deteriorate with ageing.

1.3.4.4. Sirtuins in CR

The role of sirtuins in nutrient sensing and longevity has also received intensive scrutiny. The mammalian sirtuin family of proteins is comprised of 7 sirtuins (SIRT1-7), and possess nicotinamide adenine dinucleotide (NAD)-dependent deacetylase activity targeting histone and non-histone substrates (Finkel, 2015). Pro-longevity effects of

sirtuins have been reported in mice, fruit flies, worms and yeast in the context of dietary restriction (reviewed in (Guarente, 2013)).

In mice, knockout of SIRT1 abolished CR-mediated lifespan extension. Similarly, in yeast, NAD and Sir2 (mammalian SIRT1 homologue) have been found to be necessary in promoting lifespan extension when subjected to CR (S. J. Lin, Defossez, & Guarente, 2000; Tissenbaum & Guarente, 2001). Conflicting findings in yeast, however, question the significance of Sir2 in mediating lifespan extension due to CR (Kaeberlein, Kirkland, Fields, & Kennedy, 2004). Furthermore, controversy ensued in lifespan studies on worms transgenically overexpressing *sir-2.1* because the dramatic lifespan extension initially observed by Guarente *et al*. was potentiated by linked mutations (Viswanathan & Guarente, 2011). Nevertheless, further studies on the effect of *sir-2.1* overexpression on lifespan showed lifespan extension in worms, but not to the same extent as initially reported (Viswanathan & Guarente, 2011).

SIRTs in mice reportedly deacetylate malonyl and succinyl moieties of components involved in TCA and fatty acid metabolism (Hirschey & Zhao, 2015). The interaction between NAD, sirtuins, TCA and fatty acid metabolism components strengthen the idea that sirtuins integrate metabolic and longevity signals. SIRT3-5 localise specifically in the mitochondria and promote mitochondrial homeostasis. Proteomic studies show that 65% of mouse mitochondrial proteins are acetylated and acetylome reprogramming is highly sensitive to nutrient availability (CR) and SIRT3, suggesting that enhanced mitochondrial homeostasis is mediated by resetting the acetylome landscape of mitochondrial proteins (Hebert et al., 2013).

1.3.4.5. Autophagy

The adenosine monophosphate (AMP) kinase (AMPK) is an energy sensor that is activated in conditions where energy resources are limited (high AMP:ATP ratio) and functions to increase energy supplies while reducing energy demand (Finkel, 2015). AMPK is necessary for lifespan extension associated with certain protocols of DR, implying that AMPK partially promotes DR-mediated lifespan extension.

AMPK promotes autophagy by positively regulating the UNC-51-like kinase (ULK51), a key regulator of autophagosome formation (Ulgherait, Rana, Rera, Graniel, & Walker, 2014). Autophagy is a mechanism for maintaining cellular homeostasis by removing cellular waste such as damaged organelles and aberrantly modified proteins (He, Lu, & Yue, 2013). Loss of proteostasis is one of the hallmarks of ageing and directly implicates autophagy as an important modulator of longevity (López-Otín et al., 2013). This is supported by findings that transgenic overexpression of autophagy-related 5 gene (Atg5) in mice significantly increases lifespan compared to non-transgenic controls (Pyo et al., 2013). Furthermore, overexpression of transcription factor EB (TFEB)-homologue *hlh-30* in *C. elegans* promotes expression of autophagic genes and is associated with lifespan extension (Lapierre et al., 2013), suggesting that increased autophagic activity is associated with pro-longevity benefits.

1.3.5. Somatic stem cell decline

Stem cells are important in maintaining tissue homeostasis due to their ability to self-renew and differentiate. On the demands of regeneration (for example, after injury) or growth, stem cells enter the cell cycle and undergo symmetric and asymmetric divisions to maintain the stem cell pool and produce committed progenitors, respectively, to replenish the differentiated tissue (Jung & Brack, 2014). The balance between stem cells

and progenitors is tightly regulated to avoid abnormal growth and inappropriate differentiation. This tight regulation is maintained by a special microenvironment called the stem cell niche, which relies on paracrine and juxtacrine signalling to maintain stem cell homeostasis (Jung & Brack, 2014). Regenerative capacity and tissue repair mechanisms of many organisms decline with age and is a major contributing factor to ageing. In fruit flies for instance, the number of germ line stem cells (GSCs) in the testis declines with age. The decline in GSC was found to be due to decreasing expression of the ligand Unpaired (Upd) by neighbouring apical hub cells within the stem cell niche. Upd activates the Janus kinase-signal transducer and activator (JAK-STAT) pathway in nearby GSCs to promote self-renewal. Overexpression of Upd specifically in hub cells delays the age-related decline of GSCs, suggesting that changes in the microenvironment impact stem cell maintenance within the niche (Boyle, Wong, Rocha, & Jones, 2007).

1.3.6. Microbiome in ageing

In recent years, studies investigating the impact of the gut microbiota on ageing have begun to emerge. Previous studies have shown that diversity of the gut microbiota decreases with age and is associated with increased frailty (O'Toole & Jeffery, 2015). Human Intestinal Tract chip (HITChip) studies showed that fecal microbiota differs significantly among centenarians, elderly and young adults. Centenarian microbiota is associated with a pro-inflammatory status, indicating that the microbiome undergoes age-related changes that might contribute to ageing (Biagi et al., 2010). To date, challenges remain in determining whether microbiome changes are due to age or other variables such as genetics and lifestyle. Furthermore, studies remain correlative and there is still uncertainty as to whether changes in the microbiota is caused by ageing or if ageing is driven by changes in the microbiota.

In *C. elegans*, there is strong evidence that diffusible molecules generated by the *E. coli* (OP50) food source affects lifespan. Gusarov *et al* (2013) showed that the signalling molecule nitric oxide (NO) is produced by OP50 bacteria and is beneficial to lifespan. Feeding with NO-deficient *Bacillus subtilis* shortened worm lifespan but is rescued by NO supplementation, suggesting that NO produced by bacteria consumed by worms can impact longevity (Gusarov et al., 2013). In another study, Virk *et al.* (2012) showed that excessive folate synthesis by *E. coli* is detrimental to worm lifespan (Virk et al., 2012). In younger worms, *E. coli* is mechanically broken down before entering the gut and therefore they do not live in the worm as a true microbiome, although bacteria invasion of the gut increases with age. Nevertheless, these studies highlight the fact that direct interspecies signaling can affect ageing and lifespan and strengthens the cause for studying the impact of the microbiota in ageing.

1.4. Insulin/IGF-1 signalling – an evolutionarily conserved anti-longevity pathway

Perhaps the most important evidence that ageing and lifespan are mediated by some genetic component is the discovery that inhibition of the insulin/IGF-1 signalling pathway promotes longevity in multiple organisms (Figure 1.1) (Guarente & Kenyon, 2000). The insulin signalling cascade is activated by binding of insulin (or IGF-1 and insulin-like peptides) to the insulin/IGF-1 receptor tyrosine kinase. Activation of the receptor stimulates a series of autophosphorylation events on tyrosine residues, which serve as a binding site for the insulin receptor substrate (IRS). Steric changes due to phosphoinositide 3-kinase (PI3K) binding to IRS reveals the catalytic site of PI3K, which then converts phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol

(3,4,5)-triphosphate (PIP3). AKT binds to PIP3 and a series of phosphorylation events downstream ultimately lead to phosphorylation of the FoxO transcription factor, which regulates transcription of longevity and stress response genes (Murphy et al., 2003; Taniguchi, Emanuelli, & Kahn, 2006). Phosphorylated FoxO cannot translocate into the nucleus to promote transcription of longevity and stress response genes, indicating that the insulin signalling cascade is an anti-longevity pathway.

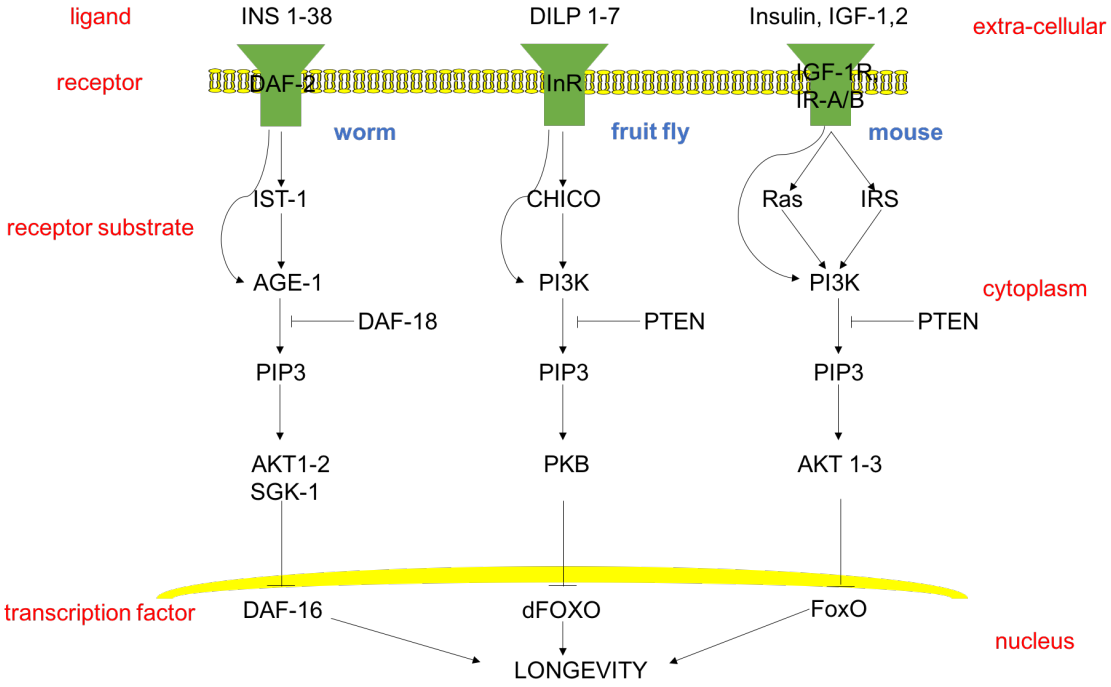


Figure 1.1: The insulin/igf-1 signalling components from worms to mammals. *Signalling cascade was modified from (Edoardo Parrella, 2010)*

That inhibition of insulin signalling extends lifespan was first observed in *C. elegans*. Kenyon *et al* (1993) showed that loss-of-function mutations in *daf-2*, a gene encoding for the insulin receptor homologue in worms (Kimura, Tissenbaum, Liu, & Ruvkun, 1997), resulted in lifespan extension that was twice as long as that of wild-type worms (Kenyon et al., 1993). Consistent with lifespan extension observed in *daf-2* mutants, inhibition of *age-1* (PI3K homologue) also increases lifespan in worms (Friedman & Johnson, 1988).

Lifespan extension is abolished when *daf-16* is mutated, indicating that pro-longevity effects mediated by insulin signalling inhibition is completely dependent on DAF-16 (K. Lin, Dorman, Rodan, & Kenyon, 1997; Murphy et al., 2003). Inhibition of insulin signalling by mutating *chico*, a gene encoding for an insulin receptor substrate homologue, extends lifespan significantly in fruit flies, substantiating that insulin signalling downregulation is beneficial to lifespan in flies, similar to worms (Clancy et al., 2001). Furthermore, heterozygous knockout of IGF-1R in mice causes lifespan extension and is also associated with increased resistance to oxidative stress (Holzenberger et al., 2003). Lastly, sequence analysis of the IGF-1R in Ashkenazi Jews, a community with a high proportion of centenarians, showed an overrepresentation of heterozygous mutations in the IGF-1R and is associated with reduced IGF-1R activity, suggesting that reduced insulin/IGF-1 activity could prolong human lifespan (Suh et al., 2008). Collectively, there is strong evidence to show that insulin signalling is an evolutionarily conserved pathway, and inhibition of the signalling cascade promotes longevity. Other biochemical pathways have also emerged as key mediators of ageing such as DNA repair, nutrient sensing, oxidative stress and many others (see section 1.3). Therefore, it is striking that a single mutation such as *daf-2* (and respective homologues) can regulate ageing, which is normally thought of as a highly complex and multifactorial process.

1.5. Epigenetics and ageing

1.5.1. Ageing is associated with changes in the epigenetic landscape

Twin studies have shown variation in longevity between genetically identical individuals, suggesting that genetic factors can only partially explain how ageing is regulated (Steves, Spector, & Jackson, 2012). An increasing amount of evidence shows that ageing is

accompanied by changes in epigenetic information of dividing and non-dividing cells. Epigenetics is broadly defined as "heritable mechanisms that occur without alterations in the underlying DNA sequence", with the epigenome being responsible for the functional use and stability of genetic material (Pal & Tyler, 2016). Epigenetic changes are sensitive to environmental stimuli and could potentially explain the interaction between genetics and external factors in regulating ageing. The reversible nature of epigenetic dynamics suggests that ageing is incredibly plastic, making epigenetic modifiers an attractive target for novel therapeutic interventions to promote healthy ageing.

In eukaryotes, DNA packaging in the nucleus is defined by the chromatin. The basic unit of chromatin is the nucleosome which is comprised of 147bp of DNA wrapped around a histone octamer comprised of a pair of histone-2A (H2A), histone-2B (H2B), histone-3 (H3) and histone-4 (H4) histones (Figure 1.2). Other proteins such as heterochromatin protein-1 (HP1) also exist to facilitate higher order heterochromatin structures.

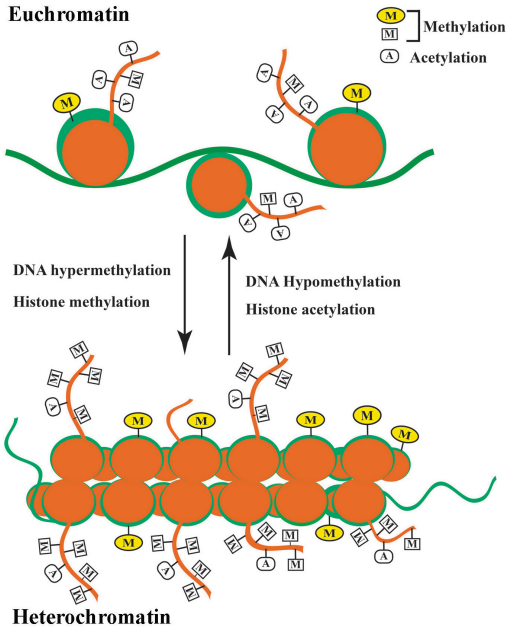


Figure 1.2: The eukaryotic chromatin. *Epigenetic modifiers mediate transcription by changing chromatin organisation. Image reproduced from (Gangisetty, Cabrera, & Murugan, 2018)*

Tight packaging of the chromatin (heterochromatin) sterically inhibits gene expression, while openly packaged chromatin (euchromatin) promotes transcription by allowing the transcriptional machinery to access the gene of interest. Chromatin dynamics are mediated by epigenetic modifications, which broadly fall into three categories: DNA methylation, non-coding RNA (ncRNA), and histone modifications. Chromatin organisation is tightly controlled in development to facilitate proper differentiation and growth of cells; however, this becomes increasingly deregulated with age. Ageing is associated with changes in the chromatin such as reduced bulk levels of core histones, altered post translational modifications and ncRNA expression, changes in DNA methylation and replacement of canonical histone variants. Such changes lead to altered local accessibility of genetic material, consequently leading to aberrant gene expression and genomic instability (for example, via increased activation of transposable elements), all of which are associated with accelerated ageing. All of this strengthens the importance of understanding how epigenetic changes affect the ageing process. Highlighted below are some key epigenetic trends and mechanisms that are associated with increasing age.

1.5.1.1. Histone loss

General loss of histones is consistently observed in ageing cells of multiple organisms (Sen, Shah, Nativio, & Berger, 2016). In replicatively aged yeast, micrococcal nuclease (MNase) digestion followed by sequencing experiments (MNase-seq) showed ~50% global reduction of nucleosomal occupancy leading to transcriptional upregulation and genomic instability. Loss of nucleosome occupancy also led to an increased range of positions that the remaining histones can adopt, thereby making the nucleosome positioning "fuzzier" with age (Hu et al., 2014). Another study showed that yeast carrying a deletion in the *ASF1* gene (histone chaperone) have significantly less histone proteins than wild-type and are short-lived. Overexpression of H2A/H2B histones in short-lived

ASF1 mutants significantly increased lifespan, suggesting that histone loss is detrimental to longevity and maintenance of histone levels through supplementation can reverse lifespan shortening effects (Feser et al., 2010). Collectively, these two studies imply that histone loss contributes to ageing.

1.5.1.2. Imbalance of activating and repressing histone modifications

Histone modifications, particularly the transcriptionally repressive histone-3 lysine-27 trimethylation (H3K27me3) and activating histone-3 lysine-4 trimethylation (H3K4me3), have been previously implicated in lifespan regulation. In *C. elegans*, knockdown of components that make up the H3K4 methyltransferase complex such as *ash-2*, *wdr-5* and *set-2* increase lifespan, while knockdown of requisite H3K4me3 demethylase *rbr-2* shortens lifespan (Greer et al., 2010). Furthermore, overexpression of *rbr-2* extends worm lifespan, suggesting that the transcriptionally activating H3K4me3 mark is limiting to worm longevity. The longevity phenotypes are likely transduced from the germline because germline loss abolishes the lifespan extending effect when *ash-2*, *wdr-5* and *set-2* are knocked down. Interestingly, lifespan extension is observed in wild-type descendants of parents deficient in *ash-2*, *wdr-5* and *set-2* for up to three generations, suggesting that epigenetic memory can be stably inherited to give a lifespan extending phenotype (Greer et al., 2011).

The levels of transcriptionally repressive H3K27me3 has been previously reported to decrease with age in *C. elegans*. Knockdown of the H3K27me3 demethylase UTX-1 increases global H3K27me3 levels as confirmed by western blot and is associated with increased lifespan compared to wild-type worms, suggesting that H3K27me3 is beneficial to longevity (C. Jin et al., 2011a; Maures, Greer, Hauswirth, & Brunet, 2011). The lifespan extension was attributed to UTX-1 having demethylase activity against

H3K27me3 marks on the *daf-2* loci, thus knocking down *utx-1* retains repressive H3K27me3 marks on *daf-2*, and effectively inhibits the insulin-signalling pathway (Maures et al., 2011). Both studies suggest that retaining H3K27me3 marks is beneficial to longevity, however confounding results from worms and other organisms challenge this hypothesis. For instance, knockdown of the H3K27 methyltransferase *mes-2* also extends worm lifespan (Ni, Ebata, Alipanahiramandi, & Lee, 2012). Knockdown of *mes-2* was also picked up independently in our lab's RNAi screen to promote lifespan extension in worms (see section 1.7). Furthermore, while H3K27me3 decreases with age in worms, H3K27me3 increases in ageing female fruit flies and killi fish (Sen et al., 2016). Additionally, H3K27me3 is elevated in SAMP8 (senescence-associated mouse prone 8) progeroid mouse models, suggesting that H3K27me3 is associated with accelerated ageing (Pal & Tyler, 2016). As such, the role of H3K27me3 in ageing is not clear-cut and further investigations are required to understand how the repressive mark impacts ageing. The finding that knockdown of H3K27me3 demethylase UTX-1 promotes lifespan through inhibition of *daf-2* transcription suggests that the impact of H3K27me3 as an anti-ageing mark is dependent on specific gene targets rather than global upregulation.

The role of activating H3 acetylation marks in ageing is perhaps the most well-understood of all epigenetic modifications. Polyamine spermidine inhibits histone acetyltransferase (HAT) activity and generates hypoacetylated chromatin states. Spermidine decreases with age and supplementation leads to increased lifespan in yeast, worms, flies and human cells, suggesting that removal of histone acetylation marks is beneficial to longevity, particularly by inducing autophagy (Eisenberg et al., 2009). Other histone acetylation marks have also been implicated in ageing. The sirtuin family of proteins, which function as NAD-dependent histone deacetylases (HDACs), are key players in

lifespan regulation. “Sirtuin” was coined from founding member *Sir2* in budding yeast where SIR stands for Silent Information Regulator and is involved in silencing heterochromatin-like regions. Sir2 removes acetyl groups from H4K16, H3K56 and H3K4 (Guarente, 1999). Deletion of *SIR2* shortens lifespan, while overexpression lengthens replicative lifespan in yeast (Kaeberlein, McVey, & Guarente, 1999). In *C. elegans*, worm homologue *sir-2.1* deacetylates H3K19 and is also involved in transcriptional silencing and heterochromatin formation. Like in budding yeast, overexpression of *sir-2.1* promotes longevity (Tissenbaum & Guarente, 2001). The result is controversial in that attempts by other groups to reproduce this data failed and lifespan extension was attributed to secondary mutations rather than *sir-2.1* itself (Viswanathan & Guarente, 2011). Studies by Mouchiroud *et al.* (2013) showed that NAD levels decrease with age due to increased activity of PARP – an NAD consuming enzyme, and that NAD supplementation increases worm lifespan in a *sir-2.1*-dependent manner (Mouchiroud et al., 2013). In mice, convincing evidence showed that overexpression of SIRT6 extended the lifespan of male mice. SIRT6 overexpressing mice also displayed lower levels of IGF-1, suggesting that lifespan extension was due to downregulation of the insulin/igf-1 signalling pathway (Kanfi et al., 2012).

The finding that loss of the activating mark H3K4me3 (and acetylation marks) and gain of the repressive mark H3K27me3 promotes longevity supports the model where heterochromatin loss promotes ageing. However, confounding results (for eg. H3K27me3 as discussed previously) might suggest that the heterochromatin loss model might not be universally applicable. It is quite possible that histone modifications could regulate lifespan by affecting specific genome pockets. Furthermore, there is no consensus as to how epigenetic modifications change with age in different species, therefore trends seen in one organism might not be applicable to another. Further

investigations are required to untangle the complexity of epigenetic modifications in the genome and how they impact ageing.

1.5.1.3. DNA methylation

DNA methylation in eukaryotes mostly occurs in the 5-carbon of cytosine residues in Cytosine-phosphate-Guanine (CpG) sites. Ageing of mammalian cells is associated with global DNA hypomethylation and local DNA hypermethylation (Sen et al., 2016). Loss of methylation in ageing, particularly at CpG islands of repetitive sequences in the genome, promotes the activity of retrotransposons and therefore leads to genomic instability (De Cecco et al., 2013). DNA hypomethylation is attributed to declining levels of DNA methyltransferase DNMT1 with age. DNA hypermethylation at specific promoters (eg. IGF-II, HIC1, CASP8) has also been previously observed, altering gene expression profiles that could promote development of age-related diseases such as cancer (Marc Jung, 2015).

1.5.2. Non-coding RNA (ncRNA)

Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but not translated into proteins. Micro RNAs (miRNA) are perhaps the most well-characterised type of ncRNA. miRNAs are ~25bp long RNA molecules and are involved in negative regulation of gene expression by targeting mRNA for degradation or inhibiting translation (Gonzalo, 2010). The involvement of miRNA in lifespan regulation was first observed in *C. elegans*. The miRNA *lin-4* and its target *lin-14* (encodes a putative transcription factor) are temporally regulated in the larval stages of the worm to promote development. Boehm and Slack (2005) showed that a loss-of-function mutation in *lin-4* leads to a shortened worm lifespan, and the lifespan phenotype completely reversed in *lin-4; lin-14* double mutants. Furthermore, *lin-14* overexpressing worms

phenocopied the short lifespan of *lin-4(lf)* mutants and overexpression of *lin-4* was beneficial to worm longevity. This suggests that *lin-4* promotes lifespan extension by repressing *lin-14* (Boehm & Slack, 2005). Lifespan extension due to *lin-14* inhibition was dependent on insulin signalling as evidenced by the lifespan extension of *lin-14(lf)* being completely abolished when subjected to *daf-16* RNAi (Boehm & Slack, 2005). Other miRNAs have also been implicated in ageing of other organisms. The miRNA miR-34 has been implicated in modulating ageing and neurodegeneration in fruit flies (Nan Liu et al., 2012), and an array of miRNAs were reportedly involved in inducing senescence of human fibroblasts (Faraonio et al., 2011). Advances in sequencing techniques indicate that 60-90% of the human genome is transcribed, leading to identification of many novel non-coding protein transcripts (Consortium, 2007). This poses an exciting avenue in investigating how ncRNAs are involved in lifespan regulation.

In summary, ageing is incredibly complex and it is not surprising that multiple molecular mechanisms have been implicated in regulating how we age. Such mechanisms have been identified to be involved in cellular senescence, changes in microenvironments such as the stem cell niche and the microbiome, DNA damage (eg. mutation accumulation and telomere attrition) and metabolism (eg. nutrient-sensing, dietary restriction, mitochondrial ageing). Changes in the chromatin also lead to transcriptional dysregulation that facilitates the development of age-related pathologies. One of the major challenges of ageing research is to dissect the interconnectedness of all these mechanisms and understand how these interactions ultimately lead to ageing. A better understanding of what drives ageing will facilitate the development of interventions that could improve healthspan and promote a life less spent in morbidity.

1.6. *C. elegans* as a model organism for ageing

Championed by Sydney Brenner as a model organism for studying development and neurobiology in the 1960s, the nematode *C. elegans* has come a long way from being a harmless neighbour to a pioneering hero in progressing scientific knowledge. *C. elegans* has been extensively used in understanding gene regulation, cell signalling, development and has even been used as models for human disease. For instance, the presenilin gene was first discovered in *C. elegans*, which was later found to have a human homologue prenesilin-1 in humans. Mutations in the presenilin gene was found to be associated with early onset familial Alzheimer's disease (AD). Similarly, worms carrying a mutation in presenilin gene *sel-12* have neuronal deficiencies, which can be rescued by ectopic overexpression of the human presenilin gene. Presenilin has since been found to be an important target of AD and has broadened our understanding of the disease progression, largely due to initial findings in *C. elegans*.

Strong conservation of molecular and cellular pathways between worms and mammals make *C. elegans* an attractive "simple" model organism for studying biological processes and certain diseases. *C. elegans* is easy to culture and maintain in large volumes in the laboratory. Once the worms hatch, they undergo development in 4 larval stages (L1 to L4) before developing into an adult (about 1mm in size), which takes about 3 days and survive for about 3 weeks under normal laboratory conditions (Figure 1.3). They can also undergo an alternative dauer pathway under stressful environmental conditions, whereby development is paused until resources become available.

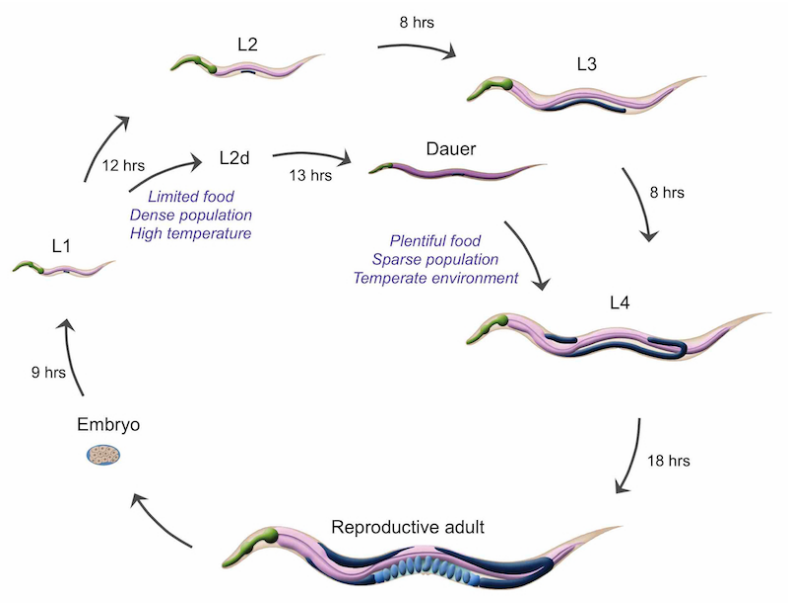


Figure 1.3: The C. elegans life cycle. *The worm goes through 4 larval stages of development before becoming a reproducing adult. The worms can undergo a developmental pause (via the dauer pathway) in stressful conditions and resume normal development once resources become available. (Image taken from www.wormatlas.org)*

Worms normally exist as self-reproducing hermaphrodites that can generate 300 isogenic progenies. However, males can arise through nondisjunction of X chromosomes, making genetic manipulation feasible. Taken together, these characteristics make the worm an ideal model organism to study ageing. The short generation time makes it practical to obtain isogenic worms in large volumes and perform lifespan analyses. The relatively short lifespan also means that more lifespan data can be generated in a limited time frame. Another advantage of using worms for ageing research is that they exhibit phenotypic and behavioural changes that are parallel to human ageing. For instance, worms move and eat less with age as evidenced by declining motility (Bolanowski, Russell, & Jacobson, 1981) and pharyngeal pumping rates (C. Huang, Xiong, & Kornfeld, 2004) respectively. The transparency of worms makes it easier to observe age-related changes in tissue integrity. Using electron micrographs and muscle GFP reporter strains, worms have been shown to undergo muscle deterioration with age, in the same way that humans do. Increased lipid accumulation in intestinal cells and compromised integrity of nuclei

in hypodermal cells were also observed in ageing worms (Herndon et al., 2002). More recently, ageing worms have also been associated with senescent pathologies such as intestinal atrophy coupled with yolk accumulation (steatosis) (Ezcurra et al., 2018), and development of uterine tumours (H. Wang et al., 2018), suggesting that tissue integrity declines with age.

A vast number of resources are available for *C. elegans* studies. For instance, the genome is fully sequenced and well-annotated (see www.wormbase.org) and a comprehensive mapping of neuronal connectivity is also accessible (www.openworm.org); a huge collection of worm mutants is available for researchers to use at the *Caenorhabditis* Genetics Center (CGC, University of Wisconsin); and gene expression is easy to downregulate through RNA interference (RNAi) – for which a library of RNAi clones is readily available (Kamath et al., 2003). There are established techniques for overexpression studies through microinjection of DNA plasmids and PCR products. A method for single-copy insertions in worms have also been developed using Mos1-mediated Single Copy Insertion (MosSCI) (Frøkjær-Jensen et al., 2008); and clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 techniques have also been adapted in worms (Friedland et al., 2013). These resources are valuable in establishing connections between gene function and phenotypic observations in ageing research as well as in the study of many other biological processes.

In 1983, Klass performed a genetic screen for mutations that gave lifespan extension in *C. elegans* and found 8 mutant candidates (Klass, 1983). Further studies conducted in Tom Johnson's laboratory eventually identified that 5 of these mutations mapped to *age-1* – the worm homologue of mammalian PI3K and a downstream component of the insulin signalling cascade (Friedman & Johnson, 1988). Indeed, the involvement of

insulin signalling in ageing was first discovered in *C. elegans*. Cynthia Kenyon showed that mutations in the insulin receptor homologue *daf-2* doubled worm lifespan and is completely dependent on the downstream target DAF-16 (a FoxO transcription factor homologue) (see section 1.4 for more details) (Kenyon et al., 1993). Components of the insulin signalling cascade have since been identified in other model organisms (eg. in mice and fruit flies) and downregulation was similarly observed to increase lifespan.

While using *C. elegans* is an excellent tool for studying the mechanisms of ageing, it is important to be mindful that there are limitations and caveats in using this model organism to understand human ageing. The worm has a simple anatomy and lacks many defined tissues such as a brain, blood, fat cells and internal organs (Tissenbaum, 2015). In some ways, the simplicity of *C. elegans* narrows down the variables to be controlled for in studying certain molecular mechanisms. For example, *C. elegans* does not have an equivalent DNA methylation mechanism for chromatin maintenance. Therefore, investigating how chromatin modifiers affect gene expression automatically removes that extra layer of complexity in the study. On the other hand, it also highlights how evolutionarily distant worms are from humans and limits how much we can extrapolate results from worm studies into human context. Furthermore, the small size of the worm means more animals must be used to generate enough material for biochemistry techniques such as immunoprecipitation (IP), RNA-sequencing and many others. In most cases, whole worm extracts are used for such experiments, which means tissue-specific information is diluted or lost. Having said that, efforts are currently being made to optimise techniques for tissue-specific studies through fluorescence-activated cell and nuclei sorting, nuclei-tagging, mRNA-tagging and more recently through polyA-tagging with sequencing (PAT-Seq) (Blazie et al., 2015).

1.7. Screen for chromatin factors involved in ageing

Previously in the lab, an RNAi screen of chromatin factors extending lifespan in *C. elegans* was conducted, which identified enzymes modulating H3K27 methylation and acetylation such as MES-2 (H3K27 methyl transferase), JMJD-3.2 (H3K27me3/2 demethylase) and CBP-1 (H3K27 acetyltransferase) (Chocian, 2015). Furthermore, another H3K27me3/2 demethylase called UTX-1 has also been reported to alter worm lifespan, collectively suggesting that the modification of H3K27 has a disproportionate effect on ageing.

An RNAi screen on histone methyltransferases and demethylases previously implicated MES-2 and UTX-1 in lifespan regulation of *C. elegans*, validating the efficacy of our screen. Specifically, knockdown of *mes-2* and *utx-1* initiated at L1 stage gave a 6% and 19% mean lifespan extension respectively compared to empty vector controls (Ni et al., 2012). Additionally, two other reports have independently shown that knockdown of *utx-1* using heterozygous mutants and RNAi resulted in significant lifespan extension and is dependent on insulin-signalling but independent of germline proliferation, corroborating that UTX-1 is involved in lifespan regulation of worms (C. Jin et al., 2011a; Maures et al., 2011).

On the other hand, downregulation of *cbp-1* was reportedly to be detrimental to "normal" lifespan and abrogates lifespan extension due to dietary restriction (Cai et al., 2017; M. Zhang et al., 2009). This disagrees with our RNAi screen results where knockdown of *cbp-1* was found to extend lifespan compared to empty vector controls

1.8. Project aims

Based on previous work in the lab, the RNAi screen on chromatin modifiers that affect lifespan identified H3K27 modifiers UTX-1 (H3K27 lysine demethylase) and CBP-1 (H3K27 acetyltransferase) to promote lifespan extension in *C. elegans*. To our surprise, we found that overexpression of *utx-1* also promotes lifespan extension in worms (see section 3.1.5). Given this confounding result, I wanted to investigate how up- and down-regulation of *utx-1* can both promote lifespan extension. In this thesis, I set out to describe how manipulation of *utx-1* levels in specific tissues affect worm lifespan and whether these lifespan outcomes (particularly where lifespan extension is observed) are dependent on insulin signalling. Furthermore, I explored the importance of the UTX-1 catalytic activity in lifespan regulation by looking at transcriptional changes mediated by the demethylase function using RNA-Sequencing (RNA-Seq). I also conducted a lifespan screen on candidate genes derived from my RNA-Seq dataset to assess whether genes that are transcriptionally regulated by UTX-1 are important in worm longevity.

While the majority of this thesis is dedicated to understanding how UTX-1 mediates lifespan in *C. elegans*, I also describe the preliminary work I did on *cbp-1*. Given that knockdown of *cbp-1* promotes lifespan extension in our screen but is reportedly detrimental to lifespan, I set out to perform some life- and healthspan analysis on *cbp-1* mutants subjected to different environments, in order to validate how *cbp-1* depletion affects worm longevity.

CHAPTER 2: MATERIALS AND METHODS

2.1. WORM HANDLING METHODS

2.1.1. *C. elegans* strains and maintenance

C. elegans strains were maintained using standard techniques as described by Brenner (Brenner, 1974). Briefly, worms were put in standard Nematode Growth Media (NGM) plates seeded with *E. coli* OP50 as food source and kept at 20°C. The Bristol N2 strain was used as wild-type for all experiments performed. Strains used for this thesis are listed below (Table 2.1) and were either generated in the lab, obtained from the *Caenorhabditis* Genetics Center (CGC), or provided by Prof. Lisa Salcini (BRIC, University of Copenhagen). CIOSK #793 was kindly gifted by Dr. Rafal Ciosk (FMI, University of Basel).

Table 2.1: Worm strains used in experiments

Strain Name	Genotype	Plasmid	Aka
N2			wild-type
CIOSK #793	*rrrSi189 II (inserted into ttTi5605 on LG II)*	*rrrSi189 = [utx-1p::FLAG-GFP-linker-TEV::utx-1ORF+3'UTR]*	*scUTX-1::GFP*
AW1166	*pAW169*	*pAW169 = rol-6(su1006)*	
ZR254 ZR255	*utx-1(tm3118) X; zrEx?*	*zrEx? = [utx-1p::utx-1::GFP + rol-6(su1006)]*	*utx-1(tm3118)+ UTX-1::GFP*
ZR256 ZR257	*utx-1(tm3118) X; zrEx??*	*zrEx?? = [utx-1p::utx-1DD::GFP + rol-6(su1006)]*	*utx-1(tm3118)+ UTX-1DD::GFP*
ZR694	*zrEx231*	*zrEx231 = [myo-3p::utx-1::GFP + rol-6(su1006) + myo-2p::RFP]*	muscle-specific *utx-1OE*

ZR695	*zrEx233*	*zrEx233 = [dpy-7::utx-1::GFP + rol-6(su1006) + myo-2p::RFP]*	hypodermis-specific *utx-1OE*
ZR698	*zrEx235*	*zrEx235 = [rab-3p::utx-1::GFP + rol-6(su1006) + myo-2p::RFP]*	neuron-specific *utx-1OE*
ZR699	*zrEx237*	*zrEx237 = [vha-6p::utx-1::GFP + rol-6(su1006) + myo-2p::RFP]*	intestine-specific *utx-1OE*
ZR314	*daf-2(e1370)ts III; utx-1(tm3118) X; zrEx?*	*zrEx? = [utx-1p::utx-1::GFP + rol-6(su1006)]*	
ZR316	*daf-2(e1370)ts III; utx-1(tm3118) X; zrEx??*	*zrEx?? = [utx-1p::utx-1DD::GFP + rol-6(su1006)]*	
ZR802	*zrEx264*	*zrEx264[utx-1p::utx-1::GFP + rol-6(su1006)]*	
CB1370	*daf-2(e1370)ts III*		
WM27	*rde-1 (ne219) V*		
NL3321	*sid-1(pk3321) V*		
VP303	*rde-1 (ne219) V; kbIs7*	*kbIs7 = [nhx-2p::rde-1 + rol-6(su1006)]*	*intestine-specific*
NR350	*rde-1 (ne219) V; kzIs20*	*kzIs20 = [hlh-1p::rde-1 + sur-5p::NLS::GFP]*	*muscle-specific*
NR222	*rde-1 (ne219) V; kzIs9*	*kzIs9 = [(pKK1260) lin-26p::NLS::GFP + (pKK1253) lin-26p::rde-1 + rol-6(su1006)]*	*hypodermis-specific*
TU3401	*sid-1(pk3321) V; uIs69*	*uIs69 = [(pCFJ90) myo-2p::mCherry + unc-119p::sid-1]*	*neuron-specific*
AW1778	*rrrSi189 II; rde-1(ne219)V*	*rrrSi189 = [utx-1p::FLAG-GFP-linker-TEV::utx-1ORF+3'UTR]*	
AW1779	*rrrSi189 II; rde-1(ne219) V; kbIs7*	*rrrSi189 = [utx-1p::FLAG-GFP-linker-TEV::utx-1ORF+3'UTR]* *kbIs7 = [nhx-2p::rde-1 + rol-6(su1006)]*	
AW1780	*rrrSi189 II; sid-1(pk3321) V*	*rrrSi189 = [utx-1p::FLAG-GFP-linker-TEV::utx-1ORF+3'UTR]*	
AW1781	*rrrSi189 II; rde-1(ne219) V; kzIs9*	*rrrSi189 = [utx-1p::FLAG-GFP-linker-TEV::utx-1ORF+3'UTR]* *kzIs9 = [(pKK1260) lin-26p::NLS::GFP + (pKK1253) lin-26p::rde-1 + rol-6(su1006)]*	
AW1782	*rrrSi189 II; sid-1(pk3321) V; uIs69*	*rrrSi189 = [utx-1p::FLAG-GFP-linker-TEV::utx-1ORF+3'UTR]* *uIs69 = [(pCFJ90) myo-2p::mCherry + unc-119p::sid-1]*	
CF1038	*daf-16(mu86) I*		

AW1708	*daf-16(mu86) I; rde-1(ne219)V; kzIs9*	*kzIs9 = [(pKK1260) lin-26p::NLS::GFP + (pKK1253) lin-26p::rde-1 + rol-6(su1006)]*	*daf-16; hypodermis-specific*
AW1709	*daf-16(mu86) I; sid-1(pk3321)V; uIs69*	*uIs69 = [(pCFJ90) myo-2p::mCherry + unc-119p::sid-1]*	*daf-16; neuron-specific*
AW1774	*daf-16(mu86) I; rde-1(ne219)V; kbIs7*	*kbIs7 = [nhx-2p::rde-1 + rol-6(su1006)]*	*daf-16; intestine-specific*
AW1776	*daf-16(mu86) I; rde-1(ne219)V*		
AW1777	*daf-16(mu86) I; sid-1(pk3321)V*		
VC1006	*cbp-1(ok1491) III/hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)*		
JK2739	*mcm-4(e1466) dpy-5(e61) I/hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)*		
AW1479	*dpy-18(e364) I/hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)*		
AW1479	*+/ hT2 [bli-4(e937) let-?(q782) qIs48] (I;III); him-5(e1490) V*		
MH2430	*cbp-1(ku258) III*		
YS2	*cbp-1(bm1) dpy-18(e364)/qC1 [dpy-19(e1259) glp-1(q339)] III*		
YS4	*cbp-1(bm2) dpy-18(e364)/qC1 [dpy-19(e1259) glp-1(q339)] III*		
AW1477	*cbp-1(bm1) dpy-18(e364)/ hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)*		
AW1478	*cbp-1(bm2) dpy-18(e364)/ hT2 [bli-4(e937) let-?(q782) qIs48] (I;III)*		

2.1.2. Bleaching

To clean contaminated stocks of worm strains, 10 gravid adults were picked onto 10 μL of 2x bleach solution (6% NaOCl, 1M NaOH). Bleach will dissolve adult worms, while the eggs remain intact. Hatched L1s are picked onto new NGM plates seeded with OP50 24 hours later and are maintained for at least 3 generations before being used in future experiments.

2.1.3. RNAi by feeding

To perform RNAi, *E. coli* HT115 transformed with L4440 vector containing the dsRNA of interest are seeded onto standard NGM plates with 25 μg/ml Carbenicillin and 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce dsRNA production. L4-stage worms are placed onto seeded plates and are observed (along with their progeny, if applicable) for phenotypes of interest. For lifespan assays, RNAi cultures are supplemented with 1mg/ml 5-Fluoro-2′-deoxyuridine (FUDR) to prevent egg-hatching. P0 animals are used for survival analysis unless otherwise stated. For every RNAi experiment initiated, a *pop-1* RNAi positive control is separately prepared to ensure that IPTG-induction is working properly. *pop-1* RNAi on L4-stage worms results in an observable lethality phenotype where eggs laid by hermaphrodites fail to hatch, showing plenty of dead eggs on the NGM plate.

2.1.4. Lifespan survival assay

5-10 L4-stage worms were picked onto plates seeded with OP50 or HT115 (for RNAi) supplemented with 1mg/ml (FUDR) to prevent egg-hatching. A population of at least 60 worms were investigated per condition tested. The animals were checked every other day and scored as dead if they are unresponsive to gentle prodding using a platinum wire.

Worms that escaped or burrowed were censored from the experiment. All lifespan experiments were performed with at least 2 independent repeats unless otherwise stated.

2.1.5. Motility Assay

5 worms were picked onto unseeded NGM plates where they were left to crawl briefly to remove excess OP50. The plates were then flooded with 50 μL of M9 (42 mM Na_2HPO_4, 22mM KH_2PO_4, 86mM NaCl, 1mM $MgSO_4$) and the worms allowed to acclimatise in the liquid for 30 seconds. The following 30 seconds was then captured using a Leica MZFII microscope and Hamamatsu digital camera (Orca-05G). Thrashing was quantified using wrMTrck (Nussbaum-Krammer, Neto, Brielmann, Pedersen, & Morimoto, 2015) on FiJi (Schindelin et al., 2012).

2.1.6. Brood Size Assay

L4-stage hermaphrodite worms were individually picked onto NGM plates seeded with OP50. The worms could lay eggs for 24 hours before being transferred onto fresh plates for 7 consecutive days. Egg laying was counted daily and total brood size determined by adding up the total number of eggs laid over the 7-day period, regardless of whether they hatched into live larvae. 10 worms per condition were assayed for this experiment.

2.1.7. Stress Resistance Assays

2.1.7.1 Oxidative Stress

L4 worms were plated onto NGM plates (seeded with OP50 or RNAi feeding clones; RNAi plates also contained IPTG and Carbenicillin) containing 10mM Paraquat Dichloride to generate reactive oxygen species (ROS). All plates were maintained at 20^{o}C. Survival was first assessed at the 22-hour time point and every 4 hours thereafter

(except for an 8 hour gap in one of the overnight time points). Worms that were unresponsive to gentle prodding with a platinum wire were scored as dead and worms that escaped the plates were censored. A population of at least 50 worms per condition were assayed for survival. All lifespan experiments were performed with at least 2 independent repeats unless otherwise stated.

2.1.7.2. Heat Stress

15 Day-5 post-L4 worms were place onto seeded plates before incubating at 35^{o}C for 6 hours. After heating, worms were transferred to freshly seeded plates and maintained at 20^{o}C. Survival was assessed daily where worms that were unresponsive to gentle prodding with a platinum wire were scored as dead and worms that escaped the plates were censored. A population of at least 40 worms per condition were assayed for this experiment.

2.1.7.3. UV Stress

10 Day-3 post-L4 worms were transferred onto unseeded plates and allowed to crawl for 10 minutes to remove excess OP50. The plates were then individually irradiated with 1000 J/m^2 UVC using a Stratalinker® UV crosslinker. Survival was assessed at least every other day where worms that were unresponsive to gentle prodding with a platinum wire were scored as dead and worms that escaped the plates were censored. A population of at least 40 worms per condition were assayed for this experiment.

2.1.8. Genetic Crosses

1 L4 hermaphrodite and 4 male worms are placed onto an unseeded NGM plate followed by a small scoop of OP50 using a platinum wire. The F1 animals are checked 3 days later, with the cross deemed to have worked when a 1:1 ratio of male and hermaphrodite

progeny was observed. F1 stage worms were either used for further crosses or allowed to self-reproduce to obtain homozygous animals. Genetic crosses are described further in the appropriate results chapters.

2.2. BACTERIAL METHODS

2.2.1. OP50

Worms were fed an uracil-deficient strain of *E. coli* called OP50 as used by Brenner (Brenner, 1974), to prevent overgrowth of the bacterial lawn when seeded onto NGM plates. To prepare the bacteria, an OP50 glycerol stock is stabbed with a pipette and inoculated into 200 ml of 2xTY liquid media. The culture is grown overnight at 37ºC with shaking before being seeded onto standard NGM plates.

2.2.2. HT115 - RNAi

Worms were fed HT115 (DE3) *E. coli*, an IPTG-inducible, RNase III-deficient strain, transformed with an L4440 plasmid containing the dsRNA sequence of interest flanked by T7 promoters. IPTG induces production of T7 RNA polymerase, which then transcribes RNA from both ends of the sequence inserted between the T7 promoters. To prepare, frozen glycerol stocks of the requisite RNAi of interest were stabbed with a pipette and streaked onto 2xTY agar plates supplemented with 50 μg/ml Ampicillin and 15 μg/ml Tetracycline. The plates were incubated at 37ºC for 12-18 hours before picking individual colonies to inoculate 5 ml of liquid 2xTY media supplemented with 50 μg/mL of Ampicillin. Bacterial cultures were incubated overnight in a 37ºC shaking incubator and subsequently seeded onto NGM plates containing 25 μg/ml Carbenicillin and 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Glycerol stocks were taken from the

Ahringer *C. elegans* RNAi feeding library (Kamath et al., 2003), except for *daf-2* (Dillin, Crawford, & Kenyon, 2002). All RNAi clones were validated by sequencing.

2.3. DNA METHODS

2.3.1. Worm lysis

Worms were individually picked onto 2 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 -80ºC for 15 minutes. The samples were then incubated at 60ºC for 20 minutes and 95ºC for 15 minutes thereafter. Samples were then used as templates for PCR reactions later.

2.3.2. PCR

PCR reactions were performed using worm lysis samples as templates. Each PCR reaction had a total volume of 25 µL and contained the following: 2 µl template, 2.5 µl 10x ThermoPol buffer (NEB), 0.125 µl Taq Polymerase (NEB), 2 µl dNTP mix (final concentration of 200 µM for each of dATP, dTTP, dCTP and dGTP), 1.25 µl forward primer (200 nM final conc.), 1.25 µl reverse primer (200 nM final conc.) and 15.875 µl PCR-grade water. Generally, each PCR reaction mix underwent the following cycles: initial denaturation at 95ºC for 30 seconds, 30 cycles: denaturation at 95ºC for 30 seconds, annealing at (Tm-5ºC) for 30 seconds, extension at 68ºC timed at 1min/kb (or 30 seconds for anything less than 1 kb) and a final extension at 68ºC for 5 minutes. Primers were designed to have little to no secondary structure, assessed using OligoCalc online calculator(Kibbe, 2007). Primer annealing temperatures were calculated using the same program. Primers used are listed below (Table 2.2).

Table 2.2: Genotyping primers used for experiments.

Primer name	Sequence	Tm (°C)	Notes
2_AG_wb_cbp-1_f	GCCCGATCTTGCAAATCTAA	54	genotyping for *cbp-1(ok1491)*
2_AG_wb_cbp-1_r	CGCATTACTGGGAGTTCCAT	56	
3_AG_MH2430_g_f	GGAGAAGCTAGATAAATCCG	56.4	genotyping *cbp-1(ku258)*; PCR product sent for sequencing
4_AG_MH2430_g_r	CTCTCGAAAGGCTCATAATC	56.4	
7_AG_cbp-1_YS2-R	ATTCATTCCACCAAGTCCAC	56.4	genotyping *cbp-1(bm1)*, paired with 3_AG_MH2430_g_f
8_AG_cbp1_YS4-F	CGCTTCTTCACGATCATGAA	56.4	genotyping for *cbp-1(bm2)*
9_AG_cbp1_YS4-R	AAACAGTGGCTAGAGTGAAG	56.4	
utx-1_g1_F	CATTGAAAGAAGCTTATGAGCAGC	56	genotyping *utx-1(tm3118)*
utx-1_g1_R	GTCCACGAATACAAGAAATAGT	52	
21_AG_rde-1_F	GGTATTTTCTTTGTAGTCGTGAG	59.2	genotyping *rde-1(ne219)*; PCR product sent for sequencing
22_AG_rde-1_R	AAGTCACTGGTAAATCGGACAG	60.1	
33_AG_sid-1genoF	CCTCAAGTAACAGCTCTGTAC	59.5	genotyping *sid-1(pk3321)*; PCR product sent for sequencing
34_AG_sid-1genoR	CCGATCCAAATGAGTCACATG	59.5	
SM147	CGTATTCTCGTGGGAAAGTG	62.1	genotyping *daf-16(mu86)*
SM155	CGTTATCAAATGCTCCTTGC	62.1	
SM156	CGGTGCCTTGTTCTTCATCT	64.2	genotyping *daf-16*
SM157	TTGTGACGGATCGAGTTCTG	64	

2.3.3. Gel electrophoresis

To resolve DNA fragments, 1% agarose gels were prepared in TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA) added with Ethidium Bromide (final conc. 0.5

μg/ml). Samples 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) and molecular markers (1kb or 100bp ladder from NEB) were loaded into the set gels and electrophoresed at 90 V for 20 to 60 minutes. DNA was visualised on a UV transilluminator and captured using a camera with AlphaImager software.

2.3.4. Sequencing

DNA fragments or plasmids of interest were amplified (via PCR and growing bacterial cultures respectively) and purified using the requisite kit – GeneClean II or Qiagen PCR cleanup kit for DNA fragments and Qiagen miniprep kit for plasmids. Samples were diluted to the appropriate concentration (10 ng/μl for PCR fragments and 100 ng/μl for plasmids) and submitted to Source Bioscience Sanger Sequencing Services. Primers to be used were also submitted at a concentration of 4 μM.

2.3.5. cDNA Preparation

RNA extracted from worms (see section 2.4.1 RNA extraction) was converted to cDNA using Evoscript Universal cDNA Master kit (Roche) as per manufacturer's instructions. Briefly, 20 μl of 5x reaction buffer was added to 1 μg of total RNA and the reaction mixture made up to 90 μL with PCR-grade water. The resulting solution was equilibrated on ice for 5 minutes before adding 10 μl of 10x enzyme mix. The reaction mixture was then heated to 42ºC for 15 minutes, 85ºC for 5 minutes and finally 65ºC for 15 minutes and kept at -20ºC until ready to be used. The 10x enzyme mix has RNase H activity, eliminating the necessity to do this as a separate step.

2.3.6. qRT-PCR

Standard PCR reactions were initially performed on qPCR primers using N2 cDNA as template to ensure that primer pairs were working properly. cDNA samples were diluted to 5ng/µl and used as template for qRT-PCR experiments. Each reaction mixture contained 2 µl of template, 12.5 µl SYBR green, 1.5 µl forward primer (final conc. 150 nM), 1.5 µl reverse primer (final conc. 150 nM) and 7.5 µl PCR-grade water. qRT-PCR was performed using Applied Biosystems StepOne Plus equipment and was analysed by the ΔΔCt method using proprietary software. *ama-1* was used as an internal control. Each sample and primer pair was performed with 6 technical replicates each. qRT-PCR primers used for this thesis are listed below (Table 2.3) and were designed using PrimerQuest tool (Integrated DNA Technologies).

Table 2.3: qRT-PCR primers used for these experiments.

Primer name	Sequence	Tm (°C)	Notes
AG_rt_fbxa-163_r	TGCACTATGATCGAAGCGTC	62.1	Target: *fbxa-163* Amplicon: 134 bp
AG_rt_fbxa-163_r	CCGAAAATTCCGAGACACTTTC	61.6	
AG_rt_hsp-1_f	AATACAAGGCTGACGATGAGG	61.9	Target: *hsp-1* Amplicon: 123 bp
AG_rt_hsp-1_r	CTGGGCTGATCTTATCCTTGAG	61.9	
SM180	CAGTGGCTCATGTCGAGT	60	Target: *ama-1* Amplicon size: 426 bp
SM181	CGACCTTCTTTCCATCATTCAT	63.9	
utx-1_rt2_f	TTCGGGTTAGGTCTTGTCTACTTG	58	Target: *utx-1* Amplicon size: 263 bp
utx-_rt2_r	TCATCATTATTCTCGTGGCTCAG	56	

2.4. RNA METHODS

2.4.1. RNA extraction

Animals were harvested from NGM plates and washed several times with M9 buffer before resuspending in 250 µl of Trizol. The samples were vortexed several times before being flash frozen in liquid nitrogen to rupture the worms. Subsequently, the samples were heated for 90 seconds at 65°C to melt the frozen solution before adding 350 µl more of Trizol and left at room temperature for 15 minutes. Each sample was transferred into phase lock tubes (Eppendorf) before adding 200 µl of chloroform, ensuring that the solution is mixed thoroughly. The samples are left at room temperature for 3 minutes and then centrifuged (12000 rpm) for 15 minutes at 4°C to separate the different phases of the mixture. The upper aqueous phase was collected into a separate tube, then mixed with 300 µl of isopropanol and left at room temperature for 10 minutes. Afterwards, the samples were centrifuged (12000 rpm) for 10 minutes at 4°C to collect the RNA pellet, which was then washed with 75% ethanol and centrifuged (9400 rpm) for another 5 minutes at 4°C. The supernatant was removed and the pellet air dried for 10 minutes before resuspending in 20 µl of RNase-free water. 2 µl of the sample was kept for checking RNA integrity on a 1% agarose gel, and the remaining 18 µl was DNase-treated (Roche) as per manufacturer's instructions.

2.4.2. RNA-Sequencing

RNA was isolated as described previously (see section 2.4.1. RNA extraction) but did not undergo the final DNase step. RNA integrity was initially checked by running the samples on a 1% agarose gel. Samples that had two distinct bands of the correct ratio of intensity was carried forward for additional quality control checks. RNA samples were quantified and quality-checked using the Agilent 2100 Bioanalyser using proprietary

Agilent kits as per manufacturer's instructions. Samples with an RNA Integrity Number (RIN) > 7 was submitted for RNA-Sequencing analysis to Beijing Genomics Institute (BGI) for transcriptomic studies. See chapter 4 for further information on experimental parameters used.

2.5. MICROSCOPY

Routine worm maintenance was performed under a low power dissection microscope fitted with a 10x objective (Nikon SMZ800). For fluorescence microscopy, 5-10 worms were anaesthetised in 25mM laevamisole before being mounted on a 2% Agarose pad. DIC and epifluorescence images were acquired using the Zeiss AxioSKOP2 microscope with Zeiss AxioCamMR and a 63x immersion oil objective. Images were exported using proprietary software and reconstructed using the Pairwise Stitching and MosaicJ plugin in Fiji (Preibisch, Saalfeld, & Tomancak, 2009; Schindelin et al., 2012).

2.6. STATISTICAL METHODS

2.7.1. Lifespan and stress resistance survival assays

Statistical significance was assessed using GraphPad Prism software with p-values calculated using the log-rank (Mantel-Cox) method.

2.7.2. Motility assay

Statistical significance was assessed using GraphPad Prism software with p-values calculated using one-way ANOVA.

2.7.3. Brood size assay

Statistical significance of total and daily brood size was determined using unpaired student's t-test and one-way ANOVA respectively in GraphPad Prism.

CHAPTER 3: TISSUE-SPECIFIC CONTRIBUTIONS OF UTX-1 TO LIFESPAN REGULATION

3.1. INTRODUCTION

3.1.1. What is UTX-1?

utx-1 encodes for the homologue of mammalian UTX (Ubiquitously-transcribed Tetratricopeptide repeats on chromosome X) – a lysine demethylase that specifically removes methyl groups from transcriptionally repressive H3K27me3 marks (and to a lesser extent H3K27me2) (Agger et al., 2007; Klose, Kallin, & Zhang, 2006). Transfection of shRNA targeting UTX in HeLa cells caused a significant increase in H3K27me3 immunostaining but did not change H3K9me3 levels, suggesting that UTX demethylates H3K27me3 specifically *in vivo*. Furthermore, bulk histones treated with recombinant human UTX showed a dose dependent decrease in H3K27me3 levels quantified using western blot, while no change was observed in H3K9me3, H3K4me3 and H3K36me3. Collectively, these results indicate that the UTX demethylase activity acts specifically on H3K27me3 marks (Agger et al., 2007).

Demethylases containing a JmjC domain remove methyl groups from lysine and arginine residues through an oxidative reaction and requires an Fe(II) ion and alpha-ketoglutarate (αKG) as cofactors for demethylation to proceed (Klose et al., 2006). UTX belongs to the KDM6 family of histone demethylases along with JMJD3 and UTY. Four members of

the KDM6 family exist in *C. elegans*: *jmjd-3.1*, *jmjd-3.2* and *jmjd-3.3* are closely related to JMJD3, while *utx-1* is a unique homologue of UTX/UTY (Agger et al., 2007). UTX and JMJD antagonises the transcriptional repression exerted by polycomb repressor complexes (PRC), which have inherent H3K27 methyl transferase activity (Agger et al., 2007).

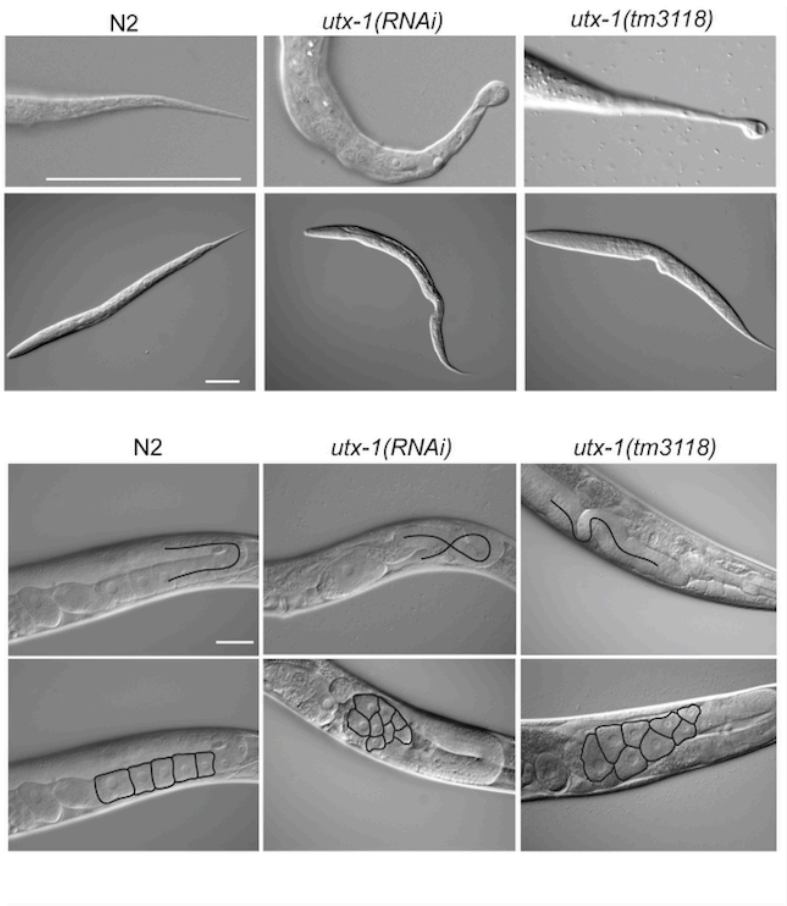
Mammalian UTX is a member of the mixed lineage leukaemia-2 (MLL2) H3K4 methyltransferase complex which contains 11 other protein members including MLL2, PTIP, ASC-2, ASH2L, RBBP5, WDR5, DPY30, matrin3, MGC4606, α- and ß-tubulin (Issaeva et al., 2007). Components of the complex have since been confirmed in *D. melanogaster* (fruit fly) orthologue Trithorax related (Trr) complex and the SET-16 complex in *C. elegans* (Mohan et al., 2011; Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a). The presence of both an H3K4 methyltransferase and H3K27me3 demethylase in the same complex suggests a dynamic interplay between H3K4 and H3K27 methylation states to regulate transcriptional gene activation (Van der Meulen, Speleman, & Van Vlierberghe, 2014). UTX was also found to promote chromatin remodeling independent of its demethylase function by interacting with the Brg-1 containing SWI/SNF complex (Miller, Mohn, & Weinmann, 2010). Furthermore, T-box factors reportedly interacted with UTX (and Jmjd3) to promote transcriptional activation of target genes involved in cell-fate decisions, differentiation and organogenesis (Miller et al., 2010). In fruit flies, dUTX was found to associate with histone acetyltransferase CBP (Creb-binding protein) and SWI/SNF complex to promote deposition of H3K27ac marks and antagonise H3K27 methyltransferase complex PRC2 (Tie, Banerjee, Conrad, Scacheri, & Harte, 2012).

3.1.2. UTX-1 is crucial for development

UTX was previously found to have roles in differentiation and development through a direct interaction with HOX genes. Studies in human embryonic teratocarcinoma NT2/D1 cells showed that retinoic-acid induces differentiation (V. M. Lee & Andrews, 1986) and is associated with upregulation of HOX genes, which is mediated by H3K27me3 dynamics (Bracken, Dietrich, Pasini, Hansen, & Helin, 2006). ChIP studies in NT2/D1 cells and mouse embryonic stem cells showed that retinoic acid treatment stimulated the binding of UTX to the HOXB1 promoter with concomitant decrease in H3K27me3 marks and an increase in HOXB1 mRNA levels (Agger et al., 2007). This suggests that UTX is involved in HOX gene regulation to promote differentiation during development. Furthermore, UTX was also found to directly interact with H3K4me3 methyltransferase MLL2 (Issaeva et al., 2007), indicating that a UTX/MLL2 complex could coordinate removal and deposition of repressive and activating marks to promote stringent gene regulation during cellular differentiation. The importance of UTX in development is corroborated by findings that loss-of-function mutations in human UTX lead to development of Kabuki syndrome, a congenital disease associated with severe mental retardation, postnatal growth impairment and facial abnormalities (Van der Meulen et al., 2014).

In *C. elegans*, UTX-1 was found to be expressed in most (if not all) nuclei from early stage embryos to adulthood, suggesting that UTX-1 plays an important role throughout worm development. Homozygous *utx-1* mutants derived from heterozygous mothers are viable but sterile, indicating that maternal contribution of *utx-1* is vital for embryogenesis but can be compensated for by maternal contribution (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a). RNAi allows for post-embryonic investigations on the effect of *utx-1* depletion in worms. Phenotypic analysis of *utx-1* mutants derived from

heterozygous *utx-1* mothers and wild-type animals subjected to *utx-1* RNAi show severe posterior defects, aberrant gonad migration and disorganised oocyte formation (Figure 3.1) (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a).



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 3.1: Loss of utx-1 causes posterior and gonadal defects. *Top shows posterior and vulval defects in utx-1 mutants and utx-1(RNAi) worms; middle panel shows aberrant gonad migration and disorganised oocyte effects of utx-1 depletion; bottom table lists quantification of defects observed. Images taken from* (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a)

Transgenic overexpression of a UTX-1::GFP construct in *utx-1* mutants rescued the sterility and gonadal defects, suggesting that lack of UTX-1 was responsible for these

defects. Transgenes are typically silenced in the germline, and the fact that UTX-1::GFP overexpression rescues the sterility phenotype suggests that UTX-1 is important in somatic gonad development rather than germline development. Interestingly, lethality and all defects exhibited by *utx-1* mutants can be rescued by transgenic overexpression of a catalytically dead version of UTX-1 (Figure 3.2), suggesting that the demethylase function of UTX-1 is dispensable for worm development.

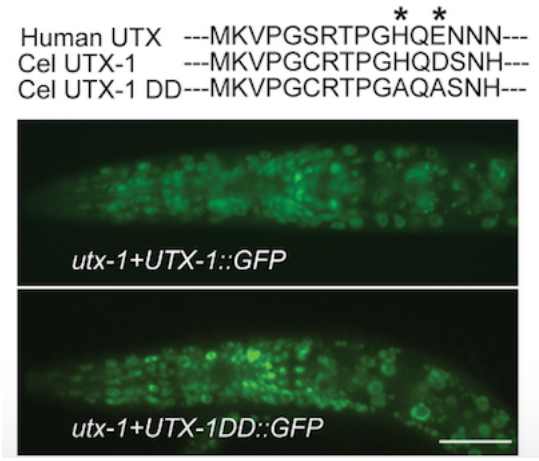


Figure 3.2: A demethylase dead version of UTX-1 rescues lethality in C. elegans. *UTX-1::GFP fusion constructs were transgenically overexpressed in utx-1 mutants. The demethylase-dead version of UTX-1 was created by mutating two key Fe(II) binding sites in the protein (marked with *). Images taken from* (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a)

The dispensability of demethylase function in UTX has been previously demonstrated in mice. UTX and UTY are homologous X and Y chromosome members of the KDM6 family of histone demethylases. *In vitro* assays have shown that UTY has lost its enzymatic activity due to sequence divergence. Shpargel *et al.* (2012) demonstrated that homozygous *Utx* mutant female mouse embryos ($X^{Utx-}X^{Utx-}$) are mid-gestational lethal. However, hemizygous male mutants carrying a mutation in *Utx* but also a wild-type copy of *Uty* ($X^{Utx-}Y^{+}$) were found to develop to term (even though they are runted), with 25% surviving postnatally and reaching adulthood (Shpargel, Sengoku, Yokoyama, &

Magnuson, 2012). This suggests a functional redundancy between UTX and UTY during mouse embryonic development. H3K27me3 ChIP on mouse embryonic fibroblasts (MEFs) of $X^{+}Y^{+}$ (controls) and $X^{Utx\text{-}}X^{Utx\text{-}}$ genotype, followed by qPCR on the *Fnbp-1* promoter (a common target of UTX and UTY) showed no significant differences in levels of H3K27me3 between *Utx* mutants and controls. On the other hand, there was a decrease in levels of H3K4me3 on the *Fnbp-1* promoter in MEFs carrying a mutation in *Utx* compared to controls (Shpargel et al., 2012). This suggests that gene regulation of *Fnbp-1* was independent of H3K27 demethylase activity of *Utx* and was more likely regulated by H3K4 methylation. Additionally, another study also found that UTX regulates mesodermal differentiation in mouse embryonic stem cells independent of its catalytic activity (C. Wang et al., 2012). Collectively, the data implies that like *C. elegans*, H3K27 demethylation is not essential for embryonic viability in mice.

In contrast to this, studies on the developmental role of fly homologue *dUtx* showed that the catalytic activity of *dUtx* is absolutely necessary for viability. *Utx* null mutants with maternal Utx contribution develop into adults but die after eclosing, but mutants lacking both maternal and zygotic contribution of *Utx* die during larval development, suggesting that *Utx* is important for viability. Analysis of *Utx* deletion mutants (Utx^{Δ}) transgenically overexpressing either wild-type ($gUtx^{wt}$) or catalytically-dead Utx ($gUtx^{cd}$) showed that viability is rescued by $gUtx^{wt}$ but not $gUtx^{cd}$, indicating that demethylase activity of *Utx* is necessary for adult viability. Unlike in mice and worms, fruit flies have *dUtx* as the sole orthologue of the KDM6 family. It is unclear if the necessity of a Utx catalytic activity for viability is an organism-specific ("private") mechanism in flies. It is also possible that the lack of other KDM6-like family members in flies means that there are no other enzymes that can compensate for the lack of Utx catalytic activity. This seems unlikely given that studies in *C. elegans* show no evidence that other KDM6 family

members compensate for the lack of UTX-1 catalytic activity (Vandamme, Lettier, Sidoli, Di Schiavi, Nørregaard Jensen, et al., 2012b). Specifically, downregulation of *jmjd-3.1*, *jmjd-3.2* and *jmjd-3.3* in *utx-1* mutants overexpressing a demethylase dead version of UTX-1 did not exhibit posterior defects as seen in *utx-1* mutants (Vandamme, Lettier, Sidoli, Di Schiavi, Nørregaard Jensen, et al., 2012b).

3.1.3. Knockdown of *utx-1* promotes lifespan extension

Two reports consistently showed that *utx-1* depletion using RNAi or heterozygous mutants gave significant lifespan extension in *C. elegans* in a germline-independent manner (C. Jin et al., 2011a; Maures et al., 2011). Analysis of temporal expression levels of *utx-1* show a bimodal pattern whereby *utx-1* levels are low between day 0 to day 5 of adulthood, but significantly rise after day 7. The increasing levels of *utx-1* is associated with decreasing levels of H3K27me3 marks with age. Knockdown of *utx-1* significantly increases global H3K27me3 levels in worms as confirmed by western blot of worm lysates subjected to empty vector or *utx-1* RNAi (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a). This led to the postulation that knockdown of *utx-1* extends lifespan by maintaining higher levels of H3K27me3, perhaps contributing to a more "youthful" chromatin state. A UTX-MLL (H3K4 methyl transferase) complex has been previously identified in mammalian cells. Simlarly in *C. elegans*, homologues of MLL components were found to interact with *utx-1*, such as *ash-2* (ASH2 homologue)), *wdr-5* (WDR5 homologue), *pis-1* (PTIP homologue), and *set-16* (MLL3/4). Knockdown of components of H3K4 methyltransferase such as *set-2*, *ash-2* and *wdr-5* have been previously shown to extend lifespan in a germline-dependent manner. Given that *utx-1* knockdown promotes longevity in a germline-independent manner, lifespan extension due to *utx-1* depletion might involve a different mechanism to that of depleting H3K4 methyl transferase components.

3.1.4. Lifespan extension due to *utx-1* depletion is dependent on insulin signalling

Knockdown of *utx-1* in long-lived *daf-2* mutants does not have an additive effect on worm lifespan (C. Jin et al., 2011a; Maures et al., 2011). Lifespan extension due to reduced insulin signalling is mediated by FoxO transcription factor homologue DAF-16, the downstream effector of DAF-2 (Kenyon et al., 1993). *daf-16* mutants are short-lived and subjecting them to *utx-1* RNAi did not give lifespan extension (C. Jin et al., 2011a; Maures et al., 2011), suggesting that lifespan extension due to *utx-1* depletion is completely dependent on the insulin signalling pathway. *utx-1* expression increases with age with concomitant decrease in H3K27me3 marks. Expression of *daf-2* also dramatically increases with age, and the timing of increase slightly lags the rise in *utx-1* expression, suggesting that *utx-1* might cause an increase in *daf-2* expression. Indeed, *utx-1* RNAi is associated with a decrease in mRNA levels of *daf-2* and other components of the insulin signalling pathway. Furthermore, *utx-1* RNAi retains transcriptionally repressive H3K27me3 levels on the *daf-2* locus, which tends to decrease with age (C. Jin et al., 2011a; Maures et al., 2011). Collectively, this indicates that knockdown of *utx-1* promotes lifespan extension by inhibiting transcription of *daf-2*, consequently downregulating the insulin signalling pathway.

3.1.5. Overexpression of *utx-1* also promotes lifespan extension

As mentioned before, an RNAi screen on chromatin modifiers that affect worm lifespan identified putative H3K27 modifiers including MES-2 (H3K27 methyl transferase), CBP-1 (histone acetyl transferase whose targets include H3K27) and UTX-1 (H3K27 demethylase) as having roles in lifespan regulation (Chocian, 2015). Given that two reports independently validate that *utx-1* downregulation promotes lifespan extension, it was expected that overexpression of *utx-1* in wild-type worms would act to shorten

lifespan (or to have no effect if normal levels of *utx-1* are saturating). Surprisingly, worms overexpressing *utx-1* are longer-lived than wild-type controls (Figure 3.3).

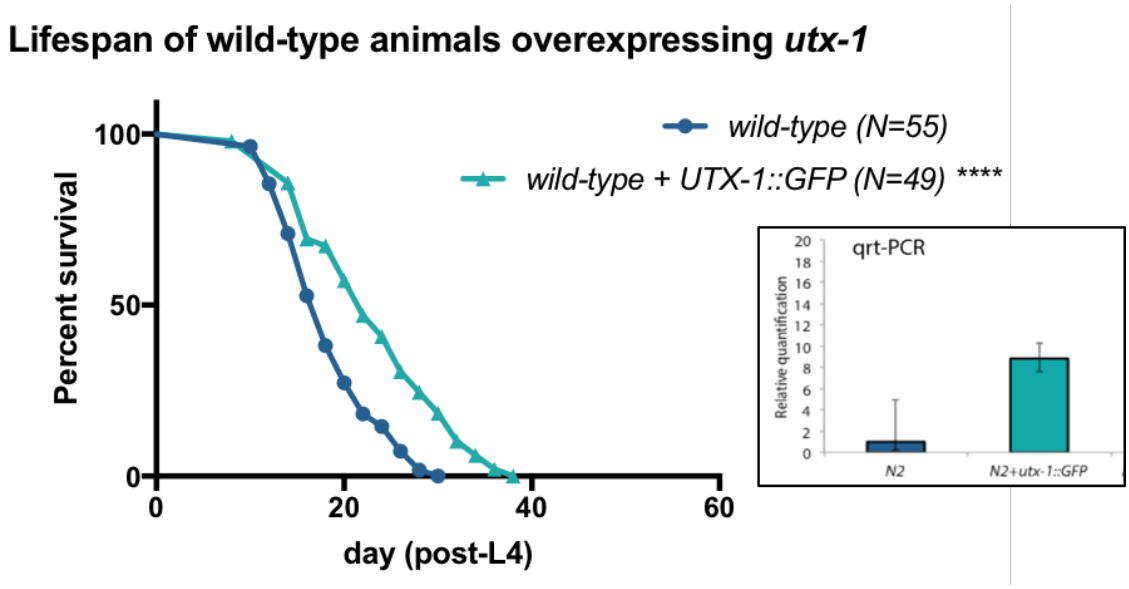


Figure 3.3: Survival of wild-type worms overexpressing utx-1. *Worms overexpressing utx-1 are significantly longer lived than wild-type controls ($p < 0.0001$). Inset: qRT-PCR confirms utx-1 overexpression in wild-type+UTX-1::GFP strain compared to wild-type. Experiment was performed by Dr Karolina Chocian in the Woollard lab (Chocian, 2015)*

The finding that overexpression of *utx-1* extends lifespan, combined with reports that *utx-1* downregulation also promotes lifespan extension highlights that the mechanism for lifespan regulation by *utx-1* is more complex than previously thought. While there is evidence to suggest lifespan extension due to *utx-1* depletion is mediated by transcriptional repression of *daf-2*, leading to downregulation of insulin signalling (see section 3.1.4), very little is known about how *utx-1* overexpression promotes lifespan extension.

3.1.6. Lifespan extension can be mediated in a tissue-specific manner

Genetic mosaic analysis of lifespan regulation mediated by *daf-2* showed that animals that lost *daf-2* in the AB lineage (epidermis, pharynx and most neurons) live twice as

long as *daf-2(+)* animals, while worms that lost *daf-2* in the P1 lineage (epidermis, pharynx, intestine, reproductive system, and body wall muscle) had modest lifespan extension (1.4x) compared to controls (Apfeld & Kenyon, 1998). This suggests that *daf-2* acts within certain cell lineages, particularly in neuronal cells, to regulate lifespan. In another study, Wolkow *et al.* (2000) showed that the long lifespan of *age-1* mutants was completely rescued back to wild-type by overexpressing *age-1* specifically in the neurons, but not when *age-1* is overexpressed in the intestine or muscle. Similarly, neuronal overexpression of *daf-2* in long-lived *daf-2* mutants rescued lifespan close to wild-type (but not completely). Muscle-specific expression of *daf-2* in *daf-2* mutants did not affect lifespan, while intestine-specific expression of *daf-2* only partially rescued lifespan (Wolkow, Kimura, Lee, & Ruvkun, 2000). This indicates that neurons (and to some extent, the intestine) are important tissues for mediating lifespan extension due to insulin-signalling. Additionally, tissue-specific expression of *daf-16* on short-lived *daf-2; daf-16* double mutants showed that intestinal rescue of *daf-16* substantially increases lifespan, implying that lifespan extension mediated by *daf-16* is regulated in the intestine (Libina, Berman, & Kenyon, 2003). Collectively, these studies highlight the neurons and intestine as crucial sites of action for mediating worm lifespan.

3.1.7. Untangling the tissue-specific effects of UTX-1 on lifespan

Spatial expression of UTX-1 has been analysed using a translational UTX-1::GFP reporter and revealed that UTX-1 is expressed in the nuclei of most, if not all cells of the worm (Vandamme, Lettier, Sidoli, Di Schiavi, Nørregaard Jensen, et al., 2012b). Given the ubiquitous expression of UTX-1 and knowing that certain tissues (ie. neurons and intestine) are important in lifespan regulation, I wanted to investigate if the lifespan extension due to up- or- downregulation of *utx-1* is context dependent. Particularly, I wanted to assess how lifespan is affected when *utx-1* levels are manipulated in a tissue-

specific manner and determine whether lifespan extension due to *utx-1* depletion and overexpression are driven by tissue-specific contributions of *utx-1*. In this chapter, I will describe my strategy for doing tissue-specific knockdown and overexpression of *utx-1* and the resulting lifespan phenotypes observed from such manipulations.

As previously mentioned, lifespan regulation due to *utx-1* knockdown was dependent on insulin signalling. Because overexpression of *utx-1* also extends lifespan, I wondered whether there exists a pro-longevity mechanism controlled by UTX-1 that is independent of the DAF-2/DAF-16 paradigm. To this end, I will also describe how I investigated the *daf-2* and *daf-16* dependence of lifespan, based on longevity outcomes that arise from my tissue-specific *utx-1* studies.

3.2. CHAPTER AIMS

- Validate the use of RNAi-insensitive mutants as a strategy to perform tissue-specific RNAi.
- Investigate the effect of tissue-specific knockdown and overexpression of *utx-1* on lifespan.
- Investigate the *daf-2* and *daf-16* dependence of lifespan regulation due to tissue-specific knockdown of *utx-1*.
- Investigate the *daf-2* and *daf-16* dependence of lifespan regulation due to tissue-specific overexpression of *utx-1*.

3.3. RESULTS

3.3.1. *utx-1* is ubiquitously expressed in *C. elegans*

Before proceeding with my hypothesis that tissue-specific perturbation of *utx-1* can have differential effects on longevity, I wanted to verify that *utx-1* is expressed ubiquitously in the worm. I looked at *utx-1* translational reporters from two sources, the first is a *utx-1* mutant strain rescued with UTX-1::GFP (called "*utx-1(tm3118)*+UTX-1::GFP" from this point onwards), a kind gift of Dr Lisa Salcini (University of Copenhagen, Denmark) (Julien Vandamme, 2013) and the second is a superficially wild-type strain carrying a single-copy insertion of UTX-1::GFP (called "*scUTX-1::GFP*" from this point onwards) kindly gifted by Dr Rafal Ciosk (ETH, Switzerland) (Seelk et al., 2016). The former was created using conventional DNA transformation and microinjection methods as described by Mello *et al.* (1991) (Mello, Kramer, Stinchcomb, & Ambros, 1991; Mello & Fire, 1995), while the latter was made using MosSCI as described by Frøkjær-Jensen *et al.* (2008) (Frøkjær-Jensen et al., 2008). Consequently, *utx-1(tm3118)+UTX-1::GFP* carries a multi-copy extrachromosomal array of the UTX-1::GFP while *scUTX-1::GFP* contains only a single copy. In both cases, *utx-1* was observed to be expressed ubiquitously in the worm (Figure 3.4A for *scUTX-1::GFP*; data not shown for *utx-1(tm3118)+UTX-1::GFP*). I did, however, see a difference in germline expression of UTX-1, where GFP was observed in *scUTX-1::GFP* (Figure 3.4B) but not in *utx-1(tm3118)+UTX-1::GFP* (Figure 3.4C). This is most likely due to transgene silencing triggered by introduction of high-copy-number extrachromosomal arrays in *utx-1(tm3118)*+UTX-1::GFP animals (Kelly, Xu, Montgomery, & Fire, 1997).

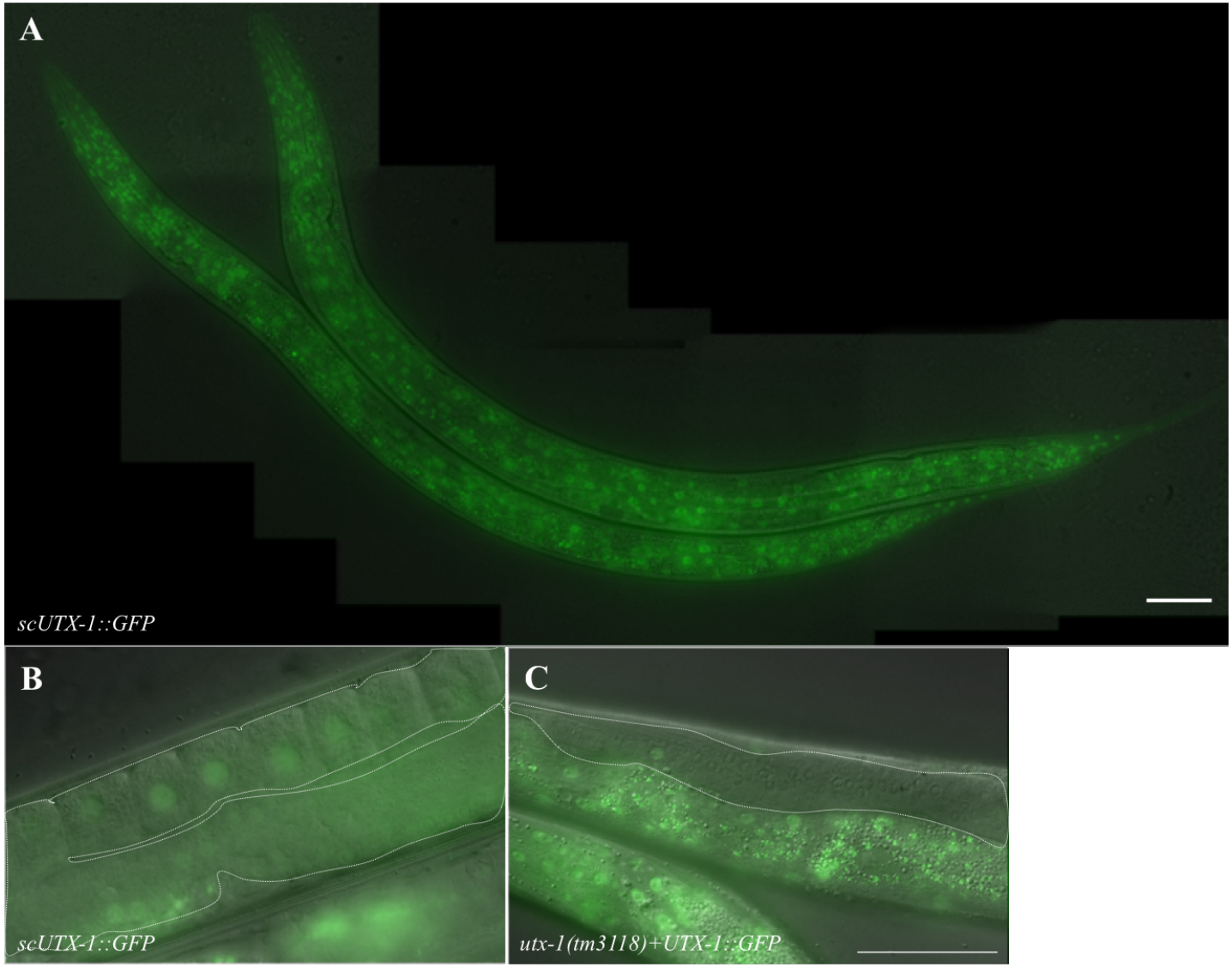
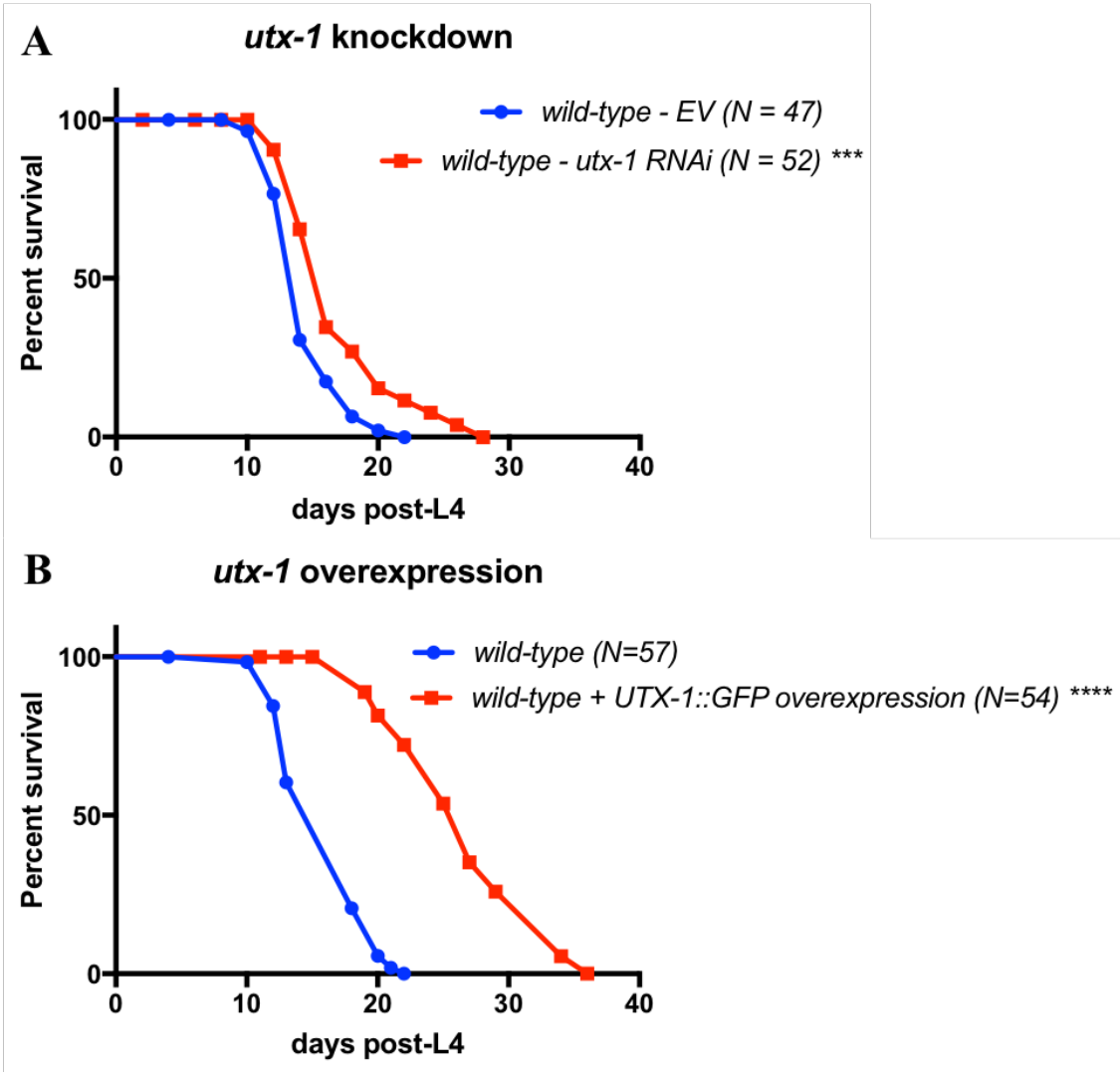


Figure 3.4: Spatial expression of utx-1. *Epifluorescence images of worms carrying either a single-* ***(A-B)*** *or multi-copy* ***(C)*** *array of utx-1p::UTX-1::GFP construct.* ***(A)*** *UTX-1::GFP was observed to be ubiquitously expressed in the nucleus of all cells in the worm;* ***(B)*** *GFP expression observed in the germline of worms expressing a single copy of UTX-1::GFP* ***(C)*** *No GFP was observed in the germline of worms transgenically overexpressing UTX-1::GFP.* White lines highlight the gonad syncytium imaged for these observations. *Scale bar = 50 µm*

My experiments consistently show that *utx-1* is expressed ubiquitously in the worm and is exclusively observed to be localised in cell nuclei. The results are consistent with published observations on the spatial expression of *utx-1* in *C. elegans* (Julien Vandamme, 2013; Seelk et al., 2016).

3.3.2. Knockdown and overexpression of *utx-1* extends worm lifespan

Knockdown of *utx-1* has been previously reported to promote longevity in *C. elegans* (C. Jin et al., 2011a; Maures et al., 2011). However, data from the Woollard lab has shown that overexpression of *utx-1* also extends lifespan (Chocian, 2015 manuscript submitted). To determine if lifespan extension is observed by overexpressing or knocking down *utx-1* by RNAi in my hands, I performed lifespan analysis on worms transgenically overexpressing *utx-1* compared to wild-type controls (Figure 3.5B), as well as animals subjected to *utx-1* RNAi compared to empty vector controls (Figure 3.5A).



Strain	RNAi Condition	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs control)
N2	EV	14.5	14	22	p = 0.0003 (***)
N2	*utx-1* RNAi	17.1	16	28	
N2	EV	16.2	15	22	p < 0.0001 (****)
N2 + UTX-1::GFP	EV	26.9	27	36	

Figure 3.5: Survival analysis showed lifespan extension upon knockdown or overexpression of utx-1. *L4-stage animals were subjected to either empty vector (EV) or utx-1 RNAi and survival was monitored at least every other day.* ***(A)*** *Lifespan analysis comparing empty vector vs utx-1 RNAi;* ***(B)*** *lifespan analysis comparing wild-type (N2) vs wild-type animals overexpressing UTX-1::GFP; qRT-PCR data confirming that utx-1 is overexpressed in the wild-type+UTX-1::GFP strain is shown in Figure 3.1. All lifespan assays were performed using HT115 bacteria as food source to ensure that results are not confounded by changing the worm diet in RNAi experiments. The accompanying table shows mean lifespans, median lifespans, maximum lifespans and p-values calculated using Mantel-Cox method.*

My results are consistent with previous assertions that *utx-1* knockdown and overexpression promotes longevity. It is interesting to note that lifespan extension due to *utx-1* overexpression is a lot more dramatic than that of *utx-1* knockdown (see maximum and median lifespans). The shape of the curve for the *utx-1* overexpressing animals are also shifted further to the right (or somewhat more "square"), suggesting that worms are dying at a much later stage compared to empty vector controls or even the animals feeding on *utx-1* RNAi. This suggests that lifespan extension due to *utx-1* up- or down-regulation might be mediated by different mechanisms. To this end, I hypothesised that parallel lifespan-extending mechanisms might arise from contributions of *utx-1* to different tissues in the worm. From this, I proceeded to come up with strategies to adjust *utx-1* levels in a tissue-specific manner and investigate how it affects lifespan.

3.3.3. Strategy for tissue-specific knockdown of *utx-1*

I first wanted to address how tissue-specific knockdown of *utx-1* affects worm lifespan by utilising RNAi-insensitive mutants. Previous reports suggest that loss-of-function mutations in genes involved in the RNA interference pathway (Figure 3.6), such as *rde-1* and *sid-1*, lead to RNAi insensitivity (Tabara et al., 1999; Winston, Molodowitch, & Hunter, 2002).

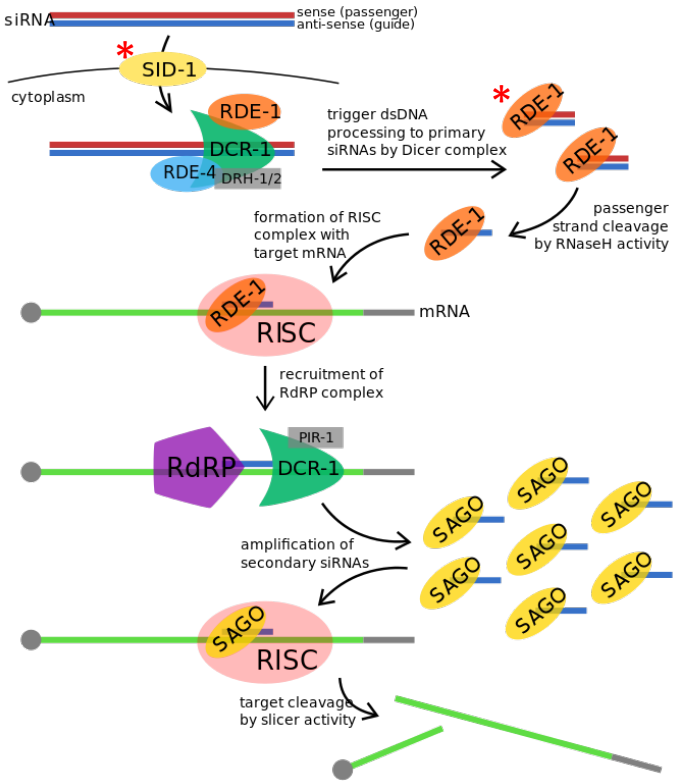


Figure 3.6: Components involved in the RNA interference pathway. *Exogenous dsRNA enters the cytoplasm through the dsRNA channel SID-1 and is processed into primary siRNA by the DICER complex, which includes piwi/argonaute protein RDE-1. RDE-1 bound to the primary siRNA facilitates formation of RNA-induced silencing complex (RISC) and recruits the RNA-dependent RNA polymerase (RdRP) to generate secondary siRNAs, inducing an amplification response. Secondary siRNAs bind to degenerate argonaute proteins, which are directly involved in transcript degradation (Tabara, Yigit, Siomi, & Mello, 2002). Figure above was taken from www.commons.wikimedia.org ("Proposed RNA interference pathway for exogenous trigger dsRNA in C. elegans").*

These mutants can be manipulated further via transgenic rescue of the gene driven by tissue-specific promoters to rescue the RNAi-insensitivity phenotype selectively in particular worm tissue (Calixto, Chelur, Topalidou, Chen, & Chalfie, 2010; Qadota et al., 2007). Using these mutants should therefore allow me to deplete *utx-1* levels of the worms in a tissue-specific manner and study lifespan changes.

3.3.3.1. *rde-1* and *sid-1* mutants are insensitive to RNAi

I first wanted to confirm whether loss-of-function alleles of *rde-1* and *sid-1*, *rde-1(ne219)* and *sid-1(pk3321)* respectively, are reliably insensitive to RNAi. To do this, I used *pop-*

1 RNAi as my test gene target. *pop-1* encodes for a member of the TCF/LEF family of transcription factors and is a downstream component of the Wnt signalling pathway, where it has important functions in cell fate determination of worm embryos (R. Lin, Hill, & Priess, 1998; R. Lin, Thompson, & Priess, 1995). Depletion of *pop-1* gives an obvious phenotype where eggs laid by the mothers fed *pop-1* dsRNA fail to hatch. I fed wild-type, *rde-1(ne219)* and *sid-1(pk3321)* L4-stage worms with bacteria expressing dsRNA that specifically targets *pop-1*, let them lay eggs for 24-hours and counted viable progeny 24 hours after the egg-laying period (Figure 3.7A).

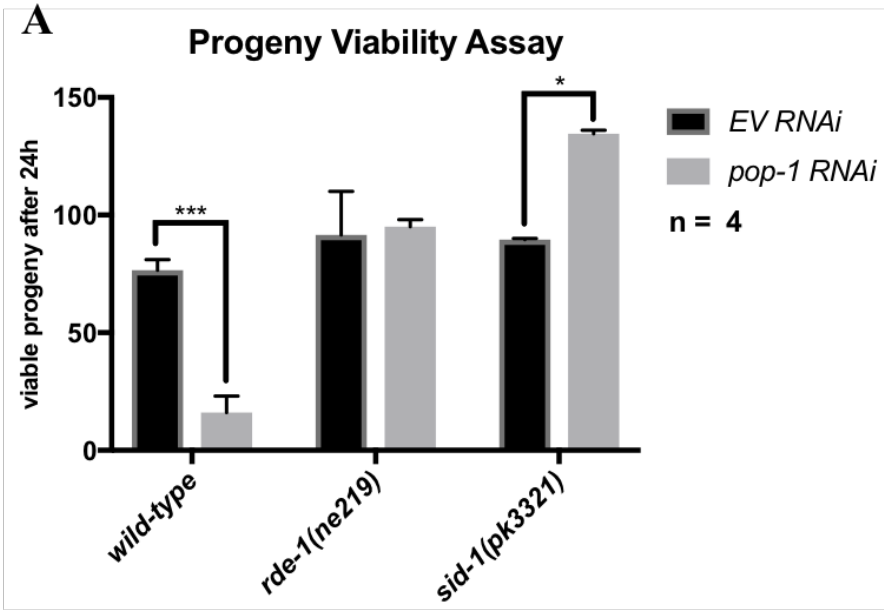
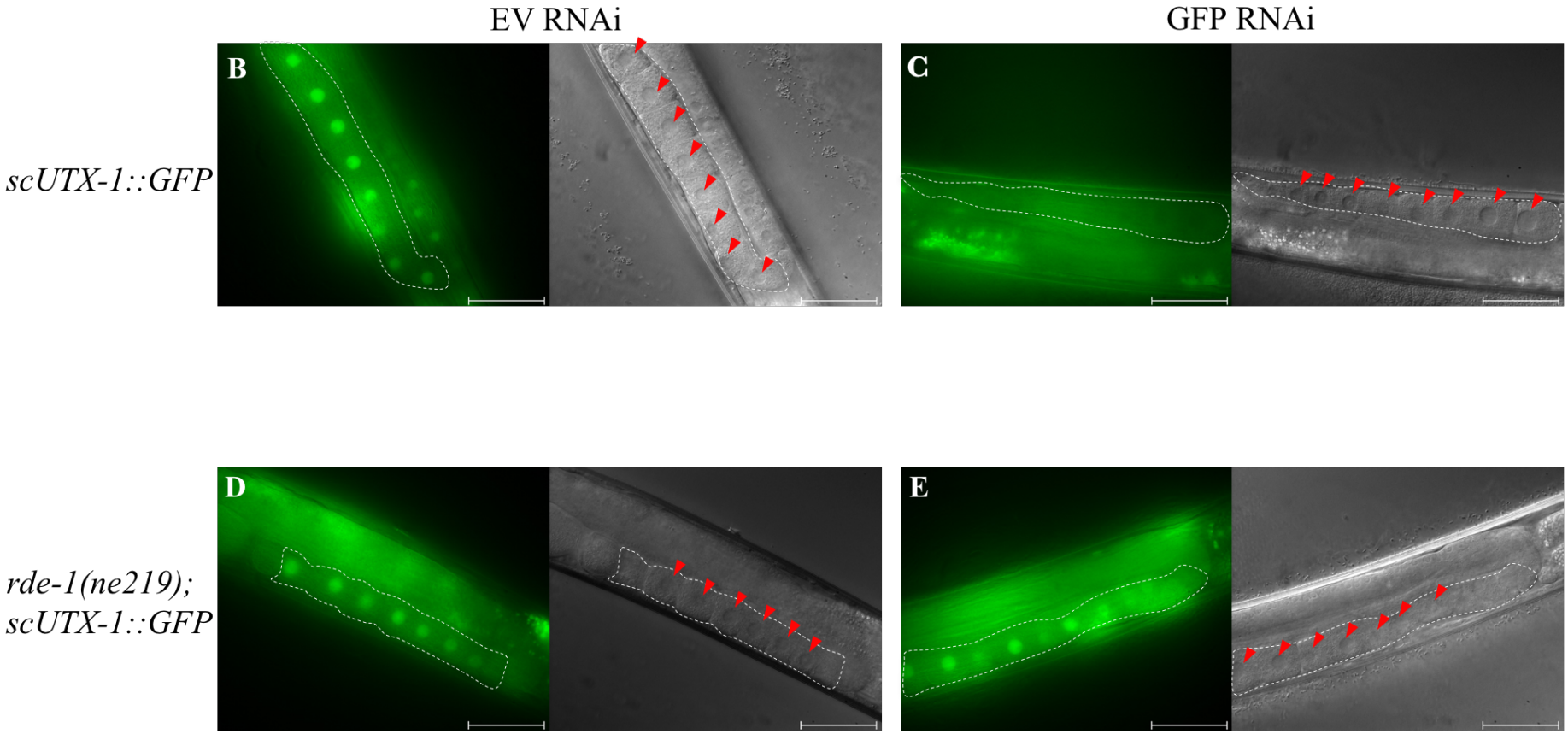


Figure 3.7: RNAi sensitivity of rde-1 and sid-1 mutants. *L4-stage animals were fed HT115 bacteria carrying either EV or pop-1 dsRNA. Worms were allowed to lay eggs for 24 hours before removing the mothers from the plate. Hatched progeny was quantified 24 hours later.* ***(A)*** *Wild-type worms had a significantly lower number of viable progeny when subjected to pop-1 RNAi compared to EV controls (p = 0.007), while viable progeny numbers of rde-1 and sid-1 animals were largely unaffected by pop-1 depletion (p = 0.02 for sid-1). Statistical significance was calculated using t-tests (to be continued)*



(cont.) L4-stage animals of each genotype were subjected to either EV or GFP RNAi and visualised using an epifluorescence microscope after 5 days. ***(B-E)*** *Representative images of worms subjected to EV and GFP RNAi; n = 15 animals were analysed per genotype. White lines show the outline of the gonad arms imaged and red arrows indicate the oocyte pronucleus;* ***(B)*** *&* ***(C)*** *EV vs GFP RNAi of scUTX-1::GFP worms;* ***(D)*** *and* ***(E)*** *are EV vs GFP RNAi of rde-1(ne219); scUTX-1::GFP worms. Scale bar = 50 µm*

Knockdown of *pop-1* in wild-type worms significantly decreased the number of viable progeny compared to empty vector controls, while knockdown of *pop-1* in *rde-1* mutants had no significant effect on progeny viability compared to controls. Interestingly, *pop-1* RNAi in *sid-1* mutants mildly increased the number of viable progeny compared to that empty vector controls. This could be due to a synthetic interaction between the *sid-1* mutation and hypomorphic levels of *pop-1* that caused an increase in the rate of egg-laying (or even total number of progeny) but this is something that I never tested. Furthermore, I also utilised the *scUTX-1::GFP* reporter strain by crossing this into the *rde-1(n219)* background and subsequently looked at how GFP levels were affected after GFP RNAi (called "*rde-1(ne219); scUTX-1::GFP*"). I compared GFP levels of the oocyte pronucleus because this was the brightest level of GFP I can observe under normal conditions, due to the large size of the nucleus. In scUTX-1::GFP animals, GFP in the oocytes disappeared upon GFP RNAi compared to empty vector controls (Figure 3.7B and C). However, in the *rde-1(ne219)* mutant background, GFP in the oocytes did not disappear after GFP RNAi, suggesting that the *rde-1(ne219)* genotype causes the animals to be insensitive to RNAi (Figure 3.7D and E). Collectively, my results corroborate the observation that *rde-1* and *sid-1* mutants causes RNAi insensitivity.

3.3.3.2. Tissue-specific rescue of *rde-1* causes tissue-specific rescue of RNAi insensitivity

rde-1 and *sid-1* mutants rescued in a tissue-specific manner have already been previously developed by other research groups as a strategy for tissue-specific knockdown studies (Calixto et al., 2010; Firnhaber & Hammarlund, 2013; McGhee et al., 2009; Qadota et al., 2007). I selected the following tissue-specific RNAi strains to use for my lifespan analysis (Table 3.1):

Table 3.1: Tissue-specific RNAi strains used for tissue-specific knockdown of utx-1.

Strain	Genotype	Integrated array	Tissue target	Reference
VP303	*rde-1(ne219) V; kbIs7*	*kbIs7 = [nhx-2p::rde-1 + rol-6(su1006)]*	intestine	
NR222	*rde-1(ne219) V; kzIs9*	*kzIs9 = [(pKK1260) lin-26p::NLS::GFP + (pKK1253) lin-26p::rde-1 + rol-6(su1006)]*	hypodermis	(Qadota et al., 2007)
NR350	*rde-1(ne219) V; kzIs20*	*kzIs20 = [hlh-1p::rde-1 + sur-5p::NLS::GFP]*	muscle	
TU3401	*sid-1(pk3321) V; uIs69*	*uIs69 = [pCFJ90 (myo-2p::mCherry) + unc-119p::sid-1]*	neuron	(Calixto et al., 2010)

I wanted conclusive evidence that *rde-1(+)* expression in a specific tissue is sufficient to rescue RNAi sensitivity only at that site. To test this, I selected the VP303 strain (intestine-specific) as a model system for tissue-specific RNAi and genetically crossed this into the scUTX-1::GFP reporter strain. I hypothesised that if *utx-1* is ubiquitously expressed and I perform *utx-1* RNAi on these animals, then GFP should only be reduced specifically in the intestine, as this is the only tissue in which *rde-1*-mediated RNAi insensitivity would be rescued. L4-stage animals were fed bacteria expressing *utx-1* dsRNA and imaged 5 days after. In scUTX-1::GFP worms, GFP in the intestinal nuclei as well as in other cells disappeared under *utx-1 RNAi* compared to empty vector controls (Figure 3.8A and B). Unsurprisingly, GFP in the intestinal nuclei did not disappear when *utx-1* RNAi was fed to *rde-1(ne219)*; scUTX-1::GFP animals with respect to controls (Figure 3.8C and D). Where *rde-1* was selectively rescued in the intestine by transgenic expression of *rde-1* driven by *nhx-2* promoter, however, GFP disappeared only in the intestine but was retained in other tissues such as the muscle and neurons as highlighted below (Figure 3.8E and F).

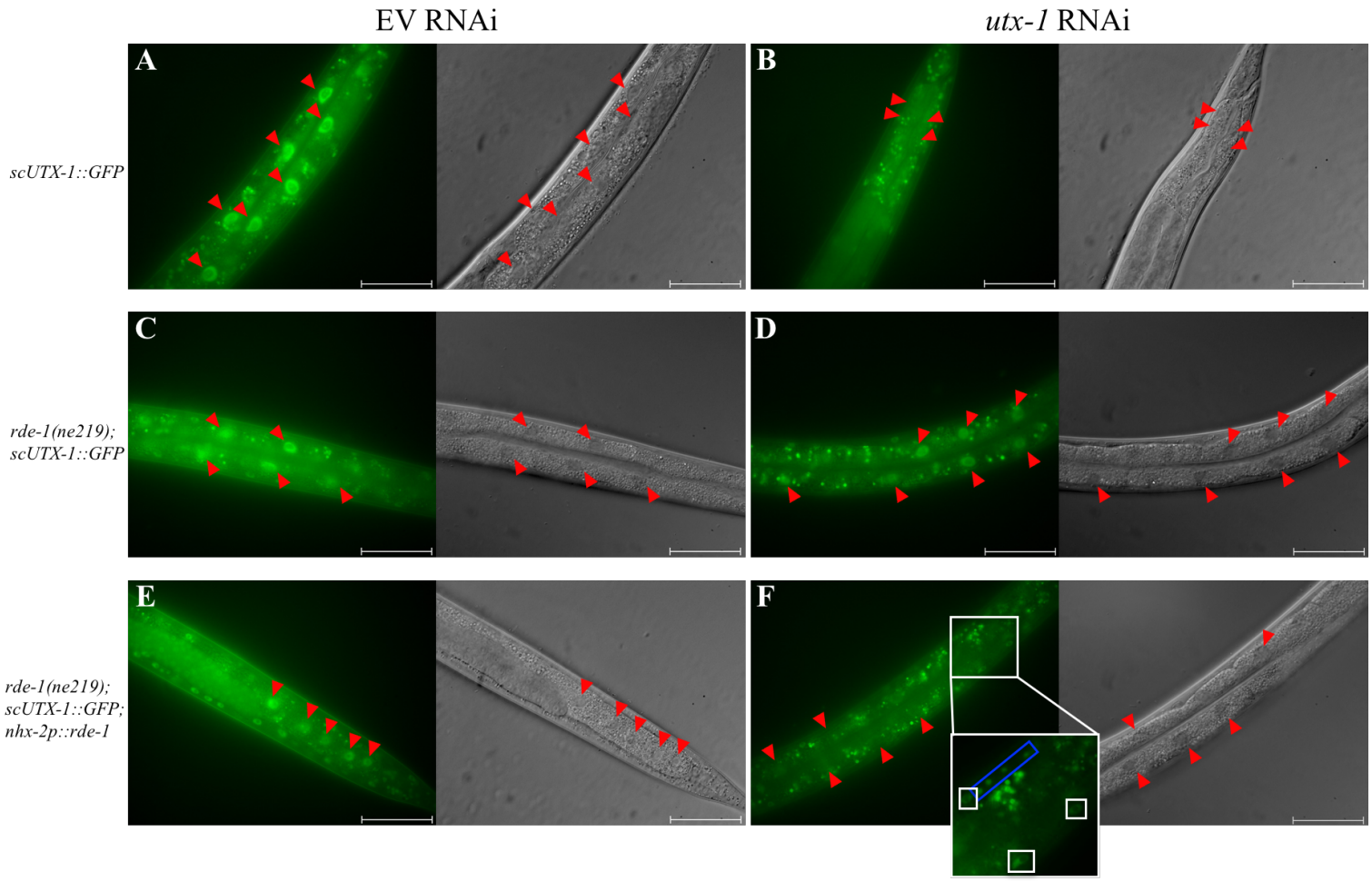


Figure 3.8: Validation of the intestine-specific RNAi strain. *L4-stage animals of each genotype were subjected to either EV or utx-1 RNAi and imaged using epifluorescence microscope 5 days later. Representative images of* ***(A)*** *&* ***(B)*** *EV vs utx-1 RNAi of scUTX-1::GFP in wild-type background;* ***(C)*** *&* ***(D)*** *EV vs utx-1 RNAi of scUTX-1::GFP in rde-1(ne219) background;* ***(E)*** *&* ***(F)*** *EV vs utx-1 RNAi of scUTX-1::GFP in rde-1(ne219) background rescued with rde-1 in the intestine. N = 10 animals imaged per condition; red arrows highlight intestinal nuclei in the worms; white boxes highlight muscle nuclei and the blue box indicates neuronal nuclei. Scale Bar =* 50 µM; *50 µM; (to be continued)*

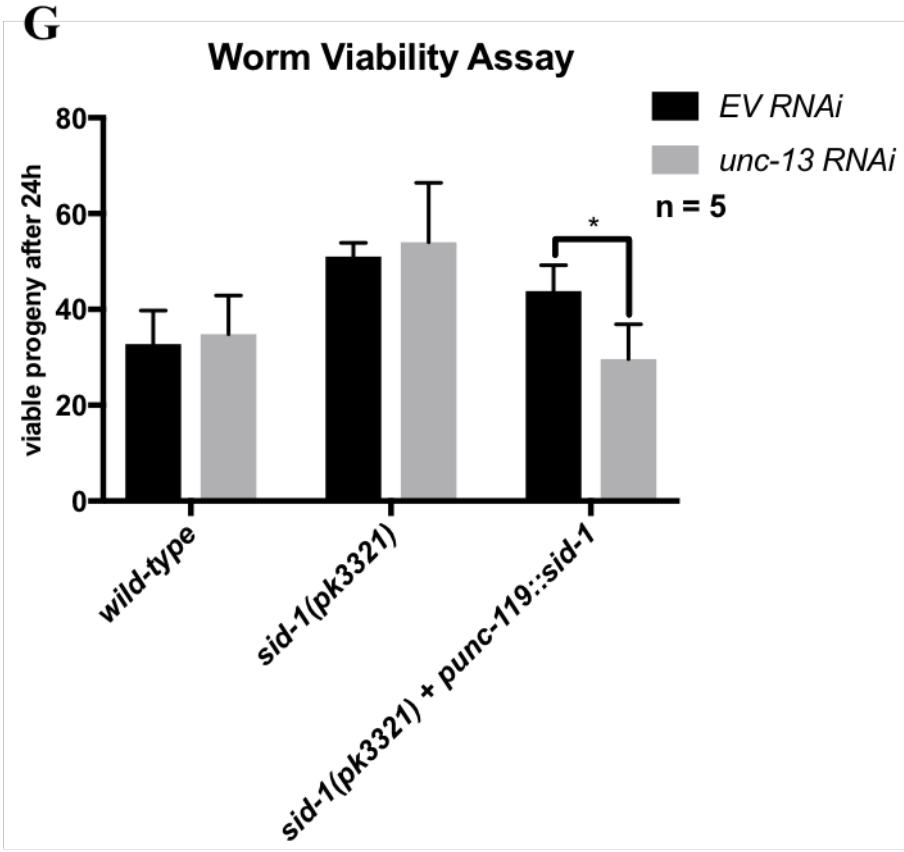


Figure 3.8 (continued): *L4-stage animals were fed HT115 bacteria carrying either EV or unc-13 RNAi. Worms were allowed to lay eggs for 24 hours before removing the mothers from the plate. Hatched progeny was quantified 24 hours later.* ***(G)*** *Viable progeny of wild-type, sid-1(pk3321) and sid-1(pk3321)+unc-119p::sid-1 animals under EV vs unc-13 RNAi conditions.*

Additionally, I also examined whether transgenic expression of *sid-1* driven by pan-neuronal *unc-119* promoter in the *sid-1(pk3321)* mutant background animals (strain TU3401) sensitised neurons to RNAi knockdown. I investigated this by knocking down *unc-13* – a neuron-specific gene that encodes for at least 5 protein isoforms involved in the regulation of neurotransmission in worms, with null mutations causing lethality. I attempted to recapitulate the observation that RNAi of *unc-13* causes a moderate lethality phenotype in *sid-1(pk3321); unc-119p::sid-1* animals (Calixto et al., 2010) (Figure 3.6G). In wild-type worms, I found no significant difference in the number of viable progeny between empty vector and *unc-13* RNAi. This is presumably due to the commonly accepted observation that neurons tend to be insensitive to RNAi techniques

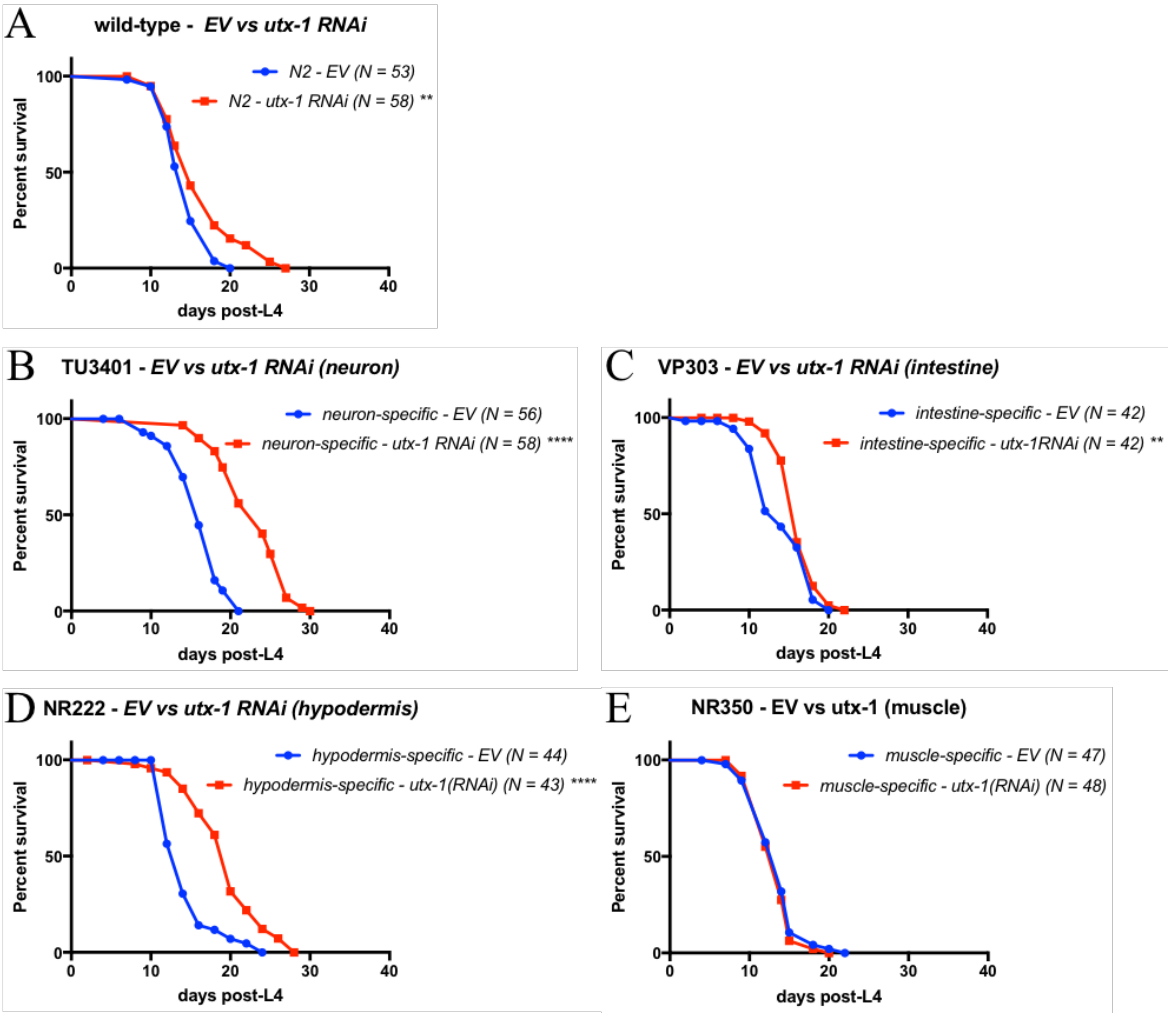
(Kamath, Martinez-Campos, Zipperlen, Fraser, & Ahringer, 2001; Timmons, Court, & Fire, 2001). *sid-1* expression is absent in most neuronal cells, leading to a refractory response to systemic dsRNA. I've previously shown that *sid-1(pk3321)* mutants are insensitive to RNAi, consistent with my data that there is no significant difference in progeny viability between EV and *unc-13* RNAi under these conditions. However, the neuronal-hypersensitive RNAi strain TU3401 showed a modest decrease in progeny upon knockdown of *unc-13* RNAi compared to empty vector controls, suggesting that neuronal-specific expression of *sid-1* sensitises worm neurons to RNAi.

I also attempted to test the tissue-specific RNAi sensitivity of the muscle-specific (strain NR350) and hypodermal-specific (NR222) RNAi strain, but was hampered by a few issues. For the muscle-specific RNAi animals, the co-injection marker used with the *hlh-1p::rde* construct was *sur-5p::NLS::GFP* to create the strain. *sur-5p::NLS::GFP* is expressed in many cells including the body wall muscle and hypodermal cells, and GFP expression is characteristically bright when observed under an epifluorescence microscope. I performed GFP RNAi on these animals, with the idea that if muscle-specific RNAi worked, GFP will disappear in the body wall muscle, but not in the hypodermal cells (data not shown). However, because *sur-5::NLS::GFP* was very bright, it was challenging to see changes in GFP expression specifically in the muscles and was therefore not pursued further. For the hypodermal-specific RNAi strain, I attempted to validate findings by Qadota *et al.*, whereby hypodermal-specific knockdown of *lin-26* caused progeny lethality in the hypodermal-specific RNAi strain and wild-type animals, but not in the *rde-1(ne219)* mutant animals (Qadota et al., 2007). Contamination in the *lin-26* RNAi glycerol stock prevented me from getting any meaningful results from the RNAi experiment and I had to abandon this in the interest of time.

Nevertheless, my findings that *utx-1* RNAi in the *rde-1(ne219); scUTX-1::GFP; nhx-2p::rde-1* genetic background depleted GFP specifically in the intestine (Figure 3.8E and F) and that *unc-13* RNAi gave a lethality phenotype only in the neuron-sensitised RNAi strain (Figure 3.8G) was sufficient proof-of-concept data that tissue-specific knockdown works. This, together with previously published results, gave me confidence that the tissue-specific knockdown strategy is a viable approach to use for exploring the effect of tissue-specific knockdown of *utx-1* on lifespan regulation.

3.3.4. Effects of tissue-specific knockdown of *utx-1* on lifespan

The lifespan extension of worms whether I knockdown or overexpress *utx-1* coupled with the ubiquitous expression of *utx-1* prompted me to investigate whether tissue-specific perturbation of *utx-1* levels had different effects in lifespan regulation. To address this, I knocked down *utx-1* in tissue-specific RNAi strains (see Table 3.1) and chronicled animal survival. Worms were subjected to RNAi from L4-stage to avoid developmental perturbations associated with *utx-1* and were exposed to this for the entire duration of their life (Figure 3.9).



Strain (target)	RNAi Condition	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs control)
N2 (systemic)	EV	14.4	15	20	p = 0.0058 (**)
N2 (systemic)	*utx-1* RNAi	16.4	15	27	
TU3401 (neuron)	EV	16.1	16	21	p < 0.0001 (****)
TU3401 (neuron)	*utx-1* RNAi	22.8	24	30	
VP303 (intestine)	EV	13.7	14	24	p = 0.0079 (**)
VP303 (intestine)	*utx-1* RNAi	16.2	16	24	
NR222 (hypodermis)	EV	14.4	14	24	p < 0.0001 (****)
NR222 (hypodermis)	*utx-1* RNAi	19.3	20	28	
NR350 (muscle)	EV	13.6	14	22	p = 0.5386 (ns)
NR350 (muscle)	*utx-1* RNAi	13.3	14	20	

Figure 3.9: Survival upon tissue-specific knockdown of utx-1. *For each strain, at least 60 L4-stage animals were subjected to either EV or utx-1 RNAi. Animals were scored as alive or dead at least every 2 days. Survival was compared between EV and utx-1 RNAi for each strain assessed.* ***(A)*** *Systemic knockdown of utx-1 vs empty vector control. Lifespan results upon tissue-specific knockdown of utx-1 in* ***(B)*** *neurons;* ***(C)*** *intestine;* ***(D)*** *hypodermis and* ***(E)*** *muscle. Accompanying table shows mean lifespans, median lifespans, maximum lifespans and p-values (vs requisite controls) calculated using Mantel-Cox method.*

Systemic knockdown of *utx-1* extends lifespan, which supports published lifespan data relating to *utx-1* depletion (Figure 3.9A) (C. Jin et al., 2011a; Maures et al., 2011), as well as my previous data. Tissue-specific knockdown of *utx-1*, which to my knowledge has never been done before, showed that neuronal-, intestinal-, and hypodermal-specific knockdown of *utx-1* promotes longevity compared to empty vector controls (Figure 3.9B-D). In contrast, muscle-specific depletion of *utx-1* has no effect on lifespan compared to empty vector (Figure 3.9E). Collectively, this suggests that the observed lifespan extension in worms upon systemic depletion of *utx-1* is most likely a net effect of spatial-dependent reduction of *utx-1*. My data suggests that the neurons and the hypodermis are the most important sites of action for increased lifespan due to *utx-1* knockdown, while the muscle is not. The intestine may also be involved in lifespan extension due to *utx-1* depletion, but to a lesser extent. It is interesting to note that lifespan extension due to neuronal- and hypodermal-specific knockdown of *utx-1* is more dramatic than that of systemic knockdown of *utx-1* (see median and maximum lifespans). It could indicate that perhaps knockdown of *utx-1* in (yet unknown) tissues or specific cells could be detrimental to worm lifespan. In addition to this, I also sought to investigate how tissue-specific overexpression of *utx-1* affects lifespan and assess whether there is a meaningful correlation with my knockdown results.

3.3.5. Effects of tissue-specific overexpression of *utx-1* on lifespan

Worms transgenically overexpressing *utx-1* in a tissue-specific manner have been kindly gifted to the lab by our collaborator, Dr Lisa Salcini (University of Copenhagen, Denmark). Mirroring tissues that I targeted for *utx-1* knockdown studies, UTX-1::GFP constructs were overexpressed using promoters that specifically act on muscle, intestine, neurons and hypodermis (Figure 3.10A). I validated my tissue-specific *utx-1* overexpression strains through epifluorescence imaging and observed that spatial

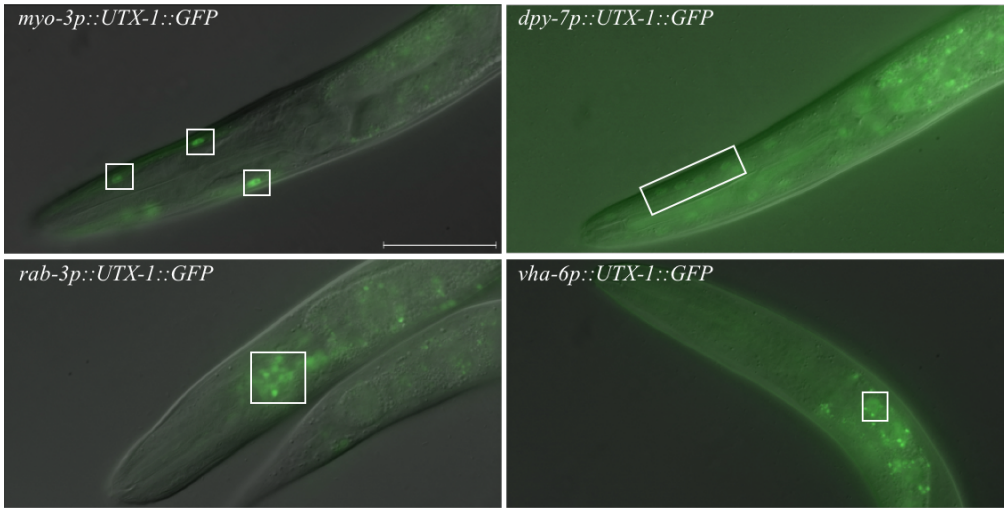
expression is consistent with where the chosen promoters are supposed to act (Figure 3.10B)

A

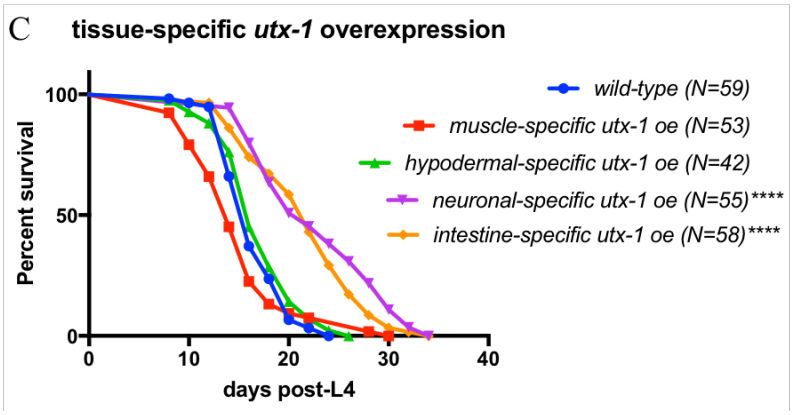


Strain	Promoter	Expression target
ZR694	*myo-3p*	muscle
ZR695	*dpy-7p*	hypodermis
ZR698	*rab-3p*	neuron
ZR699	*vha-6p*	intestine

B



C



Strain	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs N2)
N2	16.5	16	24	
ZR694 (muscle)	15.1	14	30	p = 0.1374
ZR695 (hypodermis)	17.1	16	26	p = 0.2704
ZR698 (neuron)	22.8	22	34	p < 0.0001 (****)
ZR699 (intestine)	21.7	22	34	p < 0.0001 (****)

Figure 3.10: Spatial expression profiling and lifespan analyses of utx-1 overexpression strains. *Wild-type animals were injected with UTX-1 constructs driven by tissue-specific promoters in order to achieve tissue-specific expression of utx-1;* ***(A)*** *List of tissue-specific promoters used for tissue-specific utx-1 overexpression;* ***(B)*** *Epifluorescence images showing the spatial expression of tissue-specific utx-1 overexpressing strains (Scale Bar = 50 μM);* ***(C)*** *Lifespan analyses of tissue-specific utx-1 overexpressing strains. At least 60 L4-stage animals were picked for each genotype and assessed for lifespan analysis. Animals were scored as alive or dead at least every 2 days. Survival of utx-1 overexpressing animals were compared against wild-type. Accompanying table shows mean lifespans, median lifespan, maximum lifespan and p-values calculated using Mantel-Cox method.*

Lifespan evaluation of these *utx-1* overexpressing strains were performed by Dr Karolina Chocian, a colleague in the Woollard research group (Figure 3.10C). Findings from this data suggests that overexpression of *utx-1* is beneficial to lifespan when expressed specifically in the neurons and the intestine, while no difference in longevity was observed when *utx-1* was overexpressed in the muscle and hypodermis compared to wild-type controls.

3.3.6. Summary of lifespan due to knockdown and overexpression of *utx-1*

A summary of the lifespan outcomes due to tissue-specific knockdown and overexpression of *utx-1* are summarised in the table below (Table 3.2):

Table 3.2: Summary of lifespan results after altering tissue-specific levels of utx-1.

Tissue target	**Knockdown of *utx-1***	**Overexpression of *utx-1***
	Lifespan change compared to requisite control	
Systemic	up	up
Neurons	up	up
Intestine	up	up
Hypodermis	up	no change
Muscle	no change	no change

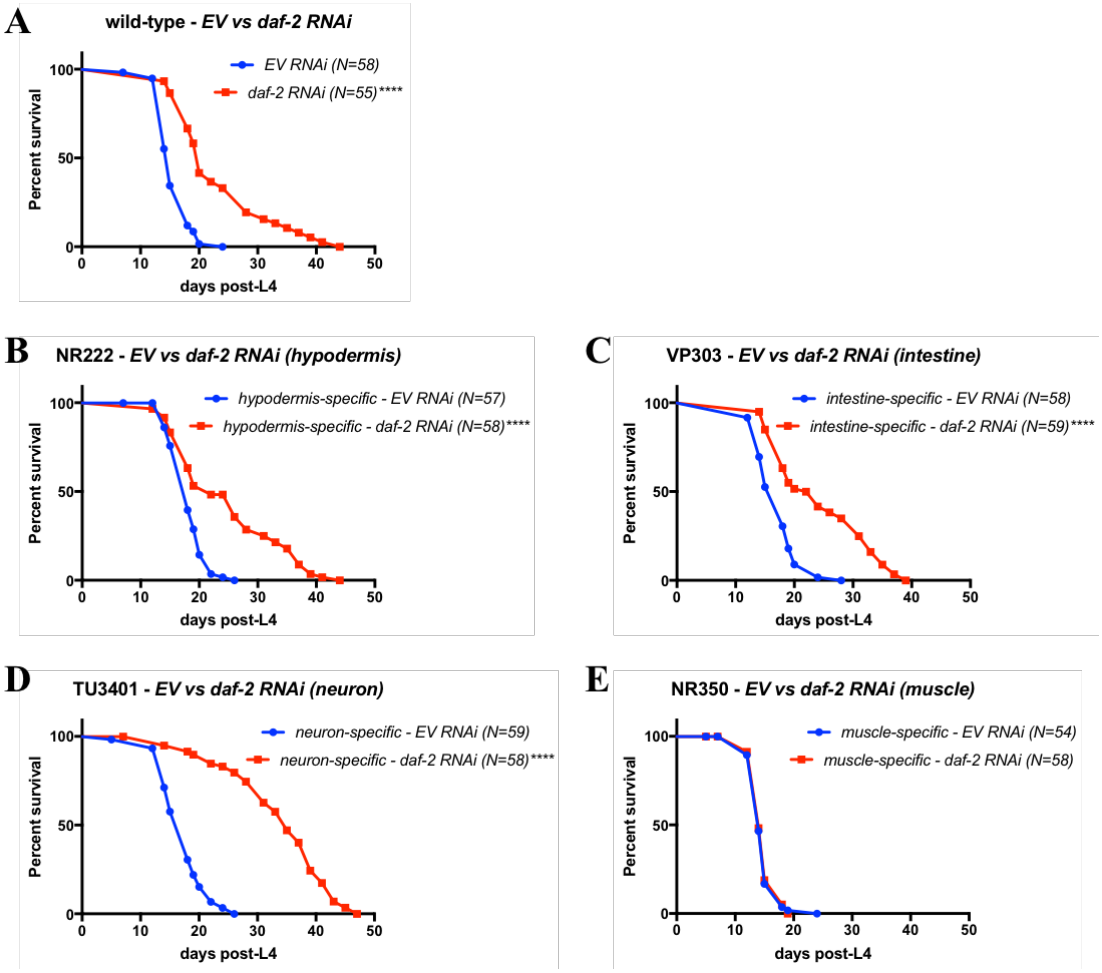
Looking at the lifespan data, I discovered that the hypodermis-specific knockdown of *utx-1* extends worm lifespan, while hypodermis-specific overexpression does not. This presents a unique site of action in which *utx-1* knockdown promotes lifespan extension. In contrast to this, both up- and down-regulation of *utx-1* in the intestine and neurons are beneficial to longevity, mirroring the lifespan outcomes when *utx-1* is knocked down or overexpressed systemically. In cases where lifespan extension was observed, I wanted to investigate whether the pro-longevity benefits were dependent on known longevity pathways. Lifespan extension due to *utx-1* depletion was reportedly due to inhibition of the insulin signalling cascade. In *C. elegans*, both *utx-1* and *daf-2* increase with age and evidence suggests that UTX-1 removes transcriptionally repressive H3K27me3 marks

from the *daf-2* locus, leading to increased transcription of *daf-2* (and increased insulin signalling activity) (C. Jin et al., 2011a). Therefore, the hypothesis is that depletion of *utx-1* retains H3K27me3 on the *daf-2* gene and inhibits transcription, thereby inhibiting the insulin signalling pathway and promoting lifespan extension. Tissue-specific rescue experiments showed that *daf-2* mediates lifespan extension in the neurons and to a lesser extent in the intestine (Wolkow et al., 2000). Furthermore, insulin signalling mediates lifespan through the downstream effector DAF-16 (FoxO transcription factor), which is reportedly necessary in the intestine for lifespan extension to occur (see section 3.1.6) (Libina et al., 2003). This highlights that the neurons and the intestine are key sites of action for lifespan extension due to downregulated insulin signalling. I have now shown that the neurons, hypodermis, and intestine are crucial sites of action for mediating lifespan extension due to *utx-1* depletion. Given that lifespan extension due to knockdown of *utx-1* is dependent on insulin signalling, I wanted to validate whether the increase in lifespan due to neuronal-, hypodermal- and intestinal- knockdown of *utx-1* is indeed dependent on components of the insulin signalling cascade.

3.3.7. Lifespan due to tissue-specific knockdown of *daf-2*

Tissue-specific knockdown of *utx-1* showed that neuron-, intestine-, and hypodermis-specific knockdown of *utx-1* is beneficial to lifespan. Maures *et al.* (2011) previously showed that universal knockdown of *utx-1* extends lifespan in an IIS-dependent manner (Maures et al., 2011), with *daf-2* transcription downregulated by retention of H3K27me3 marks due to reduction of demethylase UTX-1. I postulated that if lifespan extension due to *utx-1* depletion is directly linked to transcriptional downregulation of *daf-2*, then lifespan subjected to tissue-specific *daf-2* RNAi should reflect lifespan results I observed when *utx-1* was knocked down in a tissue-specific manner. I performed tissue-specific *daf-2* knockdown and lifespan analysis in the same way that I did for *utx-1* lifespan

experiments previously – exposing worms at L4 stage to the bacteria expressing the dsRNA of interest and maintaining RNAi throughout their lifetime (Figure 3.11).



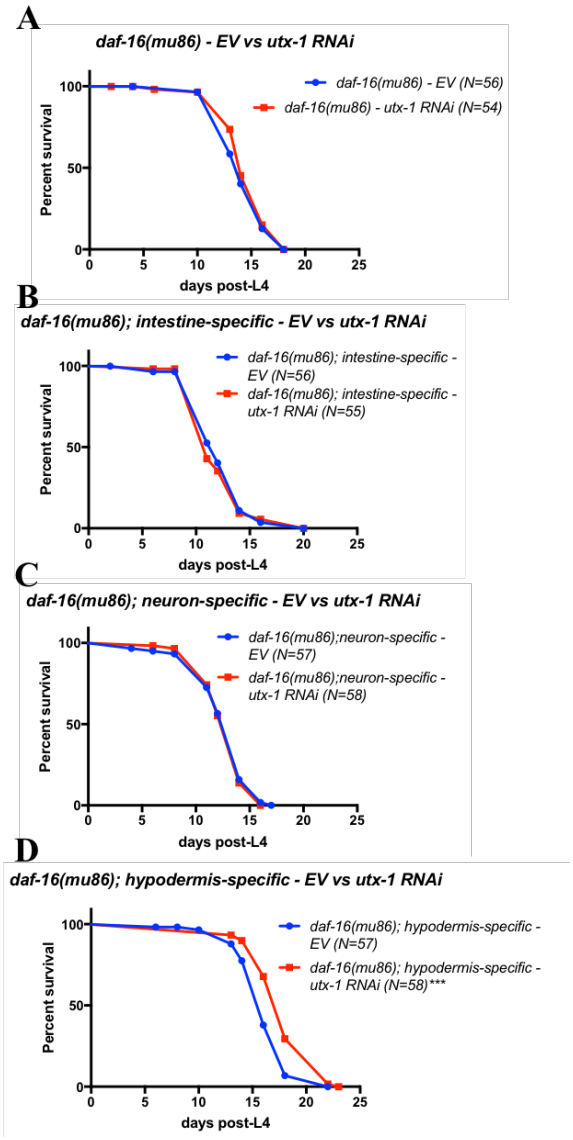
Strain	RNAi Condition	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs EV control)
N2	EV	15.7	15	24	$p < 0.0001$ (****)
	daf-2 RNAi	22.3	20	44	
NR222 (hypodermis)	EV	18.2	18	26	$p < 0.0001$ (****)
	daf-2 RNAi	24.4	22	44	
VP303 (intestine)	EV	16.9	18	28	$p < 0.0001$ (****)
	daf-2 RNAi	24.3	23	39	
TU3401 (neuron)	EV	17.2	18	26	$p < 0.0001$ (****)
	daf-2 RNAi	33.7	35	47	
NR350 (muscle)	EV	14.9	14	24	$p = 0.8217$ (ns)
	daf-2 RNAi	14.9	14	19	

Figure 3.11: Survival under tissue-specific knockdown on daf-2. *For each strain, at least 60 L4-stage animals were subjected to either EV or daf-2 RNAi. Animals were scored as alive or dead at least every 3 days. Survival was compared between EV and daf-2 RNAi for each strain assessed.* ***(A)*** *Systemic knockdown of daf-2; Tissue-specific knockdown of daf-2 in* ***(B)*** *hypodermis,* ***(C)*** *intestine,* ***(D)*** *neurons and* ***(E)*** *muscle. Mean lifespans, median lifespan, maximum lifespan and p-values are given in the accompanying table. Statistical significance was calculated using Mantel-Cox method. Lifespan experiment was only performed once.*

Systemic knockdown of *daf-2* significantly increased lifespan compared to empty vector controls as expected (Figure 3.11A). I also found that neuron-, intestine- and hypodermis-specific knockdown of *daf-2* promotes lifespan extension, absolutely mirroring the lifespan outcomes I observed when *utx-1* was knocked down in a tissue-specific manner, most likely because of *daf-2* downregulation at the tissue site of action (Figure 3.11B-D). Muscle-specific knockdown of *daf-2* does not affect lifespan, agreeing with previous reports that muscles are not crucial in mediating *daf-2*-dependent lifespan extension (Wolkow et al., 2000). Collectively, my lifespan data relating to tissue-specific depletion of *daf-2* is consistent with Maures *et al.*'s findings that lifespan extension due to *utx-1* depletion is dependent on insulin signalling (Maures et al., 2011).

3.3.8. *daf-16* dependence of lifespan on tissue-specific knockdown of *utx-1*

Pro-longevity phenotypes due to depletion of *daf-2* requires the downstream target, *daf-16* (FOXO transcription factor), to regulate lifespan (Dorman, Albinder, Shroyer, & Kenyon, 1995; Kenyon et al., 1993; K. Lin et al., 1997). I wanted to investigate whether lifespan extension due to tissue-specific knockdown of *utx-1* is dependent on *daf-16* as well. To do this, I crossed the neuron-, intestine- and hypodermis-specific RNAi strains (tissues that I previously found to be involved in lifespan extension when *utx-1* is knocked down) into the short-lived *daf-16(mu86)* animals and performed *utx-1* RNAi and subsequent survival analysis (Figure 3.12).



Strain	RNAi Condition	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs EV control)
daf-16(mu86)	EV	14.5	14	18	p = 0.3842 (ns)
	utx-1 RNAi	14.7	14	18	
daf-16(mu86); rde-1(ne219); intestine-specific	EV	12.5	12	16	p = 0.7605 (ns)
	utx-1 RNAi	12.4	11	16	
daf-16(mu86); rde-1(ne219); neuron-specific	EV	12.8	14	17	p = 0.7890 (ns)
	utx-1 RNAi	13.0	14	16	
daf-16(mu86); rde-1(ne219); hypodermis-specific	EV	16.3	16	22	p = 0.0001 (***)
	utx-1 RNAi	18.3	18	23	

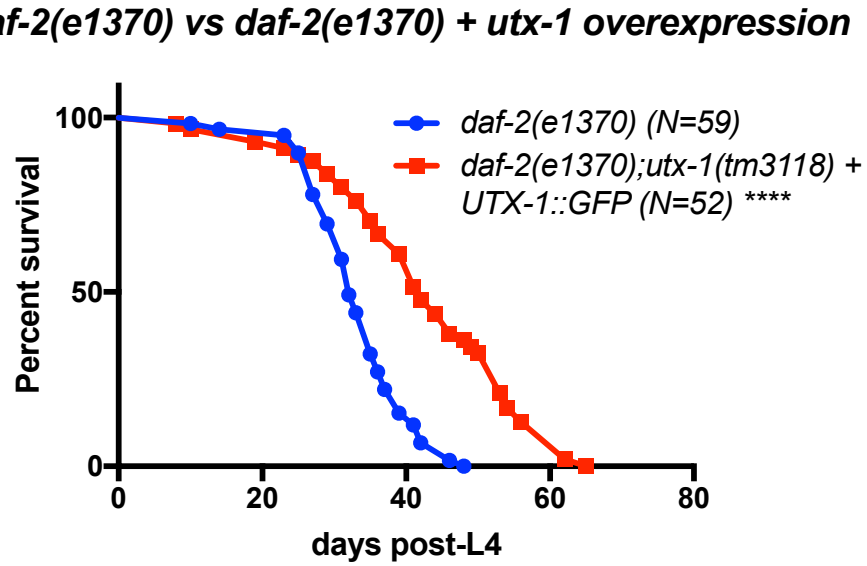
Figure 3.12: daf-16 dependence of lifespan extension due to tissue-specific utx-1 depletion. *For each strain, at least 60 L4-stage animals were subjected to either EV or utx-1 RNAi. Animals were scored as alive or dead at least every 2 days. Survival was compared between EV and utx-1 RNAi for each strain assessed. utx-1 was knocked down* ***(A)*** *globally; and tissue-specifically in the* ***(B)*** *intestine,* ***(C)*** *neurons and* ***(D)*** *hypodermis in daf-16(mu86) animals. Mean lifespan, Median lifespan, maximum lifespan and p-values are listed in the accompanying table. Statistical significance was calculated using Mantel-Cox method.*

No lifespan extension was observed in *daf-16* mutants where *utx-1* was knocked down in a neuron- and intestine-specific manner, suggesting that lifespan extension due to neuron- and intestine-specific knockdown of *utx-1* is completely *daf-16* dependent. However, I did observe a moderate lifespan extension when *utx-1* was specifically knocked down in the hypodermis in *daf-16(mu86)* animals, indicating that lifespan increase due to hypodermis-specific *utx-1* depletion is partially independent of *daf-16*. This could potentially mean that lifespan regulation by *utx-1* depletion in the hypodermal tissue could, at least in part, be mediated by a longevity mechanism parallel to the *daf-2/daf-16* paradigm that require further studies.

3.3.9. *daf-2* dependence of lifespan on *utx-1* overexpression

In parallel to my mechanistic studies on the *daf-2*/*daf-16* dependence of lifespan due to *utx-1* knockdown, I investigated if overexpression of *utx-1* relies on IIS. Reduction-of-function mutations in the *daf-2* gene are widely known to be long-lived and stress-resistant compared to wild-type worms (Gems et al., 1998; Kenyon et al., 1993) and I wanted to study if *daf-2* mutations causing lifespan increase has an additive effect to the longevity caused by *utx-1* overexpression. Previously, Dr Karolina Chocian (a colleague at the Woollard lab) performed qRT-PCR experiments to measure *daf-2* mRNA levels in young adults of *utx-1* mutants overexpressing wild-type *utx-1* (genotype: *utx-1(tm3118)+ utx-1p::UTX-1::GFP*; strain ZR254) compared to wild-type and found that *daf-2* expression in the overexpressors is 3x more than that of wild-type (data not shown; (Chocian, 2015)). This indicates that overexpression of *utx-1* upregulates transcription of *daf-2*. This also corroborates reports by Jin *et al.* (2011) that UTX-1 promotes transcriptional regulation of *daf-2* by removing transcriptionally repressive H3K27me3 marks from the *daf-2* locus (C. Jin et al., 2011a). If *utx-1* overexpression upregulates the transcription of *daf-2*, and yet I found that *utx-1* overexpression promotes lifespan extension (as in Figure 3.5B), then pro-longevity benefits due to *utx-1* overexpression

could be occurring independently of *daf-2*. To test this, I performed a lifespan analysis of long-lived *daf-2(e1370)* mutants compared to *utx-1(tm3118)+utx-1p::UTX-1::GFP* animals in the *daf-2* mutant background (Figure 3.13).



Genotype	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs *daf-2*)
daf-2(e1370)	33.0	32	48	p < 0.0001 (****)
daf-2(e1370); utx-1(tm3118) + UTX-1::GFP	42.2	42	65	

Figure 3.13: daf-2 dependence of lifespan extension due to utx-1 overexpression. *At least 60 L4-stage animals were picked for each genotype and assessed for lifespan analysis. Animals were scored as alive or dead at least every 3 days. Survival of daf-2 mutants overexpressing utx-1 was compared against daf-2 animals. Accompanying table shows mean lifespan, median lifespan, maximum lifespan and p-values calculated using Mantel-Cox method. Statistical significance was calculated using Mantel-Cox method. Lifespan experiment was only performed once.*

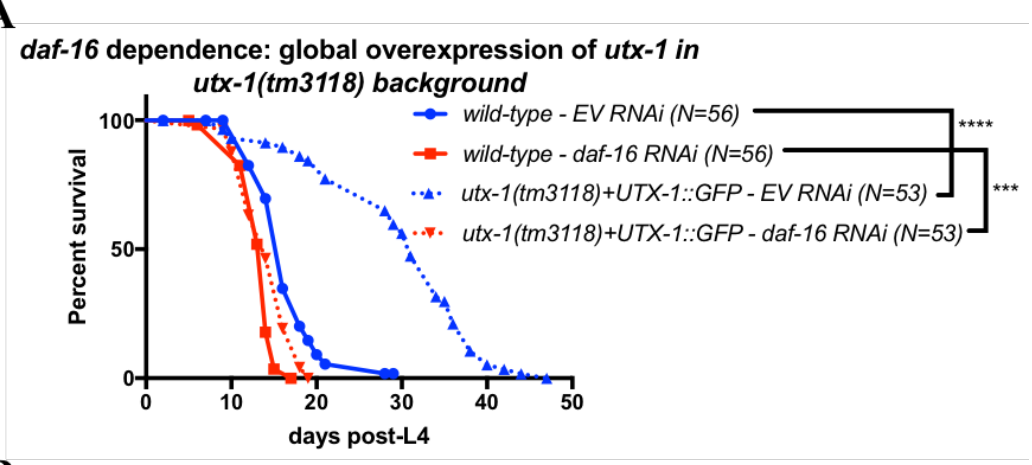
My data showed that lifespan of long-lived *daf-2(e1370)* mutants was further improved by overexpression of *utx-1*. The additive effect suggests that the pro-longevity phenotype caused by overexpressing *utx-1* is at least partially independent of *daf-2* mediated lifespan extension.

3.3.10. *daf-16* dependence of lifespan on tissue-specific overexpression of *utx-1*

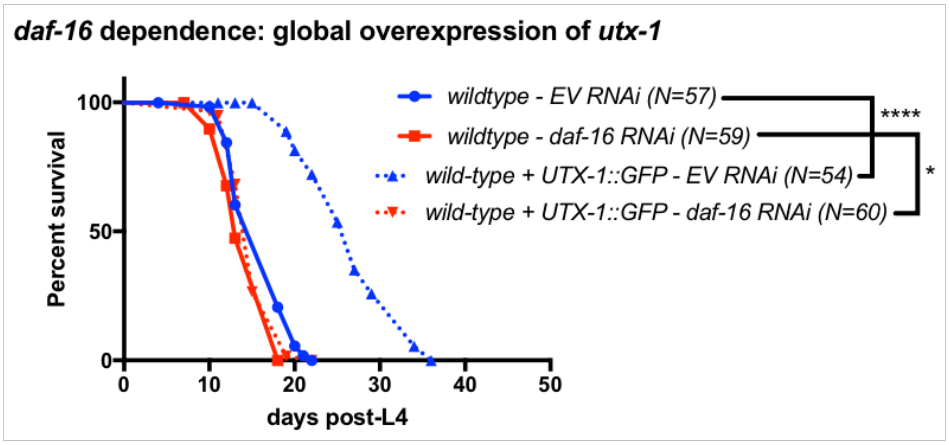
daf-16 dependence of lifespan extension due to *utx-1* overexpression was investigated to assess whether lifespan is dependent on the downstream components of the insulin signalling cascade. Lifespan analyses were performed by knocking down *daf-16* in worms overexpressing *utx-1* ubiquitously (Figure 3.14A – wild-type background and B – *utx-1(tm3118)* mutant background) and in a tissue-specific manner (Figure 3.14C - intestine and D - neurons). It should be noted that the lifespan of wild-type worms subjected to *daf-16* RNAi (med/max lifespan: 13/18 days and med/max lifespan: 14/17 days as seen on Figure 3.14A and B respectively) was comparable to *daf-16(mu86)* mutants (med/max lifespan: 14/18 days as seen on Figure 3.12A), suggesting that *daf-16* RNAi gives a similar lifespan shortening effect as that of *daf-16* mutants.

I have previously shown that intestine- and neuron-specific overexpression of *utx-1* causes lifespan extension compared to wild-type worms (Figure 3.10), and these strains were therefore assessed for the impact of *daf-16* on lifespan (Figure 3.14).

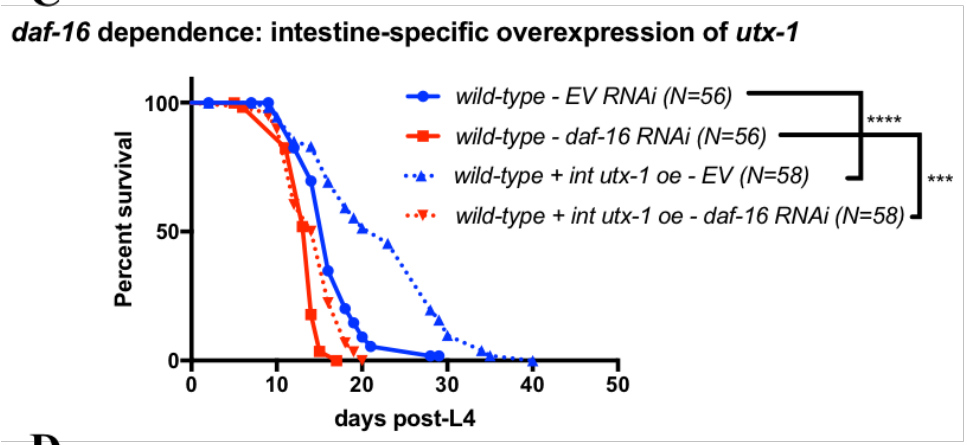
A


daf-16 dependence: global overexpression of utx-1 in utx-1(tm3118) background
wild-type - EV RNAi (N=56)
wild-type - daf-16 RNAi (N=56)
utx-1(tm3118)+UTX-1::GFP - EV RNAi (N=53)
utx-1(tm3118)+UTX-1::GFP - daf-16 RNAi (N=53)
****
***
Percent survival
days post-L4

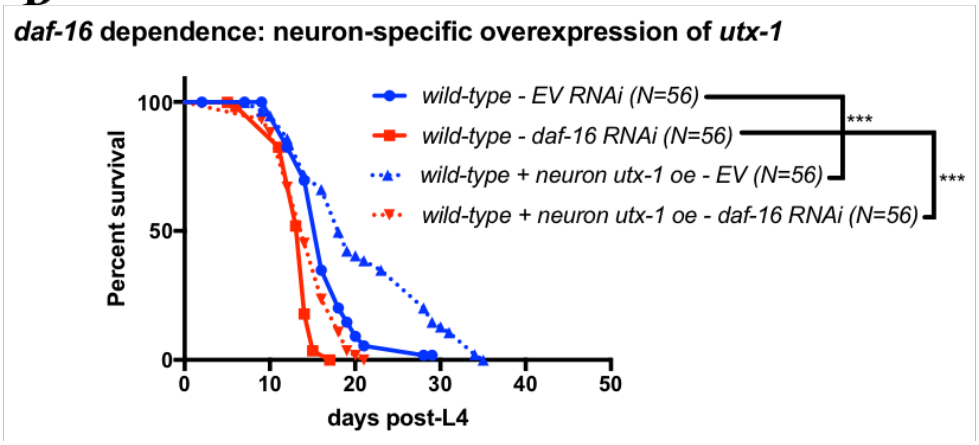
B


daf-16 dependence: global overexpression of utx-1
wildtype - EV RNAi (N=57)
wildtype - daf-16 RNAi (N=59)
wild-type + UTX-1::GFP - EV RNAi (N=54)
wild-type + UTX-1::GFP - daf-16 RNAi (N=60)
****
*
Percent survival
days post-L4

C


daf-16 dependence: intestine-specific overexpression of utx-1
wild-type - EV RNAi (N=56)
wild-type - daf-16 RNAi (N=56)
wild-type + int utx-1 oe - EV (N=58)
wild-type + int utx-1 oe - daf-16 RNAi (N=58)
****
***
Percent survival
days post-L4

D


daf-16 dependence: neuron-specific overexpression of utx-1
wild-type - EV RNAi (N=56)
wild-type - daf-16 RNAi (N=56)
wild-type + neuron utx-1 oe - EV (N=56)
wild-type + neuron utx-1 oe - daf-16 RNAi (N=56)
***
***
Percent survival
days post-L4

Strain/genotype	RNAi Condition	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance
N2 (wild-type)	EV	16.3	16	29	
utx-1(tm3118) + utx-1p::utx-1::GFP (systemic)	EV	29.9	31	47	p < 0.0001 (****) [a]
N2 + vha-6p::utx-1::GFP (intestine)	EV	22.4	23	40	p < 0.0001 (****) [a]
N2 + rab-3p::utx-1::GFP (neuron)	EV	20.7	18	35	p < 0.0001 (****) [a]
N2 (wild-type)	*daf-16* RNAi	13.3	14	17	
utx-1(tm3118) + utx-1p::utx-1::GFP	*daf-16* RNAi	14.1	15	19	p = 0.0005 (***) [b]
N2 + vha-6p::utx-1::GFP (intestine)	*daf-16* RNAi	14.5	16	20	p = 0.0001 (***) [b]
N2 + rab-3p::utx-1::GFP (neuron)	*daf-16* RNAi	14.5	14	21	p = 0.0002 (***) [b]
N2 (wild-type) [c]	EV	16.2	18	22	
N2 + utx-1p::utx-1::GFP [c]	EV	26.9	27	36	p < 0.0001 (****) [a]
N2 (wild-type) [c]	*daf-16* RNAi	14.8	13	18	
N2 + utx-1p::utx-1::GFP [c]	*daf-16* RNAi	15.4	15	22	p = 0.0291 (*) [b]

Figure 3.14: daf-16 dependence of lifespan extension due to overexpression of utx-1. *For each strain, at least 60 L4-stage animals were subjected to either EV or daf-16 RNAi. Animals were scored as alive or dead at least every 2 days. Survival of utx-1 overexpressing strains subjected to daf-16 RNAi were compared against wild-type animals subjected to daf-16 RNAi (and similarly with EV conditions).* ***(A)*** *and* ***(B)*** *show the lifespan curves of systemic utx-1 overexpressors in the utx-1(tm3118) mutant background and wild-type, respectively;* ***(C)*** *intestine- and* ***(D)*** *neuronal-specific overexpression of utx-1. All lifespan analyses for EV vs daf-16 RNAi conditions were consistently statistically significant (p < 0.0001, ****). [a] statistical significance compared to wild-type EV condition; [b] statistical significance compared to wild-type daf-16 RNAi condition; [c] data from (B) are independent from (A), (C) and (D) dataset. Statistical significance was calculated using Mantel-Cox method.*

daf-16 RNAi on worms ubiquitously expressing *utx-1* have shortened lifespan compared to empty vector controls. However, they are slightly longer-lived than wild-type worms on *daf-16* RNAi (Figure 3.14A (p = 0.0005 '***') and B (p = 0.0291 '*')). Collectively, the reduced lifespan in *daf-16* RNAi suggests that lifespan extension due to *utx-1* overexpression is mostly *daf-16* dependent. However, there is a slight difference in the extent of lifespan shortening, where *utx-1* overexpressing strains in *daf-16* RNAi were slightly better off than wild-type in *daf-16* RNAi. This might indicate that there is a *daf-16* independent mechanism that regulates lifespan in situations where the level of *utx-1* is high. There is a possibility that the reason why *utx-1* overexpressing strains subjected

to *daf-16* RNAi are slightly better off than wild-type animals in *daf-16* RNAi conditions is because it is simply too difficult to bring long lifespans back to *daf-16* levels. On the other hand, there are plenty of instances where it does. For instance, loss of *daf-16* in long-lived *daf-2* mutants completely abolishes lifespan extension (Kenyon et al., 1993); and the lifespan increase due to overexpression of *hsf-1* was completely abolished (back to *daf-16* levels) when animals were subjected to *daf-16* RNAi (Hsu, Murphy, & Kenyon, 2003). Therefore, I would favour the interpretation where lifespan extension due to *utx-1* overexpression is only partially *daf-16* dependent.

daf-16 RNAi on tissue-specific *utx-1* overexpression strains are short-lived compared to empty vector controls. Consistent with the results in worms overexpressing *utx-1* ubiquitously, tissue-specific *utx-1* overexpressing animals are longer lived than wild-type under *daf-16* RNAi conditions (Figure 3.14C and D). The lifespan difference in our tissue-specific *utx-1* overexpression compared to wild-type analyses, is arguably more convincing than that of *daf-16* depletion in ubiquitously overexpressing *utx-1* animals, supporting the interpretation that lifespan due to *utx-1* overexpression is partially independent of *daf-16*.

3.3.11. Summary of *daf-2* and *daf-16* dependence due to knockdown and overexpression of *utx-1*

The table below summarises the lifespan outcomes due to tissue-specific up- and down-regulation of *utx-1* (Table 3.3). Lifespan outcomes due to the tissue-specific knockdown of *daf-2* mirrors that of tissue-specific *utx-1* RNAi in terms of the key tissues that mediate longevity (Figure 3.11). This, coupled with the Maures *et al.*'s findings that *utx-1* knockdown transcriptionally downregulates *daf-2* suggests that lifespan extension due to *utx-1* depletion is dependent on *daf-2* (Maures et al., 2011). Furthermore, epistatic

analysis with *daf-16* showed me that lifespan extension due to knockdown of *utx-1* is largely dependent on insulin signalling, apart from hypodermal knockdown of *utx-1*. In contrast, lifespan extension due to overexpression of *utx-1* occurs independently of *daf-2*, as evidenced by epistasis analysis (Figure 3.13), but partially converges on *daf-16* (Figure 3.14). This suggests that *utx-1* overexpression could promote longevity through a yet unknown parallel mechanism.

Table 3.3: daf-2 and daf-16 dependence of lifespan extension due to tissue-specific changes in utx-1. *[a] based on the correlation of lifespan between tissue-specific utx-1 and daf-2 knockdown (Figure 3.11).*

Tissue target	**Knockdown of *utx-1***			**Overexpression of *utx-1***		
	Lifespan change compared to requisite control	***daf-2 independent?***	***daf-16* independent?**	**Lifespan change compared to requisite control**	***daf-2 independent?***	***daf-16* independent?**
Systemic	up	no[a]	no	up	yes	partially
Neurons	up	-	no	up	-	partially
Intestine	up	-	no	up	-	partially
Hypodermis	up	-	partially	no change	-	-
Muscle	no change	-	-	no change	-	-

3.4 DISCUSSION

In this chapter, I validated previous findings in the lab where both overexpression and knockdown of *utx-1* promotes lifespan extension. I hypothesised that since *utx-1* is ubiquitously expressed in the worms, there is a possibility that the lifespan extension due to systemic up- and downregulation of *utx-1* is a net effect of differential tissue-specific contributions of *utx-1* on lifespan regulation. To this end, I targeted the four main somatic tissues of *C. elegans* (intestine, hypodermis, neurons and muscle) to assess if each tissue contributes differently to *utx-1* mediated lifespan extension. I decided not to include the germline because it has been previously established that lifespan extension due to *utx-1* knockdown occurs independently of germline signalling (C. Jin et al., 2011a; Maures et al., 2011). First, germline-specific knockdown of *utx-1* (using *rrf-1* mutants) did not extend lifespan; and second, *utx-1* RNAi on worms lacking a germline (*glp-1* mutants) still promoted lifespan extension (C. Jin et al., 2011a). Furthermore, I showed that worms overexpressing UTX-1::GFP displayed ubiquitous expression in somatic tissues, but expression was silenced in the germline (see Figure 3.4). Overexpression of UTX-1::GFP in either wild-type or *utx-1* mutant background gave lifespan extension, suggesting that the germline is dispensable for lifespan extension due to *utx-1* overexpression.

3.4.1. *utx-1* knockdown mediates lifespan extension in the intestine, neurons and hypodermis

I manipulated the levels of *utx-1* in specific tissues and discovered that the intestine and the neurons are important sites of action for lifespan extension due to *utx-1* depletion or overexpression. Additionally, I also found that the hypodermis is an important tissue for enhanced longevity due to knockdown of *utx-1*. Muscle-specific up- or downregulation of *utx-1* did not affect worm lifespan, suggesting that muscles do not play a role in *utx-*

1-mediated lifespan regulation. This seems particularly striking, given that muscles are prone to senescent pathologies (atrophy) with increasing age (Herndon et al., 2002).

3.4.2. Lifespan extension due to neuronal- and intestine-specific knockdown of *utx-1* is dependent on insulin signalling

Inhibition of components of the insulin signalling, including the insulin receptor homologue DAF-2, leads to hypophosphorylation of the FoxO transcription factor DAF-16. Hypophosphorylated DAF-16 can translocate to the nucleus and promote transcription of longevity and stress resistance genes. Lifespan increase due to *utx-1* downregulation was reportedly mediated by *daf-2*, where UTX-1 demethylase function was implicated in removing transcriptionally repressive H3K27me3 marks from the *daf-2* locus (C. Jin et al., 2011a). Hence, inhibition of *utx-1* promotes lifespan extension by transcriptionally inhibiting *daf-2*, which consequently reduces insulin signalling activity and promotes nuclear localisation of DAF-16 to promote transcription of pro-longevity genes. This is corroborated by findings that knockdown of *utx-1* in long-lived mutants *daf-2* mutants does not have an additive effect on lifespan, indicating that *utx-1* and *daf-2* downregulation are involved in the same pathway to promote lifespan extension (C. Jin et al., 2011a; Maures et al., 2011).

Tissue-specific rescue of *daf-2* in long-lived *daf-2* mutants showed that neuron- and to a lesser extent intestine-specific expression of *daf-2* rescues lifespan close to wild-type, suggesting that the neurons and intestine are important tissues for *daf-2*-mediated lifespan extension (Wolkow et al., 2000). The tissues implicated in lifespan extension due to *daf-2* inhibition are the same tissues that I found to be important in increased longevity due to *utx-1* knockdown. The fact that both *utx-1* and *daf-2* act on the intestine and the neurons to improve longevity supports the finding that *utx-1* depletion promotes lifespan

extension through the same pathway as *daf-2*. Similar to hypodermal-specific knockdown of *utx-1*, I found that hypodermal knockdown of *daf-2* also promotes lifespan extension, which to my knowledge has never been directly tested before. This suggests that the hypodermis is also an important tissue for mediating lifespan extension due to *daf-2* inhibition. The fact that the hypodermis is implicated in lifespan extension due to downregulation of both *daf-2* and *utx-1* further supports that *utx-1* depletion promotes lifespan extension in a *daf-2*-dependent manner.

To assess whether lifespan extension due to tissue-specific *utx-1* depletion is indeed dependent on insulin signalling, I examined whether longevity was dependent on the most downstream effector of the pathway, *daf-16.* I knocked down *utx-1* in a tissue-specific manner in *daf-16(mu86)* mutants compared to empty vector controls and found that increased longevity due to neuronal- and intestine-specific knockdown of *utx-1* is completely abolished in the *daf-16(mu86)* mutant background. This indicates that, at least in the neurons and the intestine, lifespan extension due *utx-1* depletion is completely dependent on the *daf-2*/*daf-16* pathway. In contrast, hypodermal knockdown of *utx-1* in the *daf-16* mutant background gave a modest increase in lifespan, suggesting that lifespan extension due to hypodermal knockdown of *utx-1* is partially independent of insulin signalling.

3.4.3. How does the hypodermis mediate lifespan regulation?

To date, there are limited studies on genes that regulate lifespan in a hypodermis-specific manner. Most longevity genes seem to regulate lifespan in the neurons, intestine and the germline (Guarente & Kenyon, 2000). Therefore, it was unexpected to find that hypodermal-specific knockdown of *utx-1* promotes lifespan extension. Even more

surprisingly, this observed lifespan extension is only partially dependent on *daf-16*, despite the fact that hypodermal-specific knockdown of *daf-2* gives lifespan extension.

3.4.3.1. The hypodermis mediates the innate immune response to pathogens

The hypodermis is the main barrier epithelia of the worm and acts as the first line of defense from pathogens and injury. The natural fungal pathogen *Drechmeria coniospora* infects *C. elegans* by breaching the cuticle and disrupting hypodermal integrity. It was found that the hypodermis induces expression of antimicrobial peptides (AMPs) (such as *nlp-29*) in response to both mechanical injury and fungal infection and is mediated by the p38-MAPK pathway (Pujol et al., 2008). This suggests that the hypodermis plays an important role in mediating innate immune responses to infection and injury. Increased sensitivity to pathogens is a hallmark of ageing and therefore increased resistance to pathogens might be a possible way for the hypodermis to regulate longevity. This is in agreement with reports that mutants such as *daf-2*, *age-1*, and germline-deficient mutants *glp-1* are long-lived and also resistant to pathogenic stress, while short-lived *hsf-1* and *daf-16* mutants are associated with increased sensitivity to pathogens (Zhou, Pincus, & Slack, 2011).

3.4.3.2. Collagen genes may be involved in longevity-assurance pathways

In another study, Ewald *et al.* (2014) showed that reduced insulin signalling (in which dauer processes were inactive) promotes longevity by activating the Nrf transcription factor, SKN-1. Comparison of expression profiles between *daf-2* and *daf-2;skn-1* mutants showed that SKN-1 upregulated transcription of several collagen genes. Knockdown of collagen genes decreased the lifespan of non-dauer *daf-2* mutants (ie. at 15^{o}C), suggesting that the increased lifespan of non-dauer permitting *daf-2* mutants are dependent on the upregulation of collagen genes (Ewald, Landis, Porter Abate, Murphy, & Blackwell,

2015). In humans, collagen remodeling decreases with age and considerable efforts have been put into developing interventions that could delay this age-related process. The finding that enhanced collagen expression promotes lifespan extension in worms suggests that collagen remodeling is an important longevity assurance pathway. Hypodermal function is best characterised in the context of collagen expression in the cuticle and molting during development, therefore the discovery that collagens promote longevity suggests a potential explanation as to how the hypodermis mediates lifespan.

3.4.3.3. The hypodermis may be involved in DR-mediated lifespan regulation

Tissue-specific expression profiling of the four major tissues in *C. elegans* (ie. neurons, intestine, hypodermis and muscle) was recently conducted by isolating tissue-specific GFP labelled cells through fluorescence-associated cell sorting (FACS) followed by RNA-Seq (Kaletsky et al., 2018). Gene ontology analysis of hypodermis-enriched genes surprisingly revealed significant association with different types of metabolic processes such as carbohydrate, amino acid, fatty acid, monosaccharide, nucleoside, ROS, co-factor, nitrogen compound, aromatic compound and heterocycle metabolism (Kaletsky et al., 2018). This indicates that the hypodermis is a key player in mediating metabolic changes in the worm, which is unprecedented. Metabolic networks are highly coupled with lifespan regulation; therefore, it is quite surprising that there are not many reports that implicate the hypodermis in mediating longevity.

Intriguingly, I only found one study that reports lifespan extension due to hypodermis-specific knockdown of a gene. In *C. elegans*, knockdown of *mekk-3*, a gene that encodes a homologue of mammalian protein kinase MEKK-3, promotes lifespan extension by inducing a DR-like state despite the presence of ample food supply (Chamoli, Singh, Malik, & Mukhopadhyay, 2014). Longevity was abolished in *eat-2* mutants (a genetic

model of DR) and *pha-4* mutants (FoxA transcription factor that promotes DR-mediated lifespan extension), suggesting that *mekk-3* acts on the DR-pathway to promote longevity. Knockdown of *mekk-3* extends lifespan even further in *daf-2* mutants and promotes lifespan extension in *daf-16* animals, suggesting that *mekk-3* mediated lifespan extension does not act in the insulin signalling pathway. Notably, tissue-specific analysis showed that *mekk-3* depletion extends lifespan specifically in the hypodermis and muscle tissue (Chamoli et al., 2014). This suggests that the hypodermis and muscle are key tissues for *mekk-3*-mediated longevity. Furthermore, this study implicates the hypodermis as a key player in regulating nutrient-sensing and metabolism, which could impact worm lifespan. In the future, it would be interesting to see if there is a direct interaction between UTX-1 and MEKK-3 to mediate lifespan extension due to hypodermal-specific knockdown of *utx-1*.

3.4.4. Intestine- and neuron-specific overexpression of *utx-1* extends worm lifespan and is partially independent of insulin signalling

Overexpression of *utx-1* also promotes lifespan extension in worms, and has a more dramatic effect on longevity than what I observed in *utx-1* knockdown studies. Tissue-specific overexpression of *utx-1* revealed that the neurons and intestine are important tissues for mediating lifespan extension due to *utx-1* overexpression. Interestingly, *utx-1* knockdown also promotes lifespan extension specifically in the intestine and neurons. However, the key difference is that while lifespan extension due to *utx-1* knockdown is largely dependent on *daf-16*, improved longevity due to *utx-1* overexpression is only partially so. Furthermore, overexpressing *utx-1* in long-lived *daf-2* mutants extended lifespan even more, suggesting that *utx-1* overexpression (at least partially) promotes lifespan regulation independently of *daf-2*, but partially converges on *daf-16*. Additionally, *utx-1* overexpressing animals are longer-lived than wild-type despite

having increased expression of *daf-2* (pers. commun. Dr Karolina Chocian). This corroborates that lifespan extension mediated by *utx-1* overexpression is only partially mediated by the insulin signalling pathway. To this end, I wanted to investigate if the UTX-1 demethylase function is important in transcriptional regulation of key target genes that promote longevity. The following chapter (Chapter 4: Gene expression profiling in *utx-1* overexpressing worms) describes this in detail where I look at transcriptional changes mediated by overexpression of UTX-1 and how a selection of these transcripts affect lifespan.

3.4.5. Are specific neurons important in *utx-1*-mediated lifespan extension?

In this chapter, I showed that knockdown and overexpression of *utx-1* promotes lifespan extension and in both cases, the neurons were implicated as an important site for improving longevity upon *utx-1* up- or downregulation. The *C. elegans* hermaphrodite has 302 neurons and I want to eventually investigate whether particular neuronal subsets are important in promoting longevity when utx-1 is knocked down or overexpressed. Tissue-specific RNAi strains that target GABAergic, cholinergic, dopaminergic and glutamatergic neurons are readily available and would be useful for assessing lifespan subjected to *utx-1* RNAi. On the other hand, transgenic animals overexpressing *utx-1* driven by subtype-specific promoters can be created to use for future lifespan studies.

3.4.6. Is *utx-1*-mediated lifespan extension dosage-dependent?

Another intriguing question to ask is if *utx-1*-mediated lifespan extension is dependent on dosage. Up- and downregulation of *utx-1* promotes lifespan extension and it is unknown whether this effect is simply due to an imbalance of *utx-1* levels, or if lifespan extension is affected in a dose-dependent manner. Dose-dependent lifespan has been previously described in *S. cerevisiae* (budding yeast), where replicative lifespan was

found to be influenced by the levels of H3 lysine 56 acetylation (H3K56ac). H3K56ac reportedly has important roles in genome integrity and replication (Celic et al., 2006). Levels of H3K56ac are significantly reduced in old cells and this is associated with increased genomic instability (Dang et al., 2009). Deletion of genes encoding for Hst3 and Hst4, the histone deacetylases that act on H3K56ac, leads to reduced replicative lifespan in budding yeast, supporting the idea that H3K56ac is beneficial for replicative lifespan (Tsuchiya et al., 2006). However, overexpression of Hst3 also shortens replicative lifespan, while addition of only one extra copy of either *HST3* or *HST4* is beneficial to longevity (Pal & Tyler, 2016). This suggests that the replicative lifespan of budding yeast is highly dependent on the dose of Hst3 or Hst4, and that a fine balance of H3K56Ac levels need to be achieved to promote longevity.

Similarly in *C. elegans*, knockdown of components of the electron transport chain (ETC) differentially affected lifespan depending on the concentration of RNAi used. For example, knockdown of *atp-3*, a gene encoding for a subunit of the ATP synthase, shortens lifespan at the highest concentration of RNAi, but promotes lifespan extension in a certain window of RNAi dilution (Rea, Ventura, & Johnson, 2007). This was attributed to the mitochondrial threshold effect theory, whereby downregulation the components of the mitochondria is beneficial to lifespan at a particular threshold (Durieux & Dillin, 2007; Rea et al., 2007). This finding suggests that lifespan is sensitive to the dosage of certain genetic perturbations.

The demethylase function of UTX-1 specifically acts on H3K27me3 and this transcriptionally repressive mark decreases with age in *C. elegans*. Worms feeding on *utx-1* RNAi have increased levels of H3K27me3 as verified by western blot (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a), and this is associated with lifespan

extension (C. Jin et al., 2011a; Maures et al., 2011). This suggests that perhaps H3K27me3 is a pro-longevity mark and promotes lifespan extension by inducing a more "youthful" chromatin state. However, this does not fit with my observation that *utx-1* overexpression is also beneficial to lifespan. Immunostaining studies probing H3K27me3 on worms overexpressing *utx-1* showed a reduction of global H3K27me3 levels (Vandamme, Lettier, Sidoli, Di Schiavi, Nørregaard Jensen, et al., 2012b). Furthermore, *utx-1* overexpression is associated with increased transcription of *daf-2* (pers. commun. Dr. Karolina Chocian). Collectively, this indicates that lifespan extension occurs despite a reduction in H3K27me3 and upregulation of *daf-2*. In this regard, I wondered whether a chromatin imbalance caused by perturbing H3K27me3 levels induce a stress response pathway that consequently prolongs lifespan. Similar to how mild levels of stress can induce protective mechanisms (called "hormesis") that promote longevity, perhaps inducing chromatin imbalance by changing the levels of *utx-1* (and therefore H3K27me3) could also be beneficial to lifespan in *C. elegans*.

To address whether lifespan extension is dependent on the dose of *utx-1*, it would be useful in the future to knockdown *utx-1* using different concentrations of RNAi and assess worm lifespan. For overexpression, I have previously used transgenic animals containing a single copy of *utx-1p::utx-1::gfp* created by *mos-1* mediated Single Copy Insertion techniques (mosSCI) (kindly gifted by Dr. Rafal Ciosk) for investigating the spatial expression of *utx-1* (called "scUTX-1::GFP"). These transgenic animals are also expressing endogenous *utx-1* and would therefore theoretically be expressing 2x more *utx-1* than wild-type. Future tudies comparing the lifespan of wild-type, scUTX-1::GFP and *utx-1* overexpressing worms would be useful in investigating whether different levels of *utx-1* overexpression differentially affect lifespan.

CHAPTER 4: GENE EXPRESSION PROFILING IN UTX-1 OVEREXPRESSING WORMS

4.1 INTRODUCTION

4.1.1. Lifespan extension due to *utx-1* depletion is largely dependent on insulin signalling

In the previous chapter, I showed that up- and downregulation of *utx-1* promotes lifespan extension in worms. Mechanistic studies into lifespan extension due to *utx-1* depletion have been previously done before, where knockdown of *utx-1* reportedly promotes improved longevity in an insulin signalling-dependent manner. UTX-1 promotes *daf-2* transcription by directly removing transcriptionally repressive H3K27me3 marks from the *daf-2* locus (C. Jin et al., 2011a). In wild-type animals, transcription of *utx-1* increases with age with concomitant increase in *daf-2* expression levels, suggesting that UTX-1 might promote DAF-2 activity. *utx-1* depletion was found to increase levels of transcriptionally repressive H3K27me3 marks on the *daf-2* locus, indicating that the demethylase function of UTX-1 is important in the transcriptional regulation of *daf-2*. Knocking down *utx-1* presumably reduces insulin signalling activity by transcriptionally repressing *daf-2*, thereby promoting lifespan extension (C. Jin et al., 2011a). My tissue-specific knockdown experiments showed that the neurons, intestine and hypodermis are important sites of action for lifespan extension due to *utx-1* depletion. Epistasis analysis with *daf-16* showed that lifespan extension due to *utx-1* knockdown is largely dependent

on the insulin signalling pathway (except for partial independence in the hypodermis), corroborating that *utx-1* knockdown promotes longevity in an insulin dependent manner.

4.1.2. Lifespan extension due to *utx-1* overexpression is partially independent of insulin signalling

On the other hand, the finding that *utx-1* overexpression also promotes lifespan extension is completely novel and nothing is really known about molecular mechanisms or pathways that could be involved in lifespan regulation mediated by overexpressing *utx-1*. Neuron- and intestine-specific overexpression of *utx-1* promoted lifespan extension, which is interesting considering that neuron- and intestine-specific knockdown of *utx-1* also extends worm lifespan. Knockdown of *daf-16* in animals overexpressing *utx-1* (either systemically or in a neuron- and intestine-specific manner) shortened lifespan but not to the same extent as that of wild-type animals feeding on *daf-16* RNAi. This indicates that lifespan extension due to *utx-1* overexpression is partially independent of the insulin signalling pathway. qRT-PCR experiments on animals systemically overexpressing *utx-1* showed an increase in *daf-2* mRNA levels (pers. commun. Dr. Karolina Chocian), and despite this, *utx-1* overexpressing worms are still long-lived. Furthermore, additional lifespan extension was observed in animals overexpressing *utx-1* in the *daf-2* mutant background compared to *daf-2* mutants, suggesting that lifespan extension due to *utx-1* overexpression is (at least partially) mediated by a different mechanism to insulin signalling.

4.1.3. Linking the catalytic activity of UTX-1 and longevity

utx-1 is critical for worm development and null mutations are homozygous lethal. Interestingly, the lethality phenotype induced by *utx-1* mutations can be rescued by transgenic overexpression of a catalytically dead version of UTX-1, suggesting that the

catalytic activity of UTX-1 is dispensable for worm development (Julien Vandamme, 2013; Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a). Lifespan analysis conducted by Dr. Karolina Chocian (Woollard lab) showed that animals overexpressing *utx-1* promotes lifespan extension (Figure 4.1). I have also validated this as shown in the previous chapter. The lifespan of worms overexpressing a demethylase-dead (DD) version of UTX-1 did not increase and was comparable to wild-type animals. This suggests that the catalytic activity of UTX-1 is important for lifespan extension mediated by UTX-1.

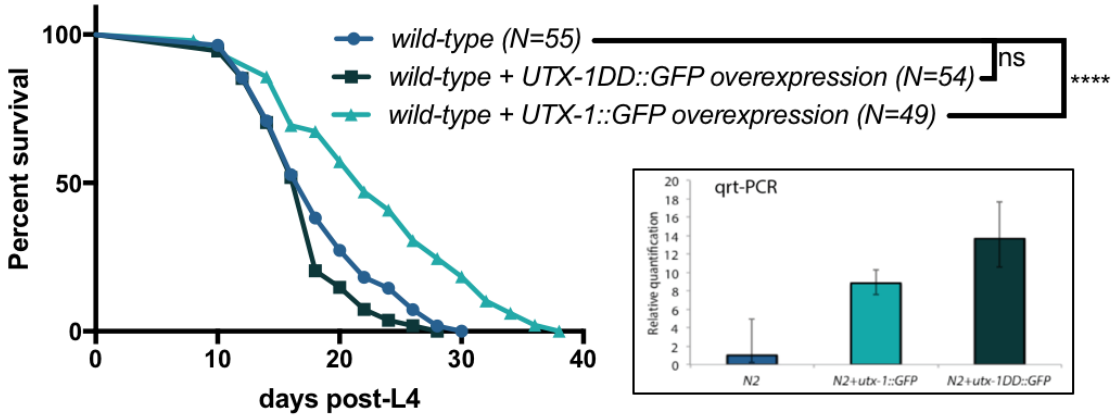


Figure 4.1: A catalytically functioning UTX-1 is required for longevity mediated by utx-1 overexpression. *Overexpression of a catalytically functional UTX-1 promotes lifespan extension (p-value < 0.0001, '****'), while overexpression of the demethylase-dead (DD) version of UTX-1 does not. Inset: qPCR probing utx-1 shows that levels of overexpression are comparable between the UTX-1 and UTX-1DD strain. Experiments shown in this figure were performed by Dr Karolina Chocian (Chocian, 2015)*

In wild-type animals overexpressing UTX-1::GFP and UTX-1DD::GFP, endogenous expression of *utx-1* would mean that a catalytically functioning UTX-1 is still present in either genotypes, which could skew the lifespan results. This was circumvented by comparing the lifespan of *utx-1(tm3118)* mutants overexpressing either UTX-1::GFP or UTX-1DD::GFP because *utx-1* mutants overexpressing UTX-1DD::GFP would be completely devoid of UTX-1 demethylase function (Figure 4.2). Because null mutations

of *utx-1* are lethal, it was necessary to use *utx-1* heterozygous mutants as appropriate controls for the experiment.

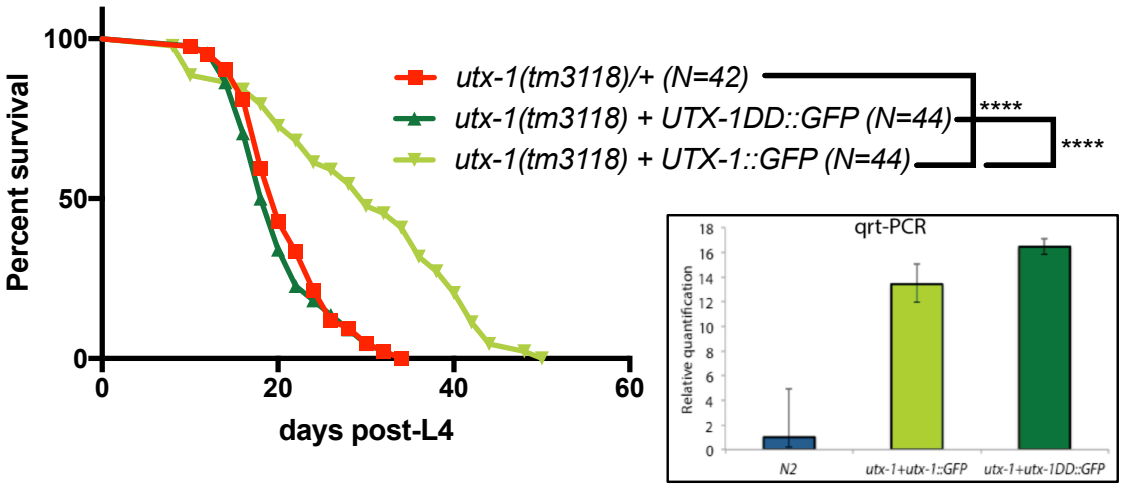


Figure 4.2: The catalytic activity of UTX-1 is crucial for lifespan extension mediated by utx-1 overexpression. *The lifespan of utx-1(tm3118)+UTX-1::GFP animals was significantly longer than either utx-1(tm3118) heterozygous mutants (p-value < 0.0001, '****') or utx-1(tm3118)+UTX-1DD::GFP (p-value < 0.0001, '****'). Inset: qPCR probing utx-1 shows that levels of overexpression are comparable between the UTX-1 and UTX-1DD strain. Experiments shown in this figure were performed by Dr Karolina Chocian (Chocian, 2015)*

utx-1 heterozygous mutants were long-lived compared to wild-type controls. This was to be expected given that *utx-1* knockdown increases worm lifespan. Overexpression of *utx-1*dd in *utx-1* mutants did not rescue lifespan back to wild-type. In other words, while UTX-1DD is able to rescue the developmental phenotype of *utx-1* mutants, it cannot rescue the longevity effect. This suggests that the demethylase function of UTX-1 is dispensable for worm development, but could be involved in mediating worm longevity. The *utx-1+UTX-1DD::GFP* animals have a comparable lifespan to heterozygous mutants *utx-1* mutants, suggesting that perhaps lifespan extension in UTX-1DD animals is driven by the *utx-1* mutation. On the other hand, overexpression of wild-type UTX-1 in the *utx-1* mutant background gave a lifespan extension that is above and beyond that of *utx-1* heterozygous mutants and UTX-1DD expressing animals, suggesting that the catalytic

activity of UTX-1, while dispensable for development, has important roles in lifespan regulation. Furthermore, there seems to be an additive lifespan extending effect between the *utx-1* mutation and *utx-1+UTX-1::GFP* overexpressing animals, indicating that lifespan extension mediated by the *utx-1* overexpression and *utx-1* haploinsufficiency is at least partially mediated by separate mechanisms. In the previous chapter, I addressed the tissue-specific contributions of *utx-1* overexpression and knockdown in lifespan regulation. Because the catalytic activity of UTX-1 plays an important role in lifespan extension in the context of *utx-1* overexpression, I hypothesised that the UTX-1 demethylase function affects the transcription of target genes that could mediate longevity in worms. This chapter describes my transcriptomic analysis pipeline and the subsequent strategies for screening candidate genes that are potentially involved in *utx-1* mediated lifespan regulation.

4.2. CHAPTER AIMS

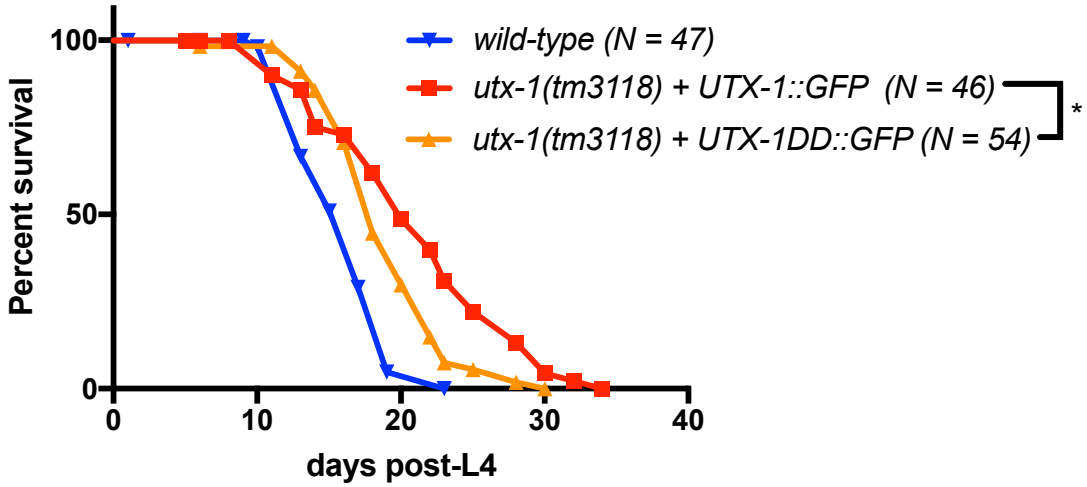
- Investigate transcriptomic changes associated with overexpression of a catalytically functional UTX-1
- Screen gene candidates that are potentially involved in lifespan regulation mediated by overexpressing a catalytically functional UTX-1 to identify pathways involved in lifespan extension due to *utx-1* overexpression

4.3. RESULTS

4.3.1. Experimental design

To analyse transcriptomic changes associated with the demethylase activity of UTX-1, I first needed to establish appropriate genotypes to use for my comparative studies. I looked at *tm3118*, an established null allele of *utx-1* rescued by transgenic overexpression of either wild-type (called "*utx-1(tm3118)+UTX-1::GFP*") or a demethylase dead (DD) (called "*utx-1(tm3118)+UTX-1DD::GFP*") version of UTX-1. The worm strains were kindly provided by our collaborator Dr. Lisa Salcini (University of Copenhagen, Denmark). The catalytic activity of UTX-1DD was inactivated by introducing point mutations in two of the three iron-binding motifs, a necessary feature of demethylases containing a JmjC-domain like UTX-1 (see Figure 3.2). The lack of catalytic activity in UTX-1DD has been verified by western blot where H3K27me2 and H3K27me3 levels of animals "rescued" with UTX-1DD were comparable to that of *utx-1* RNAi conditions, where H3K27me2/3 were significantly increased compared to wild-type (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a). Furthermore, whole worm immunostaining studies showed that animals overexpressing UTX-1DD (in a wild-type background) had comparable H3K27me3 levels to wild-type animals, while worms overexpressing a catalytically functional UTX-1 reduced global H3K27me3 compared to wild-type (Vandamme, Lettier, Sidoli, Di Schiavi, Nørregaard Jensen, et al., 2012b). Both UTX-1 and UTX-1DD rescued worm strains have similar levels of *utx-1* overexpression (see qRT-PCR data in Figure 4.2) and anecdotally have similar rates of development and brood sizes. Collectively, these two strains make for the best worms to use in investigating transcriptomic changes due to the demethylase activity of UTX-1.

Prior to the RNA-sequencing experiment, I repeated lifespan analyses comparing *utx-1* mutants overexpressing either a catalytically active or dead version of UTX-1 to validate findings that the catalytic activity of UTX-1 is important for lifespan regulation (Figure 4.3).



Genotype	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance
wild-type	15.6	17	23	
utx-1(tm3118)+UTX-1::GFP	20.8	20	34	$p < 0.0001$ (****) vs *wild-type* $p = 0.0117$ (*) vs *utx-1(tm3118)+UTX-1DD::GFP*
utx-1(tm3118)+UTX-1DD::GFP	18.9	18	30	$p < 0.0001$ (****) vs *wild-type*

Figure 4.3: Validation experiment showing that the catalytic activity of UTX-1 is crucial for lifespan extension due to utx-1 overexpression. *At least 60 L4-stage animals were picked for each genotype and assessed for lifespan analysis. Animals were scored as alive or dead at least every 2 days. Survival of utx-1(tm3118)+UTX-1::GFP animals were compared against wild-type and utx-1(tm3118)+UTX-1DD::GFP worms. Accompanying table shows mean lifespan, median lifespan, maximum lifespan and p-values calculated using Mantel-Cox method.*

My analysis showed that *utx-1* mutants overexpressing a wild-type version of UTX-1 is longer lived than animals overexpressing the catalytically dead version (Figure 4.3; $p = 0.0117$), thus independently recapitulating previous findings that the catalytic activity of UTX-1 is a key mediator of lifespan extension in the context of *utx-1* overexpression (as shown in Figure 4.2). Observations from this lifespan analysis gave me confidence to

pursue subsequent RNA-sequencing experiments to investigate how overexpression of a catalytically active UTX-1 affects gene transcription in worms.

I performed RNA-sequencing (RNA-Seq) on whole-worm extracts of *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP* to quantify the worm transcriptome of each genotype and determine transcripts that are differentially expressed (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008). The experimental approach allows me to determine an unbiased list of differentially expressed candidate genes without *a priori* assumptions on what genes the demethylase activity of UTX-1 affects. The pipeline for sample preparation to sequencing is graphically outlined as follows (Figure 4.4):

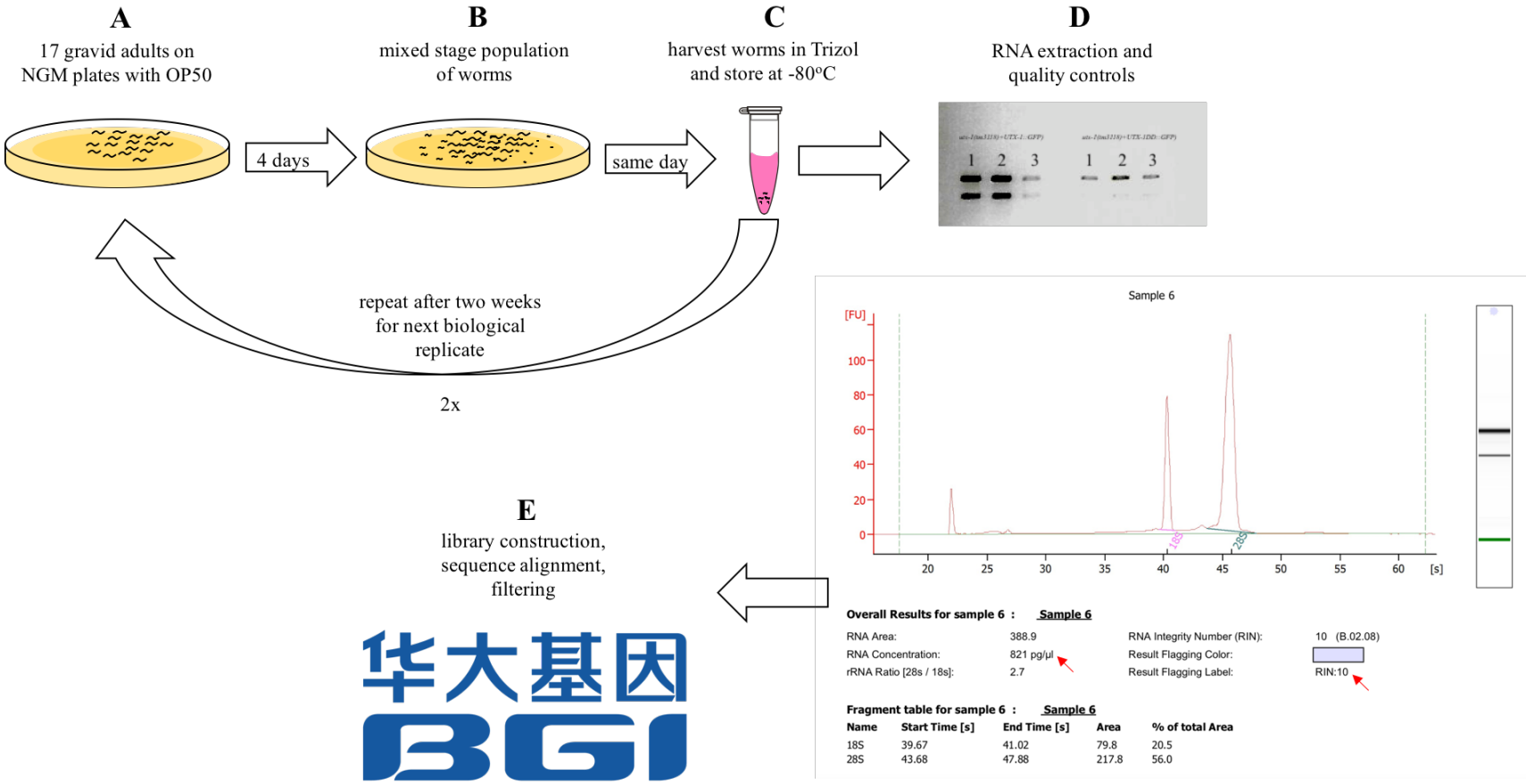


Figure 4.4: Experimental design for RNA-sequencing. ***(A)*** *3 x 90mm plates with 3 gravid adults were prepared for each biological replicate of utx-1(tm3118)+UTX-1::GFP and utx-1(tm3118)+UTX-1DD::GFP;* ***(B)*** *Animals were allowed to reproduce over 4 days in preparation for* ***(C)*** *RNA extraction using Trizol method;* ***(D)*** *top: RNA samples on agarose gel, bottom: representative image of bioanalyser readout for one of the RNA samples, red arrows indicate concentration and RNA integrity number (RIN);* ***(E)*** *RNA samples that pass quality control tests get sent to BGI for sequencing.*

Briefly, I transferred 17 gravid adults of each genotype on 3 x 90 mm NGM plates seeded with OP50 and let them develop and lay eggs at 20^{o}C for 4 days. The 4-day time-point was sufficient to obtain a good mix of worms at different developmental and adulthood stages to harvest RNA from. The percentage of different worm stages was comparable by eye for each genotype, which was important to ensure that gene expression data will not be biased towards a specific developmental time-point of the worms, but the population mix was not rigorously quantified. In the future, population distribution among the larval and adult stages of the worm will be monitored more rigorously to ensure a comparable mix of worm developmental/adult stages of each genotype assessed. I deliberately used a mixed population of worms in the first instance because I wanted to obtain a set of data that best captures transcriptomic changes due to the catalytic activity alone and avoid biases in synchronized populations due to small differences in synchrony. I extracted 3 biological replicates of RNA samples for each genotype. RNA was initially checked for degradation by gel electrophoresis before proceeding with more stringent RNA quality controls using a bioanalyser, where RNA integrity numbers (RINs) were determined. Samples with RIN > 7 were considered good quality and sent to the Beijing Genomics Institute (BGI) for RNA-Sequencing using single-end sequencing technology (termed "SE50" by BGI. BGI performed transcriptome library construction, sequencing and quality control (trimming and filtering) of clean reads which are sent back to us as fastq files (summarised in Table 4.1). Principal component analysis (PCA) of the data set showed that samples generally clustered according to genotype, apart from one biological repeat of the demethylase-dead sample (Figure 4.5). I nevertheless included this in subsequent analysis presented in this chapter, in order to introduce more stringency into the analysis.

Table 4.1: Summary of RNA-sequencing results from BGI. *nonDD samples = utx-1(tm3118)+UTX-1::GFP and DD samples = utx-1(tm3118)+UTX-1DD.*

Sample	Sequencing Strategy	Raw data size (bp)	Raw reads number	Clean data size (bp)	Clean reads number	Clean data rate (%)
nonDD_1	Single-end (50bp)	1,203,167,700	24,063,354	1,193,728,000	23,874,560	99.21
nonDD_2	Single-end (50bp)	1,203,912,200	24,078,244	1,195,499,600	23,909,992	99.30
nonDD_3	Single-end (50bp)	1,202,296,450	24,045,929	1,194,074,450	23,881,489	99.31
DD_1	Single-end (50bp)	1,203,238,600	24,064,772	1,194,375,550	23,887,511	99.26
DD_2	Single-end (50bp)	1,202,983,700	24,059,674	1,194,031,700	23,880,634	99.25
DD_3	Single-end (50bp)	1,203,227,050	24,064,541	1,192,664,150	23,853,283	99.12

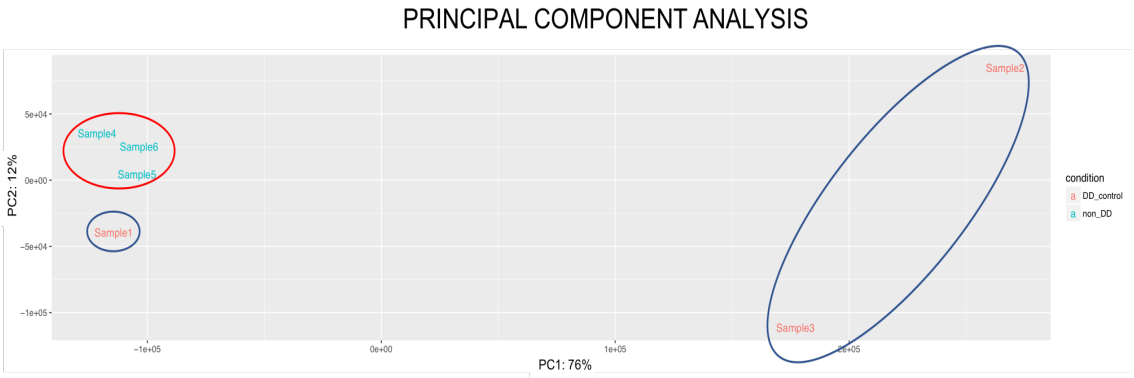


Figure 4.5: Principal component analysis of RNA-sequencing samples. *Red text ("DD_control") = utx-1(tm3118)+UTX-1DD::GFP; green text ("non_DD") = utx-1(tm3118)+UTX-1::GFP.*

Even though RNA extraction steps were meticulously followed to ensure that sample preparation was as identical as possible, variation was still observed, strikingly so in the case of UTX-1DD biological replicates. Given that I only prepared 3 biological replicates per genotype, it is difficult to assess whether 'Sample 1' in UTX-1DD control animals was an outlier or not (Figure 4.5: 'Sample 1'). At the time the experiments were performed, 3 biological replicates were chosen (and not more) due to limitations in available resources. Previous studies on a highly replicated two-condition RNA-sequencing experiment in *S. cerevisiae* (comparing wild-type and *Δsnf2* mutants) lead to

a final recommendation of using at least six biological repeats per genotype as a 'gold standard' for RNA-sequencing and differential gene expression analysis (Schurch et al., 2016). Future RNA-sequencing experiments will incorporate at least 6 biological replicates per genotype for a more robust experimental design.

4.3.2. Transcriptomic analysis pipeline

There are three general steps required to implement differential gene/transcript expression analysis: alignment of reads to an annotated genome, expression modelling to obtain transcript abundance estimates and statistical analysis for comparison of sample groups to get differentially expressed transcripts. There is an overwhelming number of software packages that can be used to perform RNA-Seq analyses such as Bowtie/Tophat/Cufflinks (Trapnell et al., 2012), STAR/RSem (Dobin et al., 2013), and Kallisto/Sleuth (Bray, Pimentel, Melsted, & Pachter, 2016; Pimentel, Bray, Puente, Melsted, & Pachter, 2017) among others (reviewed by (C. R. Williams, Baccarella, Parrish, & Kim, 2017)). Most read aligners and expression modelers have been shown to have comparable performance metrics, with heterogeneity in RNA-Seq analysis typically coming from the choice of differential expression analysis tool (Teng et al., 2016; C. R. Williams et al., 2017). Driven by my constraints in time and computing power resources, I decided to use Kallisto and the accompanying differential expression analysis software Sleuth for our data set (Figure 4.6).

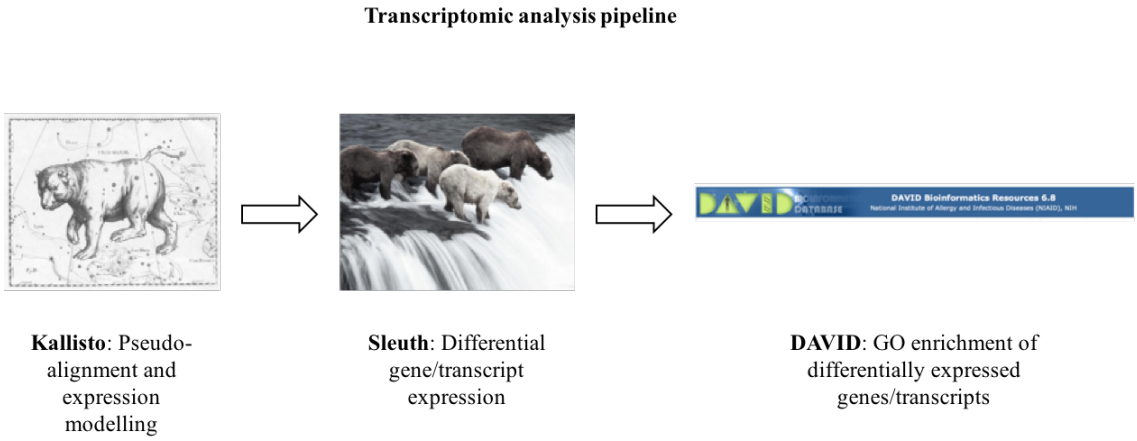


Figure 4.6: Transcriptomic analysis pipeline. *Pseudoalignment and expression modelling was performed using Kallisto version 0.43.1 (https://pachterlab.github.io/kallisto/). Transcripts were analysed for differential expression using Sleuth (https://pachterlab.github.io/sleuth/) and the resulting list was clustered based on gene ontology annotation.*

Kallisto works by pseudo-aligning clean reads from RNA-Seq to a reference genome and produces a list of compatible transcripts that they may be coming from (Bray et al., 2016). Pseudo-alignment avoids having to align every single base pair to a reference genome, making the "alignment" process significantly faster. The need for less computing power also makes Kallisto accessible even when using standard laptop computers. Furthermore, Kallisto has also been independently tested to have comparable accuracy to other alignment software (Costa-Silva, Domingues, & Lopes, 2017; Teng et al., 2016; C. R. Williams et al., 2017), giving me confidence that it will be suitable for my analysis. I used Sleuth for my differential transcript expression analysis, which uses analogous variation estimation (shrinkage) and between-sample normalisation algorithms similar to other popular differential expression tools like DESeq (Anders & Huber, 2010) (see Appendix for coding script). Gene ontology (GO) enrichment of differentially expressed transcripts were investigated using Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) (D. W. Huang, Sherman, & Lempicki, 2009b; 2009a).

4.3.3. RNA-Seq results

I analysed the transcriptomic data of *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP* and identified 202 genes that were differentially expressed between the two genotypes at a false discovery rate (FDR or q-value) ≤ 0.01. From the 202 genes, 100 genes were upregulated in *utx-1(tm3118)+UTX-1::GFP* with respect to *utx-1(tm3118)+UTX-1DD::GFP* (demethylase-dead controls), and the remaining 102 genes were downregulated (subset shown in Figure 4.7A; see Appendix for full list of transcripts). Upregulation of 2 genes from my transcript list (*C08E3.6 (fbxa-163)* and *F26D10.3.2 (hsp-1)*) was validated by qRT-PCR (Figure 4.7B). I recognised that the upregulation of *hsp-1* is not as striking in my validation experiments compared to results obtained from transcriptomic data. The transcriptomic data suggests that of the 2 isoforms of *hsp-1*, only F26D10.3.2 is upregulated and my primer pair cannot distinguish between the two, which could possibly mask the extent of upregulation. To understand the biological relevance of my RNA-seq results, I used DAVID to classify differentially expressed transcripts based on annotation in the GO database, emphasising importance on biological processes (BP) and molecular function (MF) sub-ontologies (Figure 4.7C and D).

A

Upregulated genes		Downregulated genes	
Transcript ID (gene)	**b-value[a]**	**Transcript ID**	**b-value[a]**
M60.2.1 (*endu-2*)	6.96	B0336.2.2 (*arf1.2*)	-6.30
T25B2.2a (*T25B2.2*)	4.42	Y116A8C.34a.2 (*cyn-13*)	-5.96
C36A4.14a (*C36A4.14*)	4.35	Y43D4A.5a (*Y43D4A.5*)	-5.44
F08H9.12 (*srz-97*)	3.66	K05C4.6a.1 (*hmp-2*)	-5.42
F26D10.3.2 (*hsp-1*)	3.63	Y43D4A.5b (*Y43D4A.5*)	-4.58
C24G7.1 (*delm-2*)	2.81	Y46D2A.2 (*Y46D2A.2*)	-2.63
C24G7.2 (*acd-1*)	2.60	K09A11.4 (*cyp-14A3*)	-1.82
T21E8.3 (*pgp-8*)	2.59	Y53G8AM.5 (*Y53G8AM.5*)	-1.69
C08E3.6 (*fbxa-163*)	2.39	T13F3.6 (*T13F3.6*)	-1.51

B

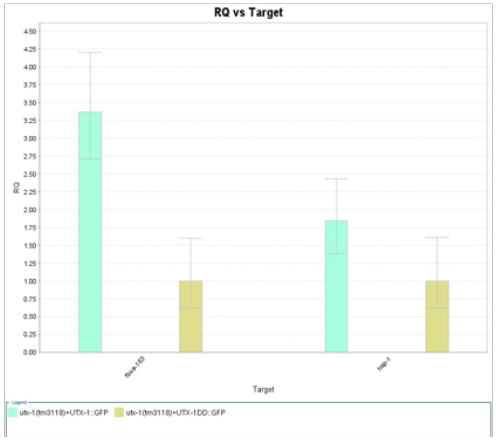


Figure 4.7: Differentially expressed transcripts based on RNA-seq experiment. (A) *Top 10 up- and down-regulated transcripts in utx-1(tm3118)+UTX-1::GFP compared to demethylase-dead controls with FDR ≤ 0.01;* ***(B)*** *qRT-PCR confirming upregulation of hsp-1 and fbxa-163 in utx-1(tm3118)+UTX-1::GFP animals compared to utx-1(tm3118)+UTX-1DD::GFP, ama-1 was used as an internal control; [a] b-value is the equivalent of log_2FC in the Kallisto/Sleuth analysis package (to be continued.)*

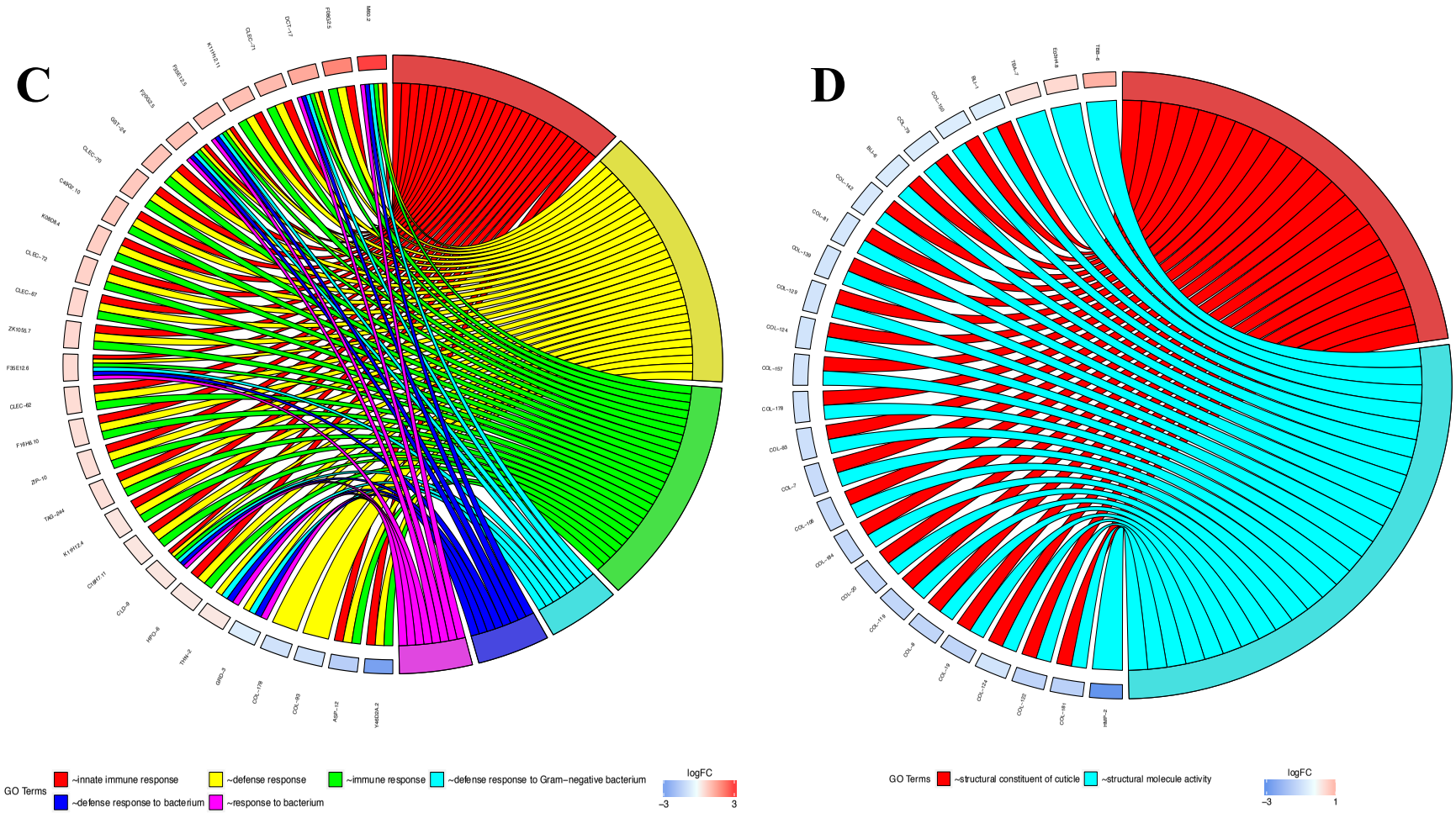


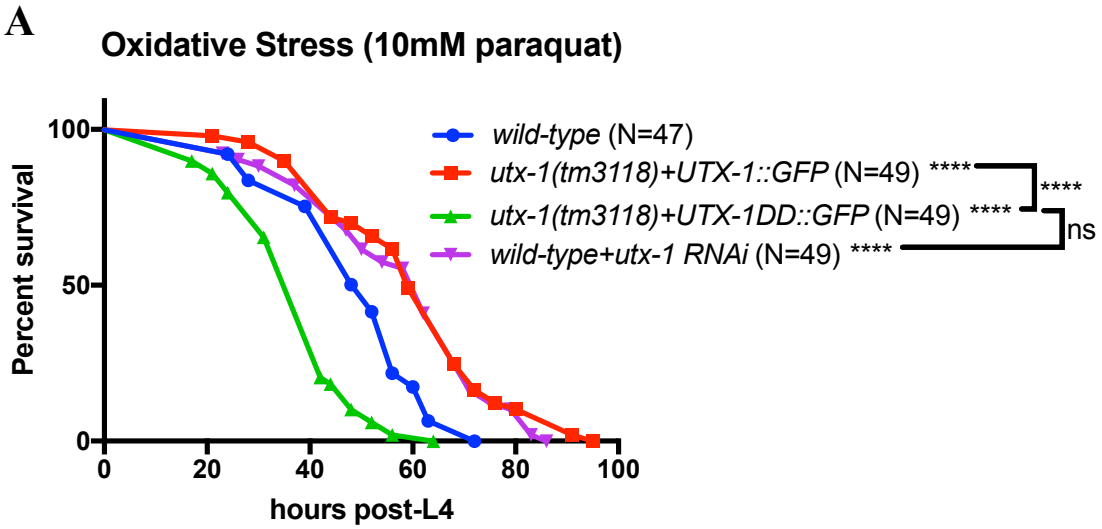
Figure 4.7 (cont.): *Differentially expressed transcripts were clustered according to* ***(C)*** *biological function (BP) sub-ontology and* ***(D)*** *molecular function (MF) sub-ontology.*

My studies revealed that differentially expressed transcripts were highly enriched for GO terms relating to immune and defense response – genes in this category were largely upregulated in the long-lived *utx-1(tm3118)+UTX-1::GFP* animals compared to demethylase-dead controls. To be more precise, GO terms that were significantly enriched (adj. pval < 0.01) were for immune (and innate) response, defense response and defense response to bacterium. Furthermore, my studies showed concomitant downregulation of genes involved in molecular functions pertaining to cuticle and structural molecule activity. Overall, my RNA-Seq and GO analysis suggest that the catalytic activity of UTX-1 possibly promotes longevity through increased expression of defense response, which is a general term for response against injury or foreign body to restrict organismal damage; and immune-response genes.

4.3.4. Stress-response of *utx-1* overexpressing worms

The transcriptomic data revealed that immune and defense response genes are broadly upregulated in the long-lived *utx-1(tm3118)+UTX-1::GFP* worms compared to *utx-1* mutants carrying the demethylase dead version. Furthermore, upregulation of genes implicated in stress-response such as *gst-23* and *hsp-1* in *utx-1(tm3118)+UTX-1::GFP* suggests that overexpression of catalytically active UTX-1 enhances resistance to stress. Long-lived worms are by and large associated with increased resistance to different stressors and pathogens, suggesting that increased ability to cope with stressful insults is important for promoting longevity (Inoue et al., 2005; Pujol et al., 2008; Yunger, Safra, Levi-Ferber, Haviv-Chesner, & Henis-Korenblit, 2017; Zhou et al., 2011). Therefore, I wanted to investigate if upregulation of defense response genes is correlated with increased stress resistance in long-lived wild-type *utx-1(+)* animals compared to *utx-1DD(+)*. The 'demethylase-dead' *utx-1(tm3118)+UTX-1DD::GFP* animals were chosen as an appropriate control, given my hypothesis that improved longevity in *utx-1*

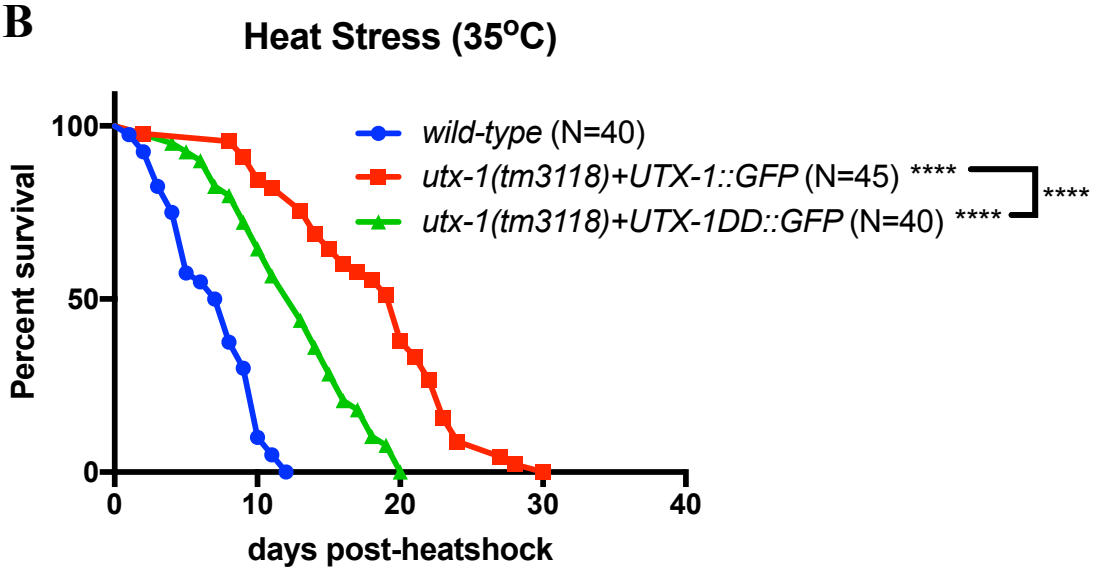
overexpressing worms is due to the catalytic activity of UTX-1. Therefore, comparing survival between *utx-1(tm3118)+UTX-1DD::GFP* and long-lived *utx-1(tm3118)+UTX-1::GFP* would allow me to investigate stress resistance that is likely due to the catalytic activity of UTX-1. I tested for 3 different stressors representing oxidative stress, heat stress and DNA damage (UV) (Figure 4.8A-C).



Oxidative Stress						
Genotype	**RNAi condition**	**Mean survival (hours)**	**Median survival (hours)**	**Maximum survival (hours)**	**Statistical significance (vs. *wild-type* in EV)**	**Statistical significance (vs. *utx-1(tm3118) +UTX-1DD::GFP*)**
wild-type	EV	49.0	52	72		
wild-type	*utx-1* RNAi	60.5	62	86	p<0.0001 (****)	
utx-1(tm3118)+ UTX-1::GFP	EV	37.9	59	95	p<0.0001 (****)	p<0.0001 (****)
utx-1(tm3118)+UTX-1DD::GFP	EV	58.9	42	64	p<0.0001 (****)	

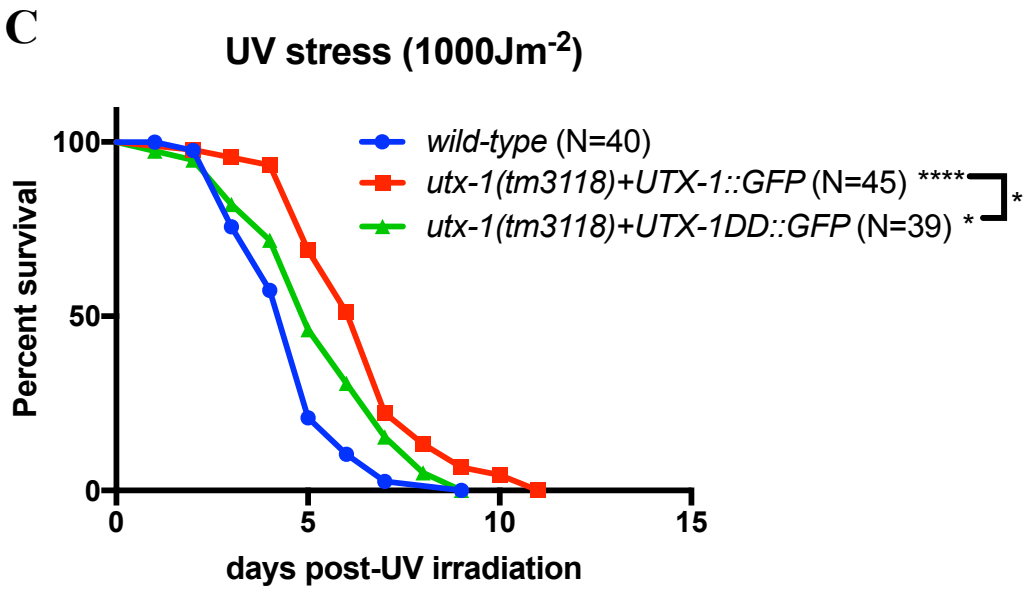
Figure 4.8: Stress resistance of utx-1 overexpressing worms. (A) *Oxidative stress: at least 60 L4-stage worms of each genotype were picked and exposed to 10mM paraquat. Animals were scored as alive or dead every 4 hours. Survival of utx-1 overexpressing animals and worms subjected to utx-1 RNAi were compared to wild-type control animals for lifespan analysis under oxidative stress conditions; (to be continued.)*

B



Heat Stress					
Genotype	**Mean lifespan (days)**	**Median lifespan (days)**	**Maximum lifespan (days)**	**Statistical significance (vs *wild-type*)**	**Statistical significance (vs. *utx-1(tm3118) +UTX-1DD::GFP*)**
wild-type	6.9	7.5	12		
utx-1(tm3118)+ UTX-1::GFP	18.0	20	30	p<0.0001 (****)	p<0.0001 (****)
utx-1(tm3118)+UTX-1DD::GFP	12.5	13	20	p<0.0001 (****)	

Figure: 4.8 (cont.): (B) *Heat stress: at least 50 day-5 adult worms of each genotype were picked and exposed 35°C for 6 hours. Animals were scored as alive or dead every day. Survival of utx-1 overexpressing worms were compared to wild-type control animals for lifespan analysis under heat stress conditions; (to be continued.)*



UV Stress					
Genotype	**Mean lifespan (days)**	**Median lifespan (days)**	**Maximum lifespan (days)**	**Statistical significance (vs *wild-type*)**	**Statistical significance (vs. *utx-1(tm3118) +UTX-1DD::GFP*)**
wild-type	4.6	5	9		
utx-1(tm3118)+ UTX-1::GFP	6.5	7	11	p<0.0001 (****)	p=0.0177 (*)
utx-1(tm3118)+UTX-1DD::GFP	5.4	5	9	p=0.0274 (*)	

Figure 4.8 (cont.): (C) *UV stress: at least 50 day-3 adult worms of each genotype were picked and exposed to 1000J/m^2 of UVC radiation. Animals were scored as alive or dead every day. Survival of utx-1 overexpressing worms were compared to wild-type control animals for lifespan analysis under UV stress conditions. Statistical significance was calculated using Mantel-Cox method for all stress assays. Heat stress was only performed once, oxidative and UV stress had 2 repeats each.*

Oxidative stress

Oxidative stress was induced by treating animals with 10 mM of paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride). Paraquat was historically used as an herbicide and functions by interfering with the electron transport chain, particularly by depleting electron-donating sources such as NADPH, to form superoxide anions (a major reactive oxygen species). I compared the survival of long-lived *utx-1(tm3118)+UTX-1::GFP* worms to wild-type and *utx-1(tm3118)+UTX-1DD::GFP* controls. I also included animals with depleted levels of *utx-1*, since downregulation of *utx-1* has also previously been reported to improve tolerance to oxidative stress (C. Jin et al., 2011a). Animals were

exposed to paraquat from L4-stage onwards and survival assessed 24 hours post-exposure at 4 hour intervals (Figure 4.8A). I found that *utx-1(tm3118)+UTX-1::GFP* animals were significantly more resistant to oxidative stress than wild-type and demethylase-dead controls. Interestingly, the *utx-1(tm3118)* mutants rescued with demethylase-dead UTX-1 were even more sensitive to oxidative stress than wild-type, which suggests that UTX-1DD acts as dominant negative with respect to oxidative stress. Furthermore, animals with depleted levels of *utx-1* are resistant to oxidative stress compared to wild-type, supporting previously published data. Mirroring the lifespan results when *utx-1* is knocked down or overexpressed (as described in chapter 3), resistance to oxidative stress is improved regardless of overexpression or knockdown of (catalytically active) *utx-1*, indicating that parallel mechanisms regulate resistance to oxidative stress.

Heat stress

Heat-shock was performed by transferring 5-day old worms from 20ºC (routine temperature for maintenance) to 35ºC for 6 hours before returning to 20ºC for the rest of the survival assay (Figure 4.8B). I deliberately performed the experiment on post-reproductive animals due to my observation that heat-shock on actively reproducing animals lead to higher incidences of bagging. My data showed that *utx-1(tm3118)+UTX-1::GFP* animals were more thermotolerant than *utx-1(tm3118)+UTX-1DD::GFP* controls, which in turn were more resistant to heat stress than wild-type worms. Although I did not include animals subjected to *utx-1* RNAi to determine how *utx-1* downregulation affects response to heat stress. However, *utx-1* depletion has been previously reported to increase resistance to heat stress compared to wild-type animals. Since my observations indicate that the catalytic activity of UTX-1 plays an important role in mediating response to heat stress as well, it is possible that parallel mechanisms are involved in regulating heat stress upon up- or down-regulation of *utx-1*.

DNA damage

Worms were staged to day-3 adults before being exposed to UVC (254 nm) radiation using the Stratalinker UV crosslinker (Stratagene) and assessed for survival (Figure 4.8C). The wavelength of UVC closely matches the maximum absorption of purine (A and G) and pyrimidine (C, T and U) nucleobases, making it the most efficient (compared to UVA and UVB) inducer of photochemical reactions within the DNA and causing DNA damage (Gentile, Latonen, & Laiho, 2003; Ravanat, Douki, & Cadet, 2001). Consistent with the pattern I observed under heat stress conditions, I established that *utx-1(tm3118)+UTX-1::GFP* is moderately more resistant to UV stress than demethylase-dead counterparts, which in turn are more resistant than wild-type worms – presumably due to the catalytic activity of UTX-1.

Transcriptomic analysis revealed that the catalytic activity of UTX-1 is associated with upregulation of immune and defense response genes. This is consistent with observations in my stress response assays, where *utx-1(tm3118)* animals rescued with a catalytically functioning UTX-1 constantly displayed increased stress resistance compared to DD-rescue as well as wild-type animals.

4.3.5. RNAi screen for genes involved in lifespan mediated by *utx-1* overexpression

Transcriptomic analysis of *utx-1* overexpressing worms led to a list of 202 differentially expressed transcripts due to the catalytic activity of UTX-1 – half of which were upregulated in the longer-lived strain overexpressing a catalytically functioning UTX-1. Given that the catalytic activity of UTX-1 demethylates transcriptionally repressive H3K27me2/3 marks specifically, there is the possibility that upregulation of certain genes in *utx-1(tm3118)+UTX-1::GFP* animals are due to removal of H3K27me2/3 marks on those particular loci (ie. direct targets). Bearing in mind that a significant proportion of

these upregulated genes are involved in defense response – a feature that is often associated with improved longevity – therein lies a potential link for how the catalytic activity of UTX-1 mediates ageing and longevity in worms. The next question I wanted to address is to determine whether there are any specific genes from my transcriptomic data that could mediate lifespan specifically through overexpression of wild-type *utx-1*. The question was tackled by conducting an RNAi screen on genes that were significantly upregulated in *utx-1(tm3118)+UTX-1::GFP* based on my transcriptomic data and assessing how lifespan is affected. I performed RNAi and survival assays on both *utx-1(tm3118)+UTX-1::GFP* and wild-type animals so that I was able to disentangle whether the gene candidates regulate lifespan generally, or specifically through the catalytic activity of UTX-1. The candidate genes in my RNAi screen were some of the most upregulated genes in *utx-1(tm3118)+UTX-1::GFP* with respect to *utx-1(tm3118)+UTX-1DD::GFP* (Table 4.2).

Table 4.2: List of genes for RNAi screen. *RNAi clones for genes on the list were verified by sequencing.* [a] *b-value is the log_2FC equivalent outputted by Kallisto software; b-values are calculated using utx-1(tm3118)+UTX-1DD::GFP as baseline controls.* [b] *Gene functions were mined from www.wormbase.org.*

Gene name	Sequence ID	b-value [a] (log_2FC)	Function [b]
delm-2	C24G7.1	2.81	Uncharacterised; predicted to have sodium ion channel activity; degenerin linked to mechanosensation
fbxa-163	C08E3.6	2.39	Uncharacterised; contains F-box motif and FTH/DUF38 motif, both predicted to mediate protein-protein interactions
parg-2	H23L24.5	1.74	Encodes a poly(ADP-ribose) glycohydrolase (PARG); orthologous to human PARG and paralogous to PARG-1; PARG-2 Exhibits poly(ADP-ribose) glycohydrolase activity *in vitro*, and is required *in vivo* for normal resistance to ionising radiation
clec-71	Y46C8AL.4	1.21	Uncharacterised; C-type lectin family
Y69A2AR.25	Y69A2AR.25	1.34	Uncharacterised
Y69A2AR.12	Y69A2AR.12	1.03	Uncharacterised
hsp-1	F26D10.3	3.63	Encodes hsp70A, a member of the heat shock family of proteins Down-regulation of *hsp-1* via RNAi results in a small reduction in the life-span of an age-1 mutant indicating that hsp-1 may play some role in regulating longevity.
K11H12.11	K11H12.11	1.11	Uncharacterised
T26H5.9	T26H5.9	1.18	Uncharacterised

srg-31	T07H8.5	1.09	Uncharacterised; predicted to have transmembrane signaling receptor activity
tbb-6	T04H1.9	1.36	Ortholog of human TUBB1 (tubulin beta 1 class VI) and TUBB8 (tubulin beta 8 class VIII); predicted to have GTP binding activity, GTPase activity, and is a structural constituent of cytoskeleton, based on protein domain information
spp-9	T25C12.2	1.47	Uncharacterised; saposin-like protein family

I wanted to conduct an RNAi screen on gene candidates with a $\log_2$FC > 1, which included 40 candidate genes for my screen. However, problems with RNAi clones in the *C. elegans* RNAi library (Source Bioscience) meant abandoning around 30 genes either due to bacterial cultures not growing or inconsistency in sequencing upon validation. [*]

4.3.6. RNAi screen results

Lifespan of animals exposed to RNAi conditions were evaluated against empty vector controls and the results broadly fell into four categories based on lifespan change observed compared to empty vector controls in both wild-type and *utx-1(tm3118)+UTX-1::GFP* (Table 4.3).

Table 4.3: Summary of RNAi screen results*. Survival assays of animals subjected to RNAi were compared to empty vector controls;* [a] *up = lifespan extension, down = lifespan shortening and nc = no change when compared to empty vector conditions.*

Phenotypic category	**RNA target**	**Lifespan change compared to empty vector controls** [a]	
		wild-type	*utx-1(tm3118)+UTX-1::GFP*
A	*clec-71, parg-2, K11H12.11*	nc	nc
B	*fbxa-163, Y69A2AR.25, srg-31, spp-9, T26H5.9*	up	up
C	*delm-2, tbb-6, Y69A2AR.12*	nc	up
D	*hsp-1*	nc	down

[*] The Ahringer RNAi library used during this study has now been "retired" and a new set was recently obtained.

For candidate genes *clec-71*, *parg-2* and *K11H12.11*, no significant lifespan change was observed between RNAi conditions and empty vector controls in both *wild-type* and *utx-1(tm3118)+UTX-1::GFP* animals, suggesting that these three genes are not involved in lifespan regulation (Figure 4.9; Table 4.4).

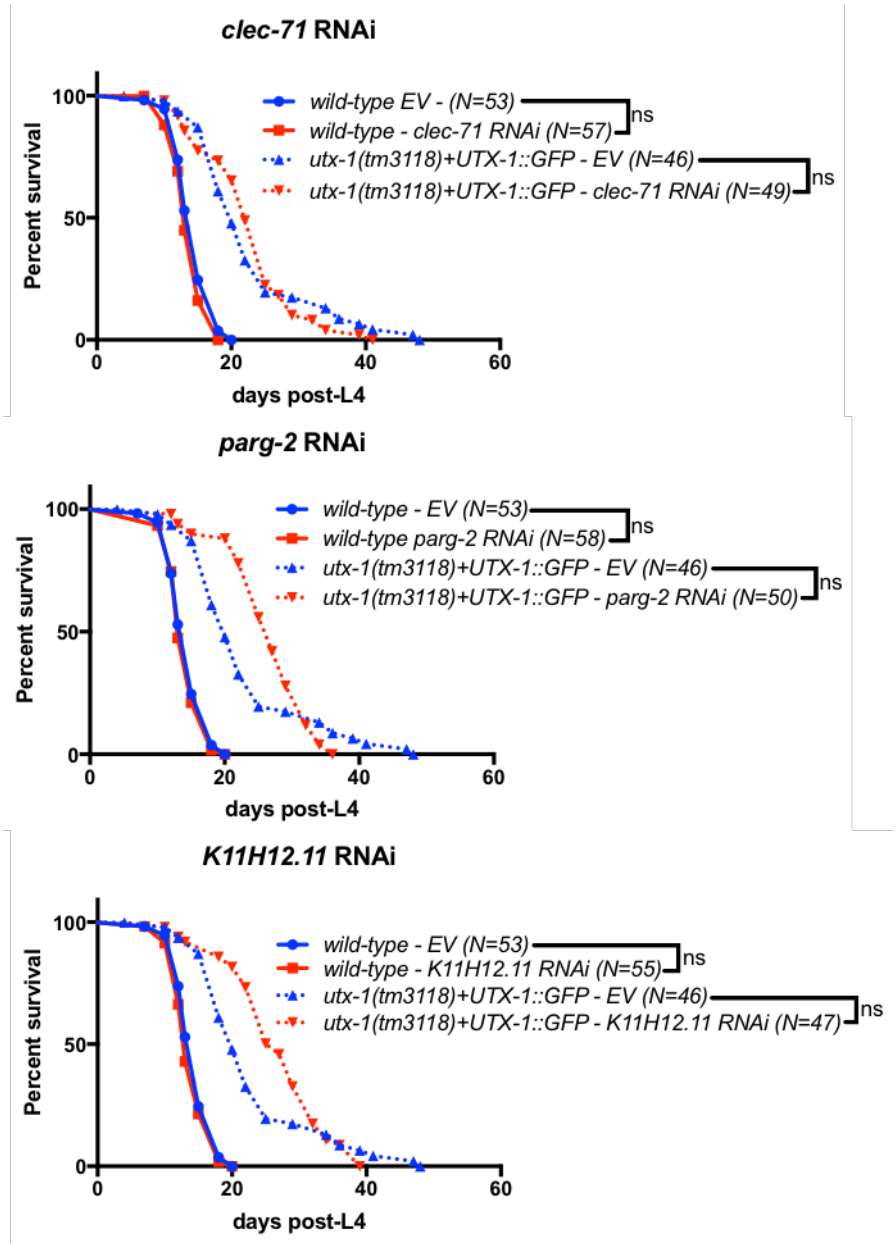


Figure 4.9: RNAi screen - phenotypic category A - no change in lifespan after RNAi of target genes. *For each strain, at least 60 L4-stage animals were subjected to either EV or candidate gene RNAi. Animals were scored as alive or dead at least every 2 days. Survival was compared between EV and candidate gene RNAi for each strain assessed. No statistical significance found on EV vs RNAi conditions for clec-71, parg-2 and K11H12.11 RNAi. See Table 4.4 for mean lifespan, median lifespan, maximum lifespan and p-values. Statistical significance calculated using Mantel-Cox method. Lifespan assays only performed once.*

Table 4.4: Maximum lifespan, median lifespan, mean lifespan and statistical significance for clec-71, parg-2 and K11H12.11 RNAi

Genotype	RNAi condition	Mean lifespan (days)	Median lifespan (days)	Maximum lifespan (days)	Statistical significance (vs EV)
wild-type	EV	14.4	15	20	p=0.1731 (ns)
wild-type	*clec-71* RNAi	13.8	13	18	
utx-1(tm3118)+ UTX-1::GFP	EV	23.1	20	48	p=0.9173 (ns)
utx-1(tm3118)+ UTX-1::GFP	*clec-71* RNAi	22.7	22	41	
wild-type	EV	14.4	15	20	p=0.3321 (ns)
wild-type	*parg-2* RNAi	14.2	13	20	
utx-1(tm3118)+ UTX-1::GFP	EV	23.1	20	48	p=0.1216 (ns)
utx-1(tm3118)+ UTX-1::GFP	*parg-2* RNAi	26.6	27	36	
wild-type	EV	14.4	15	20	p=0.3632 (ns)
wild-type	*K11H12.11 RNAi*	13.9	13	20	
utx-1(tm3118)+ UTX-1::GFP	EV	23.1	20	48	p=0.0826 (ns)
utx-1(tm3118)+ UTX-1::GFP	*K11H12.11 RNAi*	26.6	27	39	

My screen also identified 5 genes – *fbxa-163*, *srg-31*, *Y69A2AR.25*, *spp-9* and *T26H5.9* – that extend lifespan in both *wild-type* and *utx-1(tm311)+UTX-1::GFP*, suggesting that these genes regulate lifespan in parallel to *utx-1* mediated longevity (Figure 4.10; Table 4.5). *fbxa-163* was previously reported to be upregulated in *wild-type*, *xpa-1* (NER-deficient mutants) and *glp-1* animals upon UVC stress, indicating a possible role in DNA damage repair (Boyd et al., 2010); while *spp-9* was reported to function in innate immune response and antimicrobial activity (Nicholas & Hodgkin, 2004). Apart from this, the 5 genes remain largely uncharacterised and my screen uncovered a possible novel role for *fbxa-163*, *srg-31*, *Y69A2AR.25*, *spp-9* and *T26H5.9* in promoting longevity.

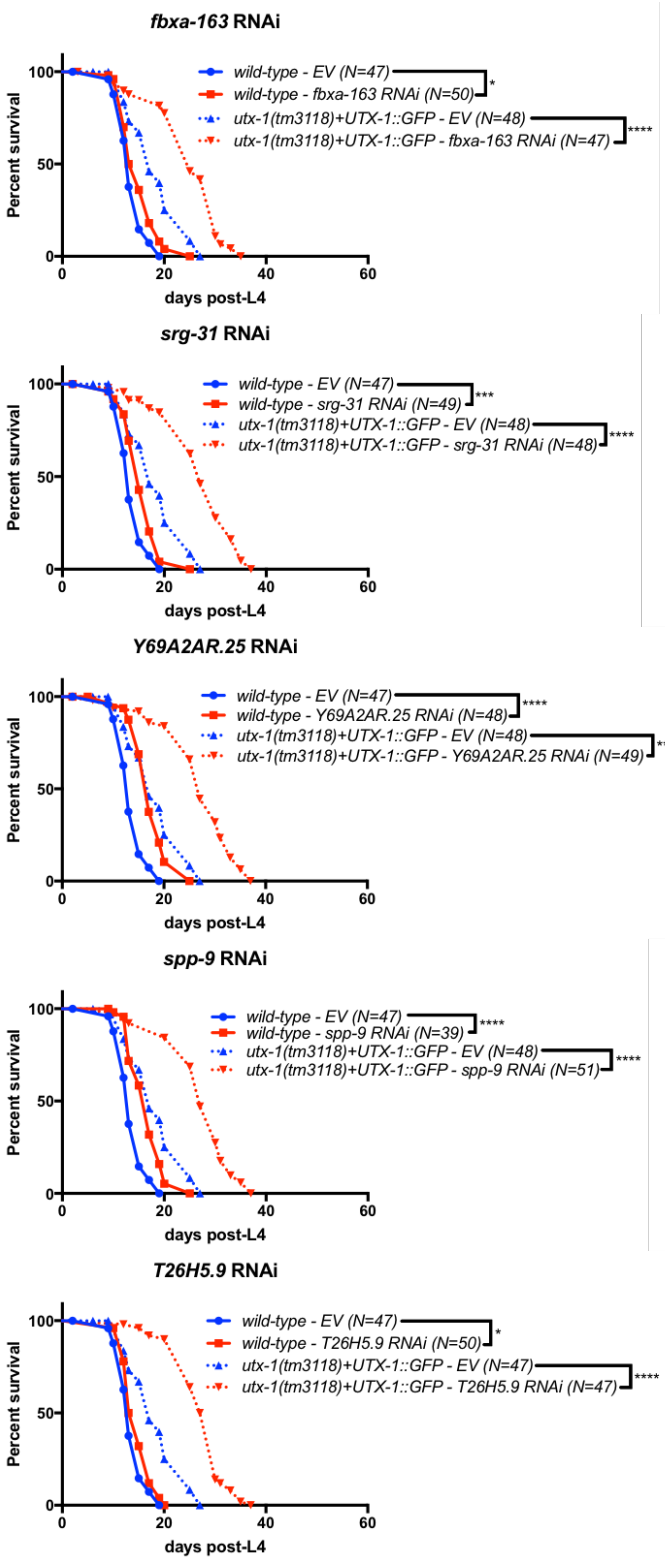


Figure 4.10: RNAi screen - phenotypic category B - lifespan extension observed in both wild-type and utx-1(tm3118)+UTX-1::GFP when subjected to RNAi of target genes. *For each strain, at least 60 L4-stage animals were subjected to either EV or candidate gene RNAi. Animals were scored as alive or dead at least every 2 days. Survival was compared between EV and candidate gene RNAi for each strain assessed. See Table 4.5 for mean lifespan, median lifespan, maximum lifespan and p-values. Statistical significance calculated using Mantel-Cox method. Lifespan assays only performed once.*

Table 4.5: Maximum lifespan, median lifespan, mean lifespan and statistical significance for fbxa-163, srg-31, Y69A2AR.25, spp-9 and T26H5.9 RNAi.

Genotype	RNAi condition	Mean lifespan (days)	Median lifespan (days)	Maximum lifespan (days)	Statistical significance (vs EV)
wild-type	EV	13.4	13	19	
wild-type	*fbxa-163* RNAi	15.0	14	25	p=0.0220 (*)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*fbxa-163* RNAi	25.0	25	35	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*srg-31* RNAi	15.5	15	25	p=0.0009 (***)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*srg-31* RNAi	27.1	27	37	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*Y69A2AR.25 RNAi*	17.5	17	25	p<0.0001 (****)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*Y69A2AR.25 RNAi*	26.9	27	37	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*spp-9 RNAi*	16.4	17	25	p<0.0001 (****)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*spp-9 RNAi*	27.3	27	37	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*T26H5.9 RNAi*	14.6	14	20	p=0.0396 (*)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*T26H5.9 RNAi*	27.5	28.5	37	p<0.0001 (****)

Another pattern that I observed from the screen was one in which RNAi of candidate genes did not affect the lifespan of wild-type worms compared to empty vector controls, but exhibited lifespan extension when knocked down in long-lived *utx-1* overexpressing strain (Figure 4.11; Table 4.6). The RNAi candidate genes showing this phenotype were *delm-2* (putative sodium ion channel), *tbb-6* (beta-tubulin) and *Y69A2AR.12*. I posit that *delm-2*, *tbb-6* and *Y69A2AR.12* may not necessarily be involved in *utx-1* mediated lifespan regulation, but could possibly reflect activation of compensatory mechanism/s induced by overexpression of *utx-1* (Dong et al., 2007). Interestingly, *tbb-6* was recently implicated as a downstream effector of the p38 signalling cascade (DLK-1 (MAP3K)-SEK-3(MAP2K)-PMK-3 (MAPK)), which is implicated in lifespan extension upon

disruption of the mitochondrial electron transport chain in worms. This suggests a possible role for *tbb-6* in mediating ageing and longevity (Munkácsy et al., 2016).

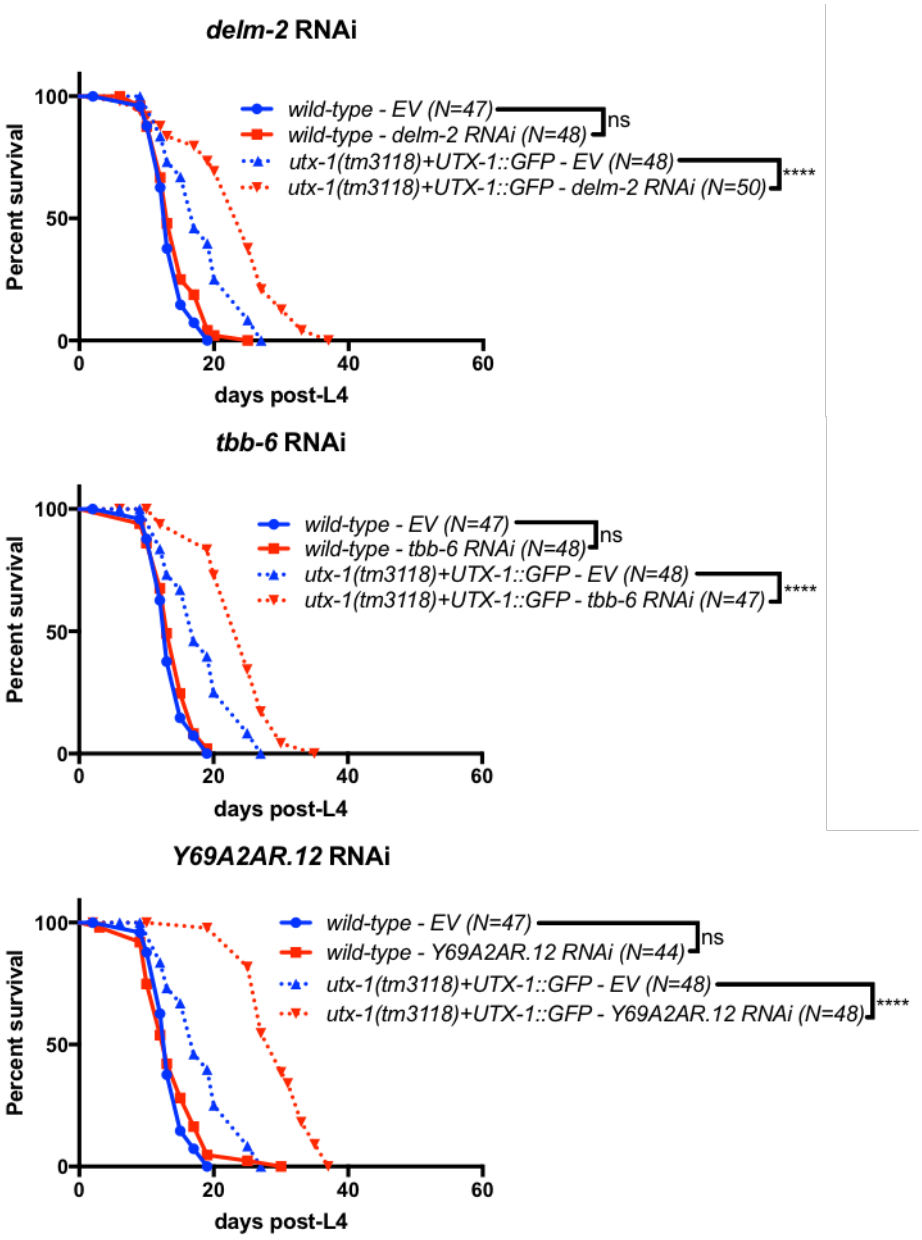


Figure 4.11: RNAi screen - Phenotypic category C - lifespan extension was observed in utx-1(tm3118)+UTX-1::GFP, but not in wild-type when subjected to RNAi conditions. *For each strain, at least 60 L4-stage animals were subjected to either EV or candidate gene RNAi. Animals were scored as alive or dead at least every 2 days. Survival was compared between EV and candidate gene RNAi for each strain assessed. See Table 4.6 for mean lifespan, median lifespan, maximum lifespan and p-values. Statistical significance calculated using Mantel-Cox method. Lifespan assays only performed once.*

Table 4.6: Maximum lifespan, median lifespan, mean lifespan and statistical significance for delm-2, tbb-6 and Y69A2AR.12 RNAi.

Genotype	RNAi condition	Mean lifespan (days)	Median lifespan (days)	Maximum lifespan (days)	Statistical significance (vs EV)
wild-type	EV	13.4	13	19	
wild-type	*delm-2* RNAi	14.4	13	25	p=0.1361 (ns)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*delm-2* RNAi	23.6	25	37	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*tbb-6* RNAi	13.9	13	19	p=0.3011 (ns)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*tbb-6* RNAi	24.4	25	35	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*Y69A2AR.12 RNAi*	13.8	13	30	p=0.4877 (ns)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*Y69A2AR.12 RNAi*	29.8	30	37	p<0.0001 (****)

Lastly, I observed that knockdown of *hsp-1* in wild-type animals had no effect on lifespan. However, knockdown of *hsp-1* in long-lived *utx-1* overexpressing worms completely abolished lifespan extension, with lifespan now comparable to wild-type worms (Figure 4.12; Table 4.7). The observed lifespan patterns were consistent over two different *hsp-1* RNAi clones used. This data implies that lifespan extension due to overexpression of a catalytically active UTX-1 requires *hsp-1*.

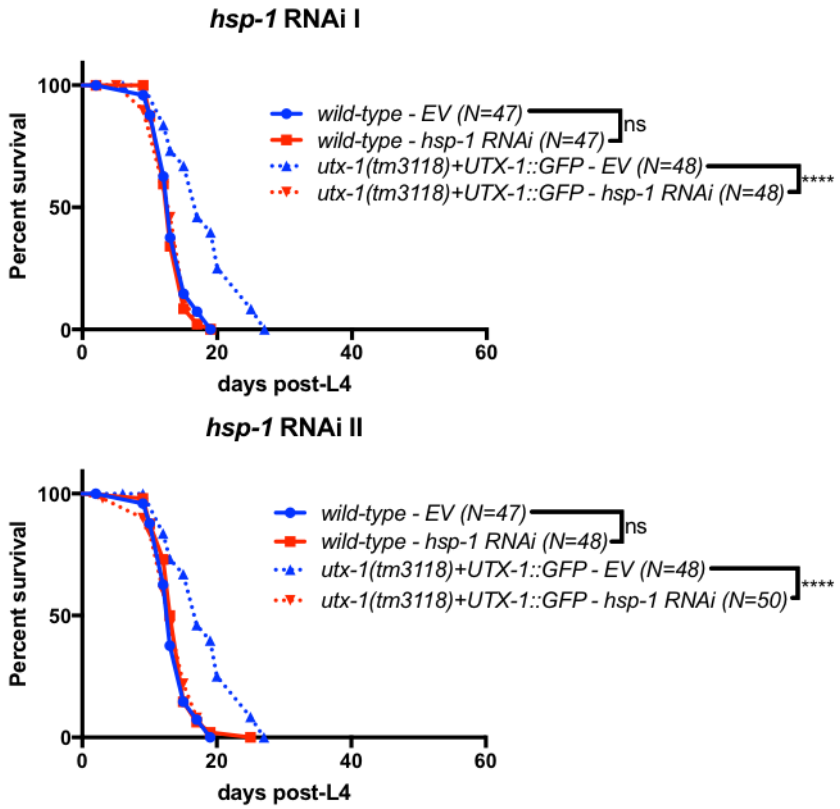


Figure 4.12: RNAi screen - Phenotypic category D - lifespan extension was abolished in utx-1(tm3118)+UTX-1::GFP when subjected to RNAi. *For each strain, at least 60 L4-stage animals were subjected to either EV or candidate gene RNAi. Animals were scored as alive or dead at least every 2 days. Survival was compared between EV and candidate gene RNAi for each strain assessed. See Table 4.7 for mean lifespan, median lifespan, maximum lifespan and p-values. Statistical significance was calculated using Mantel-Cox method. Lifespan assays only performed once. Note that both experiments were performed at the same time, therefore the empty vector controls used for each hsp-1 RNAi tested is identical.*

Table 4.7: Maximum lifespan, median lifespan, mean lifespan and statistical significance for hsp-1 RNAi.

Genotype	**RNAi condition**	**Mean lifespan (days)**	**Median lifespan (days)**	**Maximum lifespan (days)**	**Statistical significance (vs EV)**
wild-type	EV	13.4	13	19	
wild-type	*hsp-1* RNAi I	13.2	13	19	p=0.4343 (ns)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*hsp-1* RNAi I	13.5	13	19	p<0.0001 (****)
wild-type	EV	13.4	13	19	
wild-type	*hsp-1* RNAi II	14.0	15	25	p=0.3923 (ns)
utx-1(tm3118)+ UTX-1::GFP	EV	18.4	17	27	
utx-1(tm3118)+ UTX-1::GFP	*hsp-1* RNAi II	13.6	13	19	p<0.0001 (****)

4.4. DISCUSSION

4.4.1. The catalytic activity of UTX-1 is crucial for longevity

Previous work by Dr Karolina Chocian showed that overexpression of a catalytically functional UTX-1 in wild-type animals promoted lifespan extension (Figure 4.1), implicating the catalytic function of UTX-1 to be involved in lifespan regulation (Chocian, 2015). Repeating the same lifespan experiments in the *utx-1(tm3118)* mutant background showed that *utx-1* heterozygous mutants are expectedly longer-lived than wild-type animals and have comparable lifespan to *utx-1(tm3118)+UTX-1DD::GFP* animals (Figure 4.2) (Chocian, 2015). While *utx-1DD* rescued the developmental phenotypes of *utx-1* mutants, it does not rescue longevity. This suggests that *utx-1* mediates lifespan and development in distinct pathways.

The increased longevity of *utx-1(tm3118)+UTX-1DD::GFP* animals compared to wild-type indicates that lifespan extension is likely mediated by the presence of the *utx-1* mutation. Furthermore, overexpression of a functional UTX-1 in *utx-1* mutants promoted lifespan extension beyond that of *utx-1* heterozygous mutants, reinforcing the idea that the catalytic activity of UTX-1 is important in lifespan regulation. The fact that there seems to be an additive lifespan extending effect between the *utx-1* mutation and UTX-1::GFP overexpression (ie. in *utx-1(tm3118)+UTX-1::GFP* animals) suggests that lifespan regulation due to *utx-1* haploinsufficiency and wild-type UTX-1 overexpression are at least partially mediated by distinct pathways. Considering my lifespan results in the previous chapter where lifespan extension due to *utx-1* knockdown is largely dependent on the *daf-2/daf-16* pathway, while *utx-1* overexpression is only partially so, further strengthens the idea that lifespan regulation due to *utx-1* up- and downregulation are mediated by independent mechanisms.

The UTX-1 demethylase has been previously shown to specifically remove transcriptionally repressive H3K27me3 marks (Agger et al., 2007). The finding that the catalytic activity of UTX-1 is important in promoting lifespan extension suggests that UTX-1 is directly involved in the transcriptional regulation of pro-longevity genes. UTX-1 is a member of the KDM6 family of H3K27 demethylases, of which there are 3 other members in *C. elegans*: JMJD-3.1, JMJD-3.2 and JMJD-3.3 (Agger et al., 2007; Klose et al., 2006). Of the three, only *jmjd-3.2* was found to be involved in lifespan regulation. Interestingly, both overexpression and downregulation of *jmjd-3.2* also promote lifespan extension (Chocian, 2015). In contrast to *utx-1*, overexpression of *jmjd-3.2* promotes lifespan extension regardless of whether the demethylase activity is functional or not, suggesting that the catalytic function of JMJD-3.2 is dispensable for lifespan regulation (Chocian, 2015). Mammalian JMJD3 and UTX are known to interact with the MLL3/4 methyltransferase complex that targets H3K4 and the SWI/SNF chromatin remodeling complex (Miller et al., 2010); and the interaction of *utx-1* has since been verified to interact with the equivalent MLL-like methyltransferase (SET-16) in *C. elegans* (Vandamme, Lettier, Sidoli, Di Schiavi, Jensen, et al., 2012a). Both JMJD3 and UTX are known to have functions that are distinct from their enzymatic activity. For instance, UTX and JMJD3 facilitate the interaction between T-box transcription factors and the SWI/SNF complex to promote chromatin remodeling and facilitate differentiation in mice (Miller et al., 2010). Since the catalytic activity of JMJD-3.2 is dispensable for lifespan extension in worms, I postulate that *jmjd-3.*2 overexpression promotes lifespan extension by facilitating protein-protein interactions with chromatin modifiers. In contrast, *utx-1* overexpression is mediated by the H3K27 demethylase function.

4.4.2. The UTX-1 catalytic activity is involved in upregulation of defense/immune response and downregulation of collagen genes

Given that the UTX-1 catalytic activity was implicated in promoting lifespan extension in the context of *utx-1* overexpression, I postulated that UTX-1 might regulate transcription of pro-longevity genes to promote lifespan extension. To investigate this, I performed RNA-Seq on *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP*; and compared their expression profiles to assess transcriptional changes mediated by the UTX-1 catalytic activity. *daf-2* expression was previously found to be upregulated in *utx-1(tm3118)+UTX-1::GFP* compared to UTX-1DD "rescued" animals (Chocian, 2015). However, I did not find *daf-2* to be upregulated in the transcriptomic analysis of long-lived *utx-1(tm3118)+UTX-1::GFP*. *daf-2* is known to be expressed in the neurons, intestine and hypodermis and is temporally regulated, therefore the fact that I used mixed-stage whole-worm extracts for my RNA-Seq experiments might mask the upregulation of *daf-2.* This highlights a particular limitation in worm transcriptomic studies. Because culturing specific cells in the worm is not straightforward, transcription profiling in worms have been largely done on whole-worm extracts. Using whole-worm extracts could potentially mask transcripts that are expressed only in specific tissues or ones that are not highly expressed in the worm to begin with. In light of improving technologies to probe tissue-specific expression profiles by using fluorescence-associated cell sorting (FACS) to collect cells of interest prior to RNA-Seq, I would opt to probe transcription profiles of *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP* worms in a tissue-specific manner. This will especially be useful given my previous findings that *utx-1* mediates lifespan extension in a tissue-specific manner.

Comparative analysis of the transcriptome of long-lived *utx-1(tm3118)+UTX-1::GFP* animals against *utx-1(tm3118)+UTX-1DD::GFP* controls showed that 202 transcripts

were differentially expressed between the two genotypes (FDR < 0.01) – half of which were upregulated in the *utx-1(tm3118)+UTX-1::GFP*. Enrichment analysis for gene ontology (GO) terms on the differentially expressed transcripts revealed that upregulated genes are enriched for defense response, immune response and response to bacteria. In contrast, many collagen genes were strikingly downregulated (Figure 4.7). I speculate that the upregulation of immune response and defense response genes in long-lived *utx-1(tm3118)+UTX-1::GFP* animals might serve as a protective mechanism in worms that might indirectly lead to lifespan extension. *C. elegans* are conventionally fed the *E. coli* strain OP50 in the lab, which is considered to be mildly pathogenic to worms. Upregulation of immune response genes mediated by *utx-1* overexpression might be protective against this pathogenicity, which could indirectly promote longevity.

Remarkably, collagen genes are largely downregulated in *utx-1(tm3118)+UTX-1::GFP* animals. Collagens have been previously implicated in lifespan regulation and is regulated by the transcription factor SKN-1. SKN-1 was found to mediate lifespan regulation due to reduction of insulin signalling in non-dauer conditions (Ewald et al., 2015) and transcriptomic analysis showed that SKN-1 promotes upregulation of collagen genes, expression of which, tend to decrease with age. Furthermore, knockdown of most collagen genes tested in long-lived non-dauer *daf-2* mutants diminished lifespan extension, suggesting that collagen genes promote longevity in these conditions (Ewald et al., 2015). The authors of study suggested that enhanced collagen transcription might promote collagen remodeling in *C. elegans*, akin to slowing down the decline of skin integrity in humans, and propose that collagen genes are involved in a longevity-assurance pathway.

Many collagen genes are expressed in the hypodermis, which suggests that transcriptional downregulation of collagens in the long-lived *utx-1(tm3118)+UTX-1::GFP* worms largely affect this tissue. In the previous chapter, I showed that hypodermis-specific knockdown of *utx-1* promoted lifespan extension (Figure 3.9), while hypodermis-specific overexpression of *utx-1* does not (Figure 3.10). Given that *utx-1* overexpression is associated with downregulation of collagen genes, I hypothesise that hypodermis-specific knockdown of *utx-1* could lead to transcriptional upregulation of collagen genes, which could promote lifespan extension particularly due to the hypodermis. To investigate the involvement of collagen genes due to hypodermal-specific knockdown of *utx-1*, I would simultaneously knockdown *utx-1* and the collagen genes that were downregulated in my transcriptomic data in the hypodermis and assess whether lifespan extension is abrogated or not. Differential transcript expression of wild-type animals subjected to *utx-1* and EV RNAi could also be useful in determining whether collagen upregulation is involved in lifespan extension due to hypodermis-specific knockdown of *utx-1*.

4.4.3. The catalytic activity of UTX-1 promotes stress resistance

Overexpression of a catalytically functional UTX-1 promotes lifespan extension and is associated with upregulation of defense and immune response genes. In light of this, I also wanted to investigate whether animals overexpressing a catalytically wild-type UTX-1 is associated with increased resistance to stressors compared to UTX-1DD worms. Increased stress resistance is often seen as a protective mechanism that could indirectly promote lifespan extension. Indeed, a lot of long-lived mutants such as *daf-2*, *age-1*, *eat-2* and *glp-1* mutants are all associated with increased resistance to certain stressors. In contrast, down regulation of longevity-associated genes such as *daf-16*, *hsf-*

1 and *skn-1* are stress-sensitive to specific stressors compared to wild-type (Zhou et al., 2011).

To test for stress-resistance, I compared the survival of long-lived *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP* when subjected to oxidative stress (using paraquat – a drug that promotes ROS production), heat-shock (by acutely subjecting worms to 35^{o}C for 6 hours) and DNA damage stress (using 1000J/m^2 UVC) (Figure 4.7). In all cases, animals overexpressing UTX-1::GFP are more resistant to stress than animals overexpressing UTX-1DD, suggesting that the catalytic activity of UTX-1 is involved in enhancing resistance to oxidative, heat and DNA damage stress. *utx-1* depletion has been previously reported to increase resistance to oxidative, heat and UV stress as well (C. Jin et al., 2011a). In my oxidative stress study, I found that worms feeding on *utx-1* RNAi are stress-resistant compared to wild-type, but are comparable to UTX-1::GFP overexpressing animals, suggesting that oxidative stress might be mediated by distinct mechanisms in *utx-1* depleted and *utx-1* overexpressing worms. Interestingly, animals overexpressing UTX-1::DD, which would have been the closest mimic to animals with depleted *utx-1* levels due to the absence of demethylase function, are more sensitive to oxidative stress compared to wild-type animals. This indicates a possible dominant negative effect of the demethylase dead UTX-1 on oxidative stress resistance. In hindsight, I would have included worms subjected to *utx-1* RNAi in my heat stress and UV stress studies to independently assess whether *utx-1* depletion promotes resistance to heat and UV stress or not, in the stress conditions that I used.

Given that long-lived *utx-1(tm3118)+UTX-1::GFP* animals are associated with upregulation of immune and defense response genes, it would be useful to test whether these long-lived animals are resistant to pathogenic stress compared to UTX-1DD

animals. Since ageing in *C. elegans* is associated with bacteria accumulation in the intestine (Garigan et al., 2002; Gems & Riddle, 2000), I could attempt to quantify bacteria colonisation in *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP* using dsRed-expressing OP50. In principle, *utx-1(tm3118)+UTX-1::GFP* animals should exhibit less accumulation of dsRed-OP50 if they are more resistant to pathogenic stress. This would also give me a chance to verify if upregulation of immune response genes in long-lived *utx-1(tm3118)+UTX-1::GFP* animals is associated with increased resistance to pathogenic stress.

4.4.4. *hsp-1* is required for lifespan extension due to *utx-1* overexpression

In an attempt to isolate single genes that might mediate lifespan extension due to the overexpression of a catalytically functional UTX-1, I decided to perform an RNAi screen on transcripts that were upregulated in my RNA-Seq experiment and assess their impact on the lifespan of *utx-1(tm3118)+UTX-1::GFP* wild-type animals. Performing the lifespan experiment on both strains allowed me to deduce whether knockdown of a gene affects worm lifespan in general (ie. if I see the same lifespan effect on both genotypes) or if the effect is specific to *utx-1(tm3118)+UTX-1::GFP* (ie. if an effect is observed in *utx-1* mutants, but not wild-type).

My RNAi screen was comprised of genes that were upregulated in *utx-1(tm3118)+UTX-1::GFP* (compared to UTX-1DD) at $\log_2 FC > 1$. This criterion gave me a list of 40 genes, RNAi clones of which were all obtained from the Ahringer library. From the 40 genes, I was only able to test 10 due to problems with some of the RNAi clones (either incorrect sequence or did not grow in culture). Notably, I discovered 5 genes (*fbxa-163*, *srg-31*, *Y69A2AR.25*, *spp-9* and *T26H5.9*) that extended lifespan in both wild-type and *utx-1(tm3118)+UTX-1::GFP* animals, suggesting that these genes have a role in lifespan

regulation. To my knowledge, these genes have never been implicated in lifespan regulation before and it would be interesting to conduct follow-up studies on them in the future. I also identified gene targets (*delm-2, tbb-6 and Y69A2AR.12*) where knockdown enhanced the lifespan extension of long-lived *utx-1(tm3118)+UTX-1::GFP* even more, but did not affect the lifespan of wild-type animals. This could be due to activation of compensatory mechanisms induced by overexpression of *utx-1*. This can be tested by knocking down each gene individually and investigating if *utx-1* levels are increased even further.

Remarkably, I found that knockdown of *hsp-1* using two different RNAi clones completely abolished the lifespan extension of *utx-1(tm3118)+UTX-1::GFP* animals back to wild-type. This implies a possible link between the UTX-1 enzymatic activity and *hsp-1* in promoting lifespan extension due to UTX-1 overexpression, however, the mechanisms by which this occurs remains unclear.

hsp-1 encodes for hsp70A, a member of the heat-shock family of proteins. hsp70 is constitutively expressed throughout worm development and is inducible upon heat shock. Heat-shock proteins have been widely established to be involved in lifespan regulation, giving precedent to my discovery that *hsp-1* depletion abolishes the lifespan extension of *utx-1(tm3118)+UTX-1::GFP* animals. For instance, a subset of small heat-shock proteins (sHSPs) have been implicated in lifespan regulation, where knockdown of *hsp-16.1*, *hsp-16.49* and *hsp-12.6* decreased lifespan in wild-type and long-lived *daf-2* mutant worms (Hsu et al., 2003). Knockdown of *hsp-1* specifically has also been shown to cause a modest lifespan reduction in long-lived *age-1(hx546)* mutants, suggesting a possible role in lifespan regulation (Morley & Morimoto, 2004).

Transcription of heat-shock proteins such as *hsp-1* is mediated by the transcription factor HSF-1 (heat shock factor-1). Overexpression of *hsf-1* has been previously reported to promote lifespan extension, while knockdown leads to lifespan shortening, indicating that *hsf-1* is important for worm longevity. Because my transcriptomic data revealed a significant upregulation of *hsp-1*, I wondered if the catalytic activity of UTX-1 is involved in the upregulation of the heat-shock response to promote longevity. Molecular chaperones such as heat-shock proteins tend to be upregulated in a concerted manner (Labbadia & Morimoto, 2015), therefore it is surprising that only *hsp-1* and no other chaperone proteins were upregulated in the long-lived *utx-1* overexpressing strain. My finding that *hsp-1* RNAi abolishes lifespan extension in *utx-1* overexpressing animals can be taken to imply that perhaps UTX-1 promotes upregulation of *hsp-1*, independently of *hsf-1*, to promote worm longevity in the context of *utx-1* overexpression. However, it remains unclear whether *utx-1* and *hsp-1* belong to the same pathway to regulate lifespan or not. In the future, it would be useful to perform chromatin immunoprecipitation (ChIP) to probe the levels of H3K27me3 at the *hsp-1* promoter on *utx-1(tm3118)+UTX-1::GFP*, wild-type and worms subjected to *utx-1* RNAi to determine if *hsp-1* is a direct target of *utx-1*.

The discovery that lifespan extension due to *utx-1* overexpression can be mediated by a single gene supports the hypothesis that *utx-1* is beneficial to longevity by modifying transcription of key pro-longevity genes. Further validation studies will need to be conducted to prove that *hsp-1* is a genuine lifespan regulator. For instance, does overexpression of *hsp-1* extend lifespan? Does the catalytic activity of UTX-1 transcriptionally regulate *hsp-1* by removing transcriptionally repressive H3K27me3 marks on the *hsp-1* locus? Answering these questions would improve our understanding of the relationship between the UTX-1 catalytic activity and longevity and will have

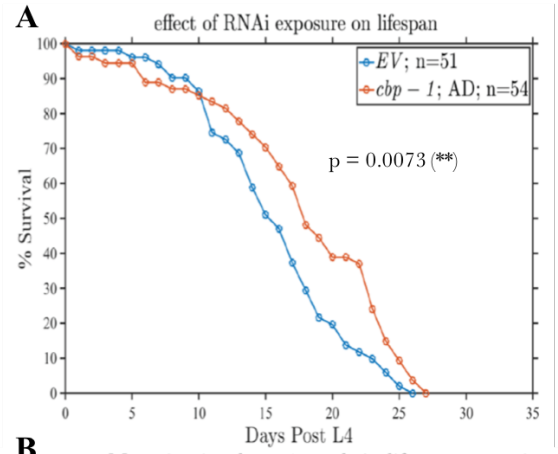
bigger implications in elucidating the role of H3K27 marks in chromatin-mediated lifespan regulation.

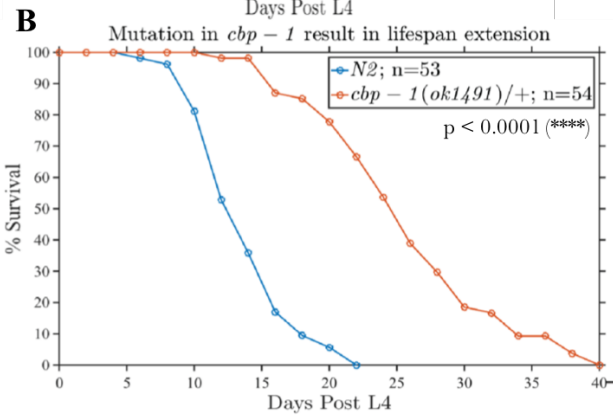
CHAPTER 5: HISTONE ACETYLTRANSFERASE CBP-1 AND LONGEVITY

5.1. INTRODUCTION

cbp-1 encodes for the mammalian homologue of the CBP/p300 lysine acetyltransferase (CBP = cAMP-response binding protein (CREB binding protein)). CBP/p300 acetylates several histone marks and perturbation of CBP/p300 significantly affects the chromatin landscape. Comparative histone acetylation studies on wild-type and CBP/p300 double knockout mouse embryonic fibroblasts (MEFs) showed reduced acetylation on H3K27 and H4K18, suggesting that CBP/p300 acts specifically on H3K27 and H3K18 (Q. Jin et al., 2011b). Additionally, overexpressing CBP/p300 by transfection in COS-7 (fibroblast-like cells from monkey kidney tissue) cells showed preferential increase in H3K14, H4K5 acetylation (Kirk J McManus, 2003). Worm homologue CBP-1 reportedly also acetylates the FOXO transcription factor DAF-16, indicating that CBP/p300 could acetylate non-histone targets (Chiang et al., 2012). The function of CBP/p300 was first described in human adenoviruses where CBP/p300 was identified as a binding factor for the E1A oncoprotein. Binding of CBP/p300 to the E1A oncoprotein N-terminus (along with coactivator E1B) was highly correlated with proliferation and differentiation in primary rodent cells, suggesting that CBP/p300 functions in cell proliferation and differentiation (Shi & Mello, 1998; van den Elsen, de Pater, Houweling, van der Veer, & van der Eb, 1982; H. G. Wang, Moran, & Yaciuk, 1995). The role of CBP/p300 in differentiation is

further corroborated in *D. melanogaster*, where loss of *dCBP* leads to lethality by inhibiting transcription of *twist* and *snail*, crucial genes for mesodermal differentiation (Akimaru, Hou, & Ishii, 1997). Furthermore, CBP/p300 haploinsufficiency in humans leads to Rubinstein-Taybi syndrome, a condition characterised by dysmorphic physical features and severe mental retardation (Kumar, Suthar, Panigrahi, & Marwaha, 2012). In *C. elegans*, CBP-1 interacts with key developmental regulators such as SKN-1 and PAL-1 to regulate mesoderm and endoderm specification in worm embryos (Shi & Mello, 1998). The histone acetyltransferase (HAT activity) is essential for *cbp-1*-mediated differentiation and null mutations of *cbp-1* cause lethality (Shi & Mello, 1998; Victor et al., 2002). Previously, an RNAi screen on chromatin modifiers that affect worm lifespan identified *cbp-1* as a potential regulator of ageing (Chocian, 2015). Results from the screen showed that lipofuscin accumulation (an age-related pigment) decreased between day 3 and day 6 adults when worms are subjected to *cbp-1* RNAi. Furthermore, downregulation of *cbp-1* using RNAi and heterozygous mutants gave a significant lifespan extension compared to controls, although the extent of lifespan extension in *cbp-1* RNAi conditions was less dramatic than that of mutants. Both findings suggest that *cbp-1* knockdown is beneficial to lifespan (Figure 5.1A and B) (Chocian, 2015).





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 5.1: Lifespan is extended upon depletion of cbp-1. ***(A)*** *cbp-1 RNAi promotes lifespan extension compared to EV;* ***(B)*** *cbp-1 heterozygous mutants are longer-lived than wild-type animals. Taken from (Chocian, 2015).*

The result that *cbp-1* knockdown extends lifespan is quite surprising given that published data overwhelmingly support the hypothesis that *cbp-1* knockdown is detrimental to worm longevity (Cai et al., 2017; Vora et al., 2013; M. Zhang et al., 2009). Additionally, *cbp-1* was found to be a crucial mediator of lifespan extension due to several dietary restriction (DR) regimes, further indicating that the absence of *cbp-1* could negatively affect lifespan. CBP/p300 also facilitates energy homeostasis in mice by upregulating gluconeogenic gene expression in fasting conditions, implicating CBP/p300 as a key modulator of the effect of dietary restriction (Yi Liu et al., 2008).

In this chapter, I sought to validate results from the RNAi screen and establish how lifespan and healthspan are affected by *cbp-1* downregulation.

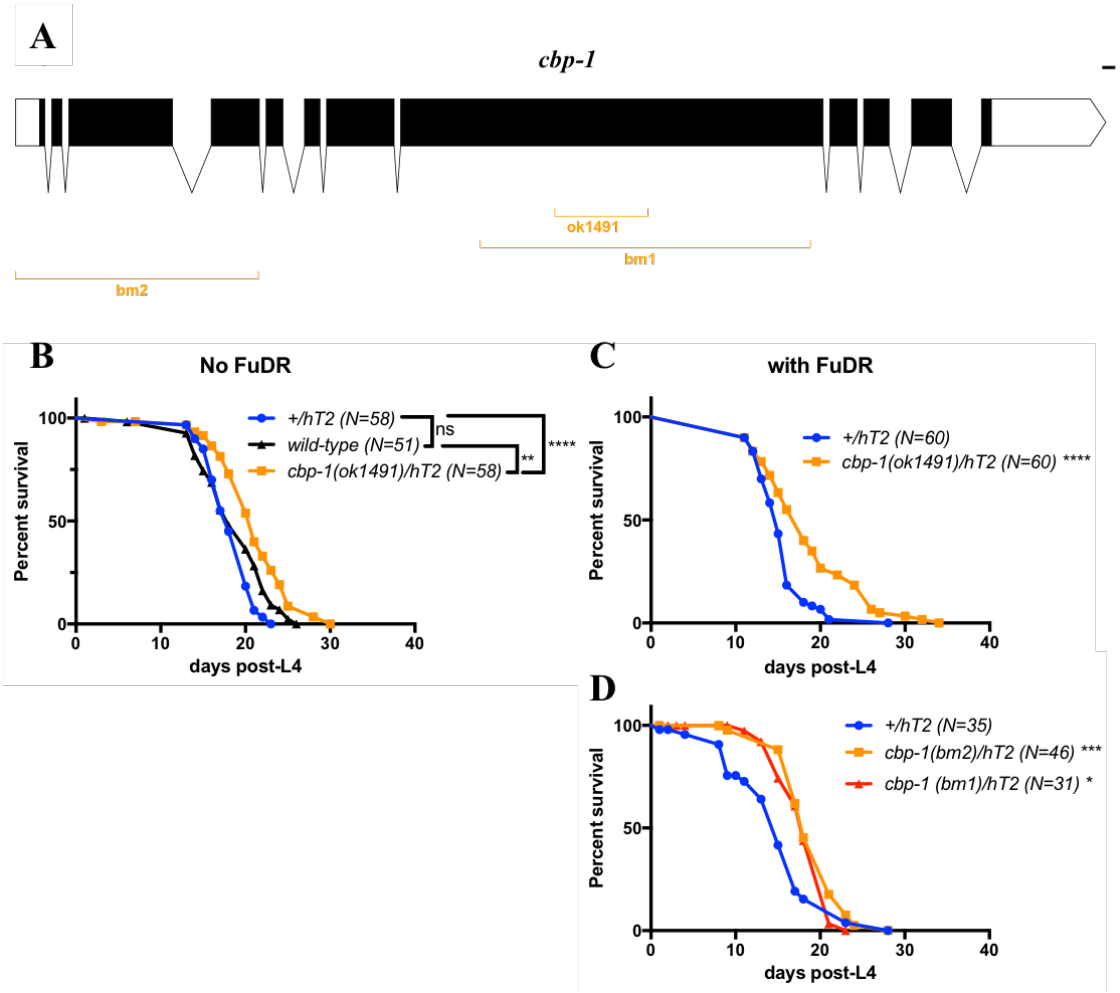
5.2. CHAPTER AIMS

- Assess life- and healthspan mediated by *cbp-1* downregulation

5.3. RESULTS

5.3.1. Hypomorphic levels of *cbp-1* improve worm lifespan in OP50

Following the screen results where knockdown of *cbp-1* reduced lipofuscin accumulation in ageing worms and improves lifespan, I sought to validate these results by investigating lifespan in *cbp-1* loss-of-function mutants available from the CGC stock centre (see Methods: section 2.1.1). All *cbp-1* alleles used carry a deletion in the *cbp-1* open reading frame (ORF) and are homozygous lethal, suggesting that all three mutations are null (Figure 5.2A). *cbp-1(ok1491)* is a 709bp out-of-frame deletion in the *cbp-1* ORF; while *cbp-1(bm1)* is an 837bp in-frame deletion that removes the histone acetyltransferase (HAT) domain and bromodomain of CBP-1, with the resulting truncated protein being stably expressed in heterozygous *cbp-1* mutants (Victor et al., 2002). *cbp-1(bm2)* is a 2470bp deletion including 851bp upstream of the *cbp-1* ORF and the first 4 exons of the gene. Immunostaining studies showed that *cbp-1* expression is completely abolished in these *cbp-1(bm2)* mutants (Victor et al., 2002). Because null mutations of *cbp-1* are homozygous lethal, it was necessary to use balanced heterozygous strains (using the balancer hT2(I;III)) for all my lifespan analyses.



Genotype	With FuDR?	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs hT2 controls)	Statistical Significance (vs wild-type)
+/hT2	no	18.2	18	23		
wild-type	no	18.2	18	26	p = 0.0532 (ns)	
cbp-1(ok1491)/hT2	no	20.8	21	30	p < 0.0001 (****)	p = 0.0040 (**)
+/hT2	yes	15.2	15	28		
cbp-1(ok1491)/hT2	yes	18.4	18	34	p < 0.0001 (****)	
+/hT2	yes	14.2	15	28		
cbp-1(bm2)/hT2	yes	19.0	18	28	p = 0.0008 (***)	
cbp-1(bm1)/hT2	yes	18.1	18	23	p = 0.0120 (*)	

Figure 5.2: Lifespan of cbp-1 loss-of-function mutants. ***(A)*** *The cbp-1 gene and the cbp-1 deletion alleles used, note that bm2 deletion begins before cbp-1 ORF (not shown), scale = 100bp; lifespan analysis of cbp-1(ok1491) without* ***(B)*** *and with FuDR* ***(C)****, note that lifespan of +/hT2 is comparable to wild-type controls;* ***(D)*** *lifespan of cbp-1(bm1) and cbp-1(bm2) heterozygous mutants. For all strains tested, at least 60 L4-stage animals were picked and assessed for lifespan analysis. Animals were scored as alive or dead at least every 2 days. All lifespan experiments were done using standard OP50 as food source. Statistical significance was assessed using Mantel-Cox method.*

Lifespan experiments were initially carried out in both FuDR and non-FuDR conditions to assess whether the sterilising drug might mask the effect of *cbp-1* knockdown on lifespan. Given that the *cbp-1* heterozygous mutants were balanced using hT2(I;III), I decided to use the balancer strain as controls for my experiment. I observed that the lifespan of the balancer (+/hT2) was comparable to wild-type animals, and was therefore an appropriate control to use (Figure 6.2B). I found that lifespan extension was consistently observed in *cbp-1(ok1491)* heterozygous worms compared to controls in both the presence or absence of FuDR (Figure 5.2 B-C). FuDR was used for subsequent lifespan experiments to reduce manual handling of worms. Furthermore, the lifespan of two other alleles *cbp-1(bm2)* and *cbp-1(bm1)* heterozygous animals were also long-lived compared to controls, suggesting that downregulation of *cbp-1* improves worm longevity (Figure 5.2D). These results disagree with current literature where *cbp-1* downregulation by RNAi has been shown to shorten lifespan.

As I mentioned before, the basis for studying how *cbp-1* mediates lifespan is because it was discovered from an RNAi screen of chromatin factors that promote lifespan extension, which was done in the Woollard lab (Chocian, 2015). All published data claiming that *cbp-1* depletion shortens lifespan have been exclusively done through RNAi feeding protocols. Knockdown of *cbp-1* in our screen was done by RNAi feeding as well. *cbp-1* RNAi was introduced post-developmentally to ensure that the lifespan phenotype was not influenced by possible developmental defects caused by the RNAi. I wondered whether the difference in lifespan outcomes between our screen and published data was due to the time-point at which RNAi was introduced to the animals. However, *cbp-1* RNAi was introduced at various time-points: post-embryonic (Chiang et al., 2012), L4-stage (M. Zhang et al., 2009) and day 1 adults (Cai et al., 2017), and still consistently

gave a lifespan shortening effect, suggesting that lifespan variation is not due to the time-point at which *cbp-1* RNAi was introduced. Given the overwhelming evidence that *cbp-1* RNAi shortens lifespan, it is possible that the efficacy of the *cbp-1* RNAi used for our screen was poor, leading to a false positive result. Another possibility is that the degree of *cbp-1* knockdown is critical for longevity, similar to how the dose of knockdown of some mitochondrial genes could differentially affect worm lifespan (Durieux & Dillin, 2007; Rea et al., 2007). *cbp-1* expression after *cbp-1* RNAi was not quantified during the screen, therefore it is difficult to establish if this is the case. Nevertheless, my data suggests that downregulation of *cbp-1* extends worm lifespan, at least in heterozygous mutants. The dominance of the lifespan extension phenotype observed in heterozygous *cbp-1* mutants indicates that perhaps haploinsufficiency in *cbp-1* promotes lifespan extension, suggestive of dose-dependent effects.

5.3.2. Healthspan is slightly improved and brood size unchanged in *cbp-1* mutants

All heterozygous *cbp-1* mutants have improved lifespan compared to their requisite controls and I wanted to assess whether lifespan extension due to downregulation of *cbp-1* is also associated with improved healthspan. Healthspan was assessed by looking at the swimming vigour of worms, where faster thrashing rates is highly correlated with better health. The motility rates of *cbp-1(ok1491)/hT2* animals were recorded and compared against +/*hT2* balancer controls to determine if downregulation of *cbp-1* positively affects healthspan (Figure 5.3A – no FuDR and B – with FuDR).

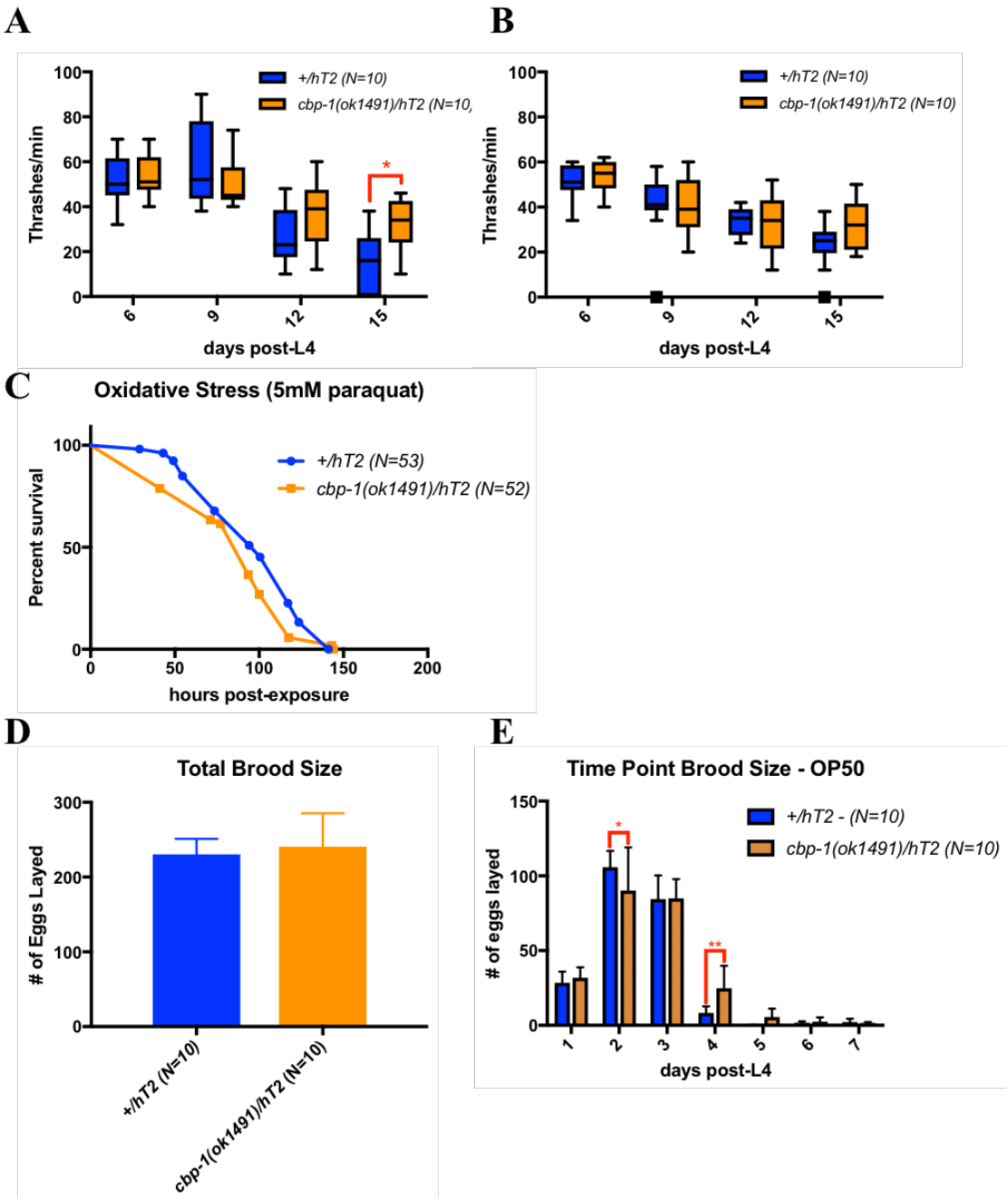


Figure 5.3: Healthspan and brood size of cbp-1 mutants. *To quantify motility, worms were placed in a drop of M9 and thrashing was counted over a 30 second period. Motility assay of cbp-1(ok1491)/hT2 and +/hT2 animals without* ***(A)*** *and with FuDR* ***(B)*** *respectively;* ***(C)*** *Oxidative stress assay was performed by exposing L4-stage animals to 5mM paraquat and monitoring survival every 4 hours (p=0.1725, ns) – experiment was only performed once;* ***(D)*** *total and* ***(E)*** *daily brood size count of cbp-1(ok1491)/hT2 and +/hT2 worms. Statistical significance for motility assay and daily brood size was assayed using one-way ANOVA and student's t-test for total brood size. Statistical significance of survival assay was assessed using Mantel-Cox method.*

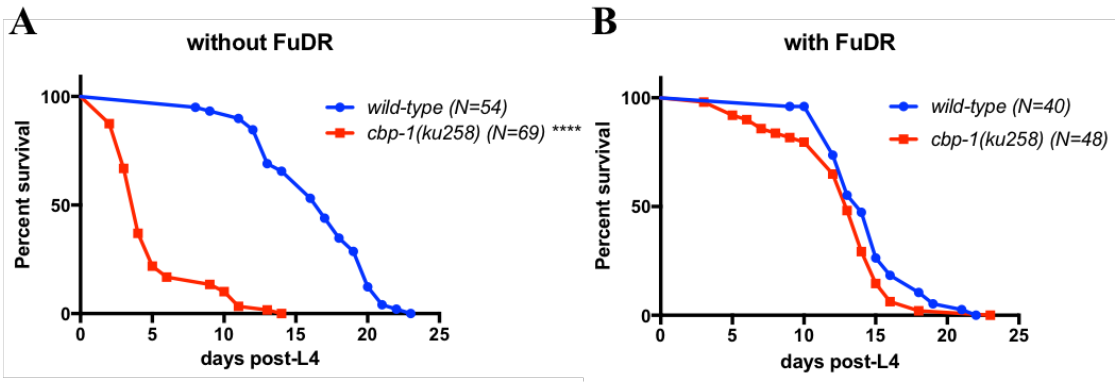
Analysis from my thrashing assay revealed that in "no FuDR" conditions, a modest improvement in motility was observed in older (day 15) *cbp-1(ok1491)/hT2* worms compared to balancer controls. In earlier time points, there is a general trend that *cbp-1(ok1491)* mutants moved better than *+/hT2*, although this did not reach statistical significance until day 15. The trend was recapitulated in FuDR conditions, however there was no statistically significant difference between *cbp-1(ok1491)/hT2* and *+/hT2*. I rationalised that the modest improvement in motility that we observed in "no FuDR" conditions might have been masked under FuDR treatment since FuDR generally makes the worms a bit sicker. Knockdown of *cbp-1* reportedly facilitated nuclear localisation of DAF-16 in worms (Chiang et al., 2012). DAF-16 is important in protecting against oxidative stress, particularly by upregulating several stress response genes such as *sod-3* and *gst-4* (Murphy et al., 2003). Since knockdown of *cbp-1* promotes nuclear localisation of DAF-16, I wanted to assess if knockdown of *cbp-1* leads to increased oxidative stress resistance. I exposed *cbp-1(ok1491)/hT2* and *+/hT2* animals to 5mM paraquat to determine whether downregulation of *cbp-1* affects resistance to oxidative stress. Survival analysis showed no statistically significant difference in survival between *cbp(ok1491)/hT2* and *+/hT2* controls, implying that downregulation of *cbp-1* does not impact oxidative stress resistance (Figure 5.3C).

cbp-1 was previously implicated as a cofactor of *prmt-5* and *cep-1* in inducing transcription of *egl-1*, a component of the programmed cell death pathway, to promote germline apoptosis (Yang et al., 2009). Therefore, I wanted to assess if downregulation of *cbp-1* affects brood size of the worms (Figure 5.3D – total brood size and Figure 5.3E – brood size per day). Brood size was assessed by counting eggs laid every 24 hours until day 7, where worms generally stopped laying eggs and total brood size calculated by

pooling egg-laying data over the entire period of the experiment. I did not count viable progeny (eggs that hatched) because null *cbp*-1 animals are not viable and would have skewed our analysis. Total brood size was comparable between +/*hT2* and *cbp-1(ok1491)/hT2* animals, suggesting that downregulation of *cbp-1* did not affect the reproductive capacity of worms (Figure 5.3C). However, I did observe a slight shift in egg-laying rates where *cbp-1(ok1491)/hT2* animals were perhaps laying eggs at a slightly later stage than +/*hT2* controls (Figure 5.3D). Overall, we showed that *cbp-1* downregulation is associated with a modest improvement in healthspan and does not impact brood size.

5.3.3. Gain-of-function *cbp-1* mutation does not affect lifespan

A *cbp-1* gain-of-function mutant *cbp-1(ku258)* was isolated and found to be involved in worm vulval induction, particularly by suppressing the multivulva (Muv) phenotype induced by activated *let-60 ras* (Dennis J Eastburn, 2005). The *cbp-1(ku258)* allele is a comprised of two guanine to adenine (G→A) substitutions that are 336bp apart, leading to a histidine to arginine (H909R) and a glutamic acid to lysine substitution (E1021K) in the HAT domain of CBP-1. *In vitro* assays showed that the recombinant CBP-1 protein containing the mutations found in the *ku258* allele acetylated purified mammalian histones about 6x more than wild-type CBP-1, indicating that mutations in the *ku258* allele enhance HAT activity (Dennis J Eastburn, 2005). I assessed the lifespan of *cbp-1(ku258)* animals to determine if survival would be complementary to what I found in *cbp-1* haploinsufficient mutants where lifespan extension was observed. Lifespan analysis was initially performed on animals without FuDR and I found *cbp-1(ku258)* to be short-lived, primarily because of high incidences in "bagging" phenotype where eggs hatch inside the mothers, leading to premature death (Figure 5.4A).



Genotype	With FuDR?	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs hT2 controls)
wild-type	no	16.1	17	23	
cbp-1(ku258)	no	4.7	4	14	$p < 0.0001$ (****)
wild-type	yes	14.4	14	22	
cbp-1(ku258)	yes	12.6	13	23	$p = 0.0753$ (ns)

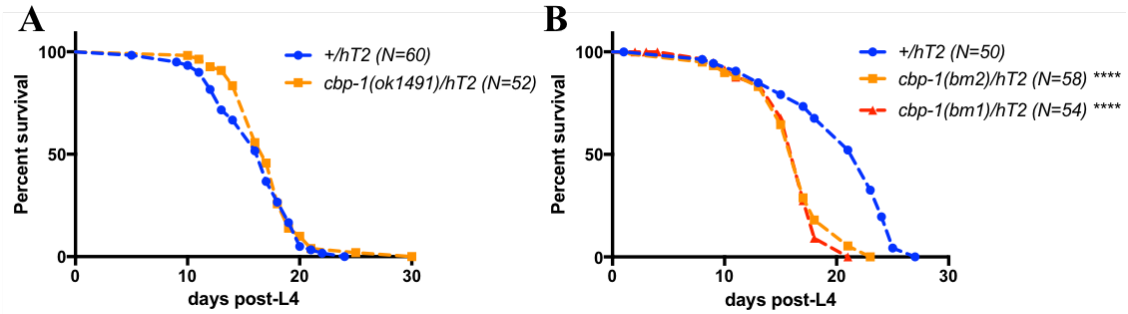
Figure 5.4: Lifespan of cbp-1 gain-of-function animals. *For all strains tested, at least 60 L4-stage animals were picked and assessed for lifespan analysis. Animals were scored as alive or dead at least every 2 days.* ***(A)*** *without FuDR;* ***(B)*** *with FuDR. Mean lifespan, median lifespan, maximum lifespan and statistical significance shown on accompanying table. Lifespan assays were only performed once. Statistical significance was assessed using Mantel-Cox method.*

Given the role of *cbp-1* in inhibiting Muv in *let-60* gain-of-function mutants, *ku258* might have been sufficient to induce a vulvaless (Vul) phenotype which could explain the cause of bagging. I repeated the experiment in the presence of FuDR to prevent egg-hatching (and therefore avoid bagging) and found that lifespan of *cbp-1(ku258)* did not significantly change compared to wild-type animals (Figure 5.4B). This implies that increased activity of *cbp-1* has no effect on worm longevity.

5.3.4. Lifespan extension in *cbp-1* hypomorphic mutants is abolished when worms are fed on HT115 bacteria

Survival of long-lived *cbp-1* heterozygous mutants was assayed in HT115 bacteria since this food source is routinely used for RNAi feeding protocols and I wanted to eventually

perform epistasis analysis with key longevity genes to determine how *cbp-1* knockdown mediates lifespan extension (Figure 5.5).



Genotype	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	Statistical Significance (vs hT2 controls)
+/hT2	16.0	17	24	
cbp-1(ok1491)/hT2	17.1	17	30	p = 0.2709 (ns)
+/hT2	20.1	23	27	
cbp-1(bm2)/hT2	16.4	17	23	p < 0.0001 (****)
cbp-1(bm1)/hT2	16.1	17	21	p < 0.0001 (****)

Figure 5.5: Lifespan of cbp-1 mutants in HT115. *For all strains tested, at least 60 L4-stage animals were picked and assessed for lifespan analysis. Animals were scored as alive or dead at least every 2 days.* ***(A)*** *cbp-1(ok1491);* ***(B)*** *cbp-1(bm2)/hT2 and cbp-1(bm1)/hT2 vs controls. Mean lifespan, median lifespan, maximum lifespan and p-values are given in the accompanying table. Statistical significance was assessed using Mantel-Cox method.*

I found that the lifespan extension observed in *cbp-1* haploinsufficient mutants fed the standard OP50 bacteria was completely abolished in HT115. For *cbp-1(ok1491)/hT2*, lifespan extension was completely abolished, while significant shortening of lifespan was seen in *cbp-1(bm2)/hT2* and *cbp-1(bm1)/hT2* animals.

Different food sources can have a substantial impact on metabolism and worm physiology. An extreme example would be the metabolic shift from glucose to fatty acid oxidation when worms are switched from ample food supply to dietary restriction conditions. Therefore, it is possible that worms have adaptive mechanisms in place to support organismal needs in response to changing diets and that failure of these adaptive

mechanisms could be detrimental to lifespan. Given the striking difference in lifespan that was observed by switching from OP50 *E. coli* B to HT115 *E. coli* K12 diet, I posit that *cbp-1* might affect survival by mediating the worm's adaptive response to particular diets. Due to time-constraints and foreseeable technical challenges associated with this project, future investigations were halted to prioritise studies on UTX-1, another H3K27 modifier.

5.4. SUMMARY

In summary, we discovered that hypomorphic levels of *cbp-1* extend worm lifespan in OP50 and is associated with a mild improvement in health, but have no effect on brood size and oxidative stress resistance. Lifespan extension is completely abolished when the food source is switched to HT115, suggesting a possible role for *cbp-1* in adaptive response mechanisms due to changing diets or nutrient sensing. Gain-of-function mutations of *cbp-1* do not significantly affect worm lifespan.

5.5. DISCUSSION

Lifespan analysis conducted on three different *cbp-1* heterozygous mutants overwhelmingly support that knockdown of *cbp-1* promotes lifespan extension in worms, at least when OP50 is used as food source. The lifespan extension of *cbp-1* mutants is presumably not due to increased resistance to oxidative stress, given that paraquat treatment did not enhance survival of *cbp-1* mutants compared to controls. In contrast, published data give compelling evidence that *cbp-1* knockdown is detrimental to longevity, although lifespan results published in the context of *cbp-1* downregulation have been exclusively conducted using RNAi feeding protocols, therefore using the canonical RNAi bacteria strain HT115 (Cai et al., 2017; Lublin et al., 2011; M. Zhang et al., 2009). Our *cbp-1* lifespan data in HT115 support this, where no lifespan extension was observed in *cbp-1* heterozygous mutants when feeding on HT115 bacteria. *cbp-1* is reportedly crucial for lifespan extension under several dietary restriction (DR) regimens and knockdown of *cbp-1* also causes DiI filling defects in amphid neurons, collectively suggesting a potential role in nutrient sensing. The fact that my results show that *cbp-1* downregulation extends lifespan in OP50 but not in HT115 and that *cbp-1* RNAi shortens lifespan according to several reports highlights that *cbp-1* downregulation could have pleiotropic effects on the worm. Such changes are likely sensitive to changing environments (such as bacterial diet) which could add complexity in determining how *cbp-1* regulates lifespan.

5.5.1. *cbp-1* mutants are long-lived but not resistant to oxidative stress

Using a *daf-16* translational reporter, Chiang *et al*. (2012) found that *cbp-1* RNAi promoted nuclear localisation of DAF-16 compared to empty vector controls (Chiang et al., 2012). When insulin signalling is inhibited, DAF-16 is hypophosphorylated and can

translocate to the nucleus to promote transcription of longevity and stress resistance genes (Murphy et al., 2003). DAF-16 is also known to translocate to the nucleus in response to oxidative and heat stress, suggesting that nuclear localisation of DAF-16 is important for increased stress resistance and enhanced longevity (Henderson & Johnson, 2001). Therefore, I expected that enhanced nuclear localisation of DAF-16 due to depletion of *cbp-1* would be beneficial to lifespan and stress resistance. This agrees with my findings that heterozygous *cbp-1* mutants are long-lived compared to wild-type. However, I also found that *cbp-1* mutants treated with paraquat (an oxidative stress mimic) had comparable survival to controls, suggesting that *cbp-1* RNAi does not increase resistance to oxidative stress. This suggests that nuclear localisation of DAF-16 is inadequate to promote stress resistance in *cbp-1* mutants. Perhaps downregulation of *cbp-1* affects acetylation of key stress resistance genes, leading to no improvement in oxidative stress resistance, despite promoting longevity. While most long-lived mutants are associated with increased resistance to certain stressors, this is not a universal observation. For instance, genetic or RNAi knockdown of the p53 homologue *cep-1* increases worm lifespan, which is dependent on *daf-16*. However, depletion of *cep-1* does not enhance resistance to heat, oxidative or UV stress, suggesting that longevity and stress resistance can be mediated by independent mechanisms (Arum & Johnson, 2007).

5.5.2. GABAergic neurons are important for lifespan extension due to *cbp-1* depletion

cbp-1 is expressed in most if not all cells of the worms, therefore lifespan phenotypes might be dependent on specific tissues where *cbp-1* levels are reduced (Dennis J Eastburn, 2005; Shi & Mello, 1998; Victor et al., 2002). Cai *et al*. (2017) performed tissue-specific RNAi of *cbp-1* and found that lifespan shortening was observed when *cbp-*

1 was knocked down in the germline, neurons (pan-neuronal) and to a lesser extent in the intestine but not in the muscle and hypodermis. Furthermore, they also identified that neuronal expression of *cbp-1* is especially important for lifespan extension mediated by axenic dietary restriction (ADR). Remarkably, further tissue-specific RNAi studies on a subset of neurons revealed that knockdown of *cbp-1* in GABAergic neurons extend lifespan but abolish lifespan extension due to ADR. They also showed that knockdown of *cbp-1* in dopaminergic, cholinergic and glutamatergic neurons do not impact normal lifespan. Given that pan-neuronal knockdown of *cbp-1* shortens lifespan and the neuronal subsets tested (GABAergic, dopaminergic, cholinergic and glutamatergic) which make up about 200 of the 302 neurons in *C. elegans* do not, this suggests that lifespan shortening upon *cbp-1* knockdown might be mediated by neurons not tested in this analysis (Cai et al., 2017). It is interesting that GABAergic neuronal knockdown of *cbp-1* is beneficial to lifespan but abolished DR-mediated lifespan extension. It is strikingly similar to my results where *cbp-1* haploinsufficiency leads to normal lifespan extension in OP50 but not in HT115. Both findings seem to suggest that tissue-targeted knockdown of *cbp-1* is beneficial to normal lifespan at the cost of impaired nutrient or environmental-sensing.

My lifespan analyses were performed on *cbp-1* mutants, likely leading to universal knockdown of *cbp-1*, whereas published lifespan studies on *cbp-1* knockdown are done through RNAi by feeding. It is established that RNAi by feeding is less effective in neurons (Kamath et al., 2001; Timmons et al., 2001). The fact that germline- and intestine-specific knockdown of *cbp-1* shorten lifespan could partially explain why systemic knockdown of *cbp-1* by RNAi feeding leads to a net effect of lifespan shortening in worms. GABAergic neuronal knockdown of *cbp-1* phenocopies lifespan extension

observed in *cbp-1* heterozygous mutants (as shown by my data, at least in OP50), suggesting that GABAergic neurons may be the site of *cbp-1* function in lifespan regulation. Given that I observed no lifespan extension in *cbp-1* mutants when OP50 was switched to HT115, I cannot rule out that lifespan extension due to systemic *cbp-1* RNAi is not only due to tissue-specific effects. It is also quite possible that an aberrant adaptive response (ie. changing from OP50 to HT115) due to *cbp-1* RNAi could mask lifespan extension. In this regard, using an RNAi-compatible OP50 strain (OP50(xu363)) to knockdown *cbp-1* and assessing lifespan might help in uncoupling these mechanisms (Xiao et al., 2015). Briefly, RNAi-competent OP50 was created by replacing the wild-type copy of OP50 RNase III with the same deletion found in the HT115 strain. An IPTG-inducible T7 RNA polymerase was also introduced to allow dsRNA production from L4440 plasmids (Xiao et al., 2015). I envision that lifespan extension in RNAi-compatible OP50 knockdown of *cbp-1* compared to controls would suggest that the lack of lifespan extension due to *cbp-1* RNAi in HT115 is likely due to impaired nutrient-sensing.

5.5.3. *cbp-1* is involved in DR-mediated lifespan regulation

cbp-1 plays an important role in lifespan extension due to bacterial dilution, genetic (*eat-2* mutants) and axenic dietary restriction protocols (M. Zhang et al., 2009). Distinct mechanisms are implicated depending on the DR regime used, for instance *hsf-1* is required for lifespan extension due to genetic DR but not *daf-16*, whereas *daf-16* is necessary for lifespan increase due to bacterial dilution DR (bDR). *cbp-1*, which is required for lifespan extension by most DR protocols, is reportedly regulated by bantam-homolog food sensor microRNA-80 (miR-80). Loss of mir-80 leads to a constitutive-DR state and promotes healthy ageing in worms. The *cbp-1* ORF contains several binding

targets of miR-80, suggesting a direct regulation. The current model suggests that abundance of food leads to increased miR-80 and consequent downregulation of *cbp-1*, while dietary restriction (or lack of food) inhibits mirR-80 leading to upregulation of *cbp-1* (Vora et al., 2013). Furthermore, *cbp-1* downregulation abolishes lifespan extension mediated by DR, corroborating *cbp-1* as a mediator of lifespan through DR (M. Zhang et al., 2009). My data showed that lifespan extension of *cbp-1* mutants in OP50 is completely abolished when experiments are repeated in HT115, indicating that *cbp-1* knockdown promotes lifespan extension in a diet-specific manner. This may or may not overlap with the possibility that *cbp-1* depletion might regulate transcriptional changes leading to impaired adaptive response to dietary change.

5.5.4. *cbp-1* might regulate lifespan in a diet-specific manner

In *C. elegans*, changes in metabolism, fat storage (Brooks, Liang, & Watts, 2009), developmental rates (MacNeil, Watson, Arda, Zhu, & Walhout, 2013) and fecundity (Soukas, Kane, Carr, Melo, & Ruvkun, 2009) have been described when animals are subjected to different food sources. My observation that *cbp-1* knockdown using heterozygous mutants are long-lived when fed OP50 but not HT115 suggests that *cbp-1* mediates lifespan in a diet-specific manner. Diet-mediated lifespan has been previously described in worms. *alh-6* mutations, a gene encoding for pyrroline-5-carboxylate dehydrogenase, confer shortened worm lifespan in OP50 but normal lifespan in HT115. ALH-6 is involved in catalysing glutamate synthesis from pyrroline-5 carboxylate (P5C), the second step in the proline catabolic pathway (proline → P5C → glutamate). OP50 is associated with increased expression of *prodh*/*B0513.5* compared to HT115. *prodh* encodes a proline dehydrogenase that catabolises proline to P5C in the proline catabolic pathway. The increased expression of *prodh* coupled with mutations in *alh-6* lead to an

increased concentration of P5C in OP50, a toxic substrate that causes premature ageing by promoting mitochondrial damage (Pang & Curran, 2014).

Gene expression changes in metabolic and gene regulatory networks have been previously observed in response to dietary change. For instance, microarray expression profiling of young adult worms feeding on *Comamonas* DA1877 and *E. coli* HT115 compared to standard OP50 bacteria identified metabolic enzymes such as short-chain acyl-CoA dehydrogenase ACDH-1 to be highly sensitive to dietary change (MacNeil et al., 2013). I wondered whether depletion of *cbp-1* could affect transcriptional regulation of key target genes that are sensitive to dietary change and can affect lifespan regulation at the same time. Because *cbp-1* depletion extends 'normal' lifespan and when specifically knocked down in GABAergic neurons, it would be informative to perform RNA-Seq analysis specifically on GABA neurons subjected to *cbp-1* RNAi (compared to EV) to investigate whether transcriptional changes are involved in diet-specific lifespan regulation.

In another study, mutations in *nmur-1* and *osm-3* were found to specifically increase lifespan in OP50 compared to wild-type, but not in HT115 (Maier, Adilov, Regenass, & Alcedo, 2010). *nmur-1* encodes for a mammalian homologue of the neuromedin U receptor, while *osm-3* encodes for a kinesin motor protein that is involved in cilia formation. *osm-3;nmur-1* double mutants were observed to have the same lifespan phenotypes as those observed in single mutants, indicating that these two genes may be working on the same pathway to mediate diet-specific lifespan extension (Maier et al., 2010). *osm-3* and *nmur-1* have been found to be co-expressed in some amphid and chemosensory neurons, indicating that these genes mediate lifespan by sensing dietary

cues from the environment. Depletion of *cbp-1* (by RNAi) was found to produce DiI filling effects on amphid neurons in worms, suggesting that *cbp-1* mediates some function on that specific site. In the future, it would be useful to investigate if *cbp-1* affects the transcriptional regulation of *osm-3* and *nmur-1*, in order to promote diet-specific lifespan regulation.

It is currently unknown whether downregulation of *cbp-1* affects adaptation to the differing nutritional content and/or metabolites produced by bacteria food sources (Pang & Curran, 2014; Virk et al., 2012). HB101 is considered a nutrient-rich food source due its ability to support growth of wild-type animals compared to OP50 (Boris Borisovich Shtonda, 2006). HT115 and OP50 are largely similar in macronutrient content apart from HT115 being a more carbohydrate-rich diet (Brooks et al., 2009). Previously, survival analysis of wild-type animals feeding on HB101, HT115, OP50 or OP50-derived DA837 were found to have comparable median and maximum lifespans to each other, suggesting that lifespan is not greatly affected by different bacterial diets (Brooks et al., 2009). In contrast, my lifespan data showed that *cbp-1* heterozygous mutants are long-lived in OP50 but not in HT115, leading me to hypothesise that *cbp-1* (at least partially) mediates lifespan through some nutrient-sensing mechanism. In the future, testing the lifespan of *cbp-1* mutants feeding on different food sources, such as HB101, DA837 and HT115 would be a useful experiment to do in order to assess if *cbp-1* is involved in diet-specific lifespan regulation.

5.5.5. Interactions between *cbp-1* and canonical longevity pathways

Investigating the interaction between *cbp-1* and genes involved in known longevity pathways might explain how *cbp-1* mediates longevity. Inhibition of insulin signalling

through downregulation of *daf-2* (insulin receptor) and *age-1* (PI3K) lead to improved lifespan and is mediated by downstream effector *daf-16* (FOXO transcription factor) (Kenyon et al., 1993). *cbp-1* RNAi downregulation dramatically reduces lifespan in long-lived *daf-2(e1370)* mutants suggesting that *cbp-1* is partially responsible for lifespan due to insulin-signalling inhibition. Furthermore, simultaneous knockdown of *cbp-1* and *daf-16* does not give an additional lifespan-shortening effect compared to animals subjected to *daf-16* RNAi only, reinforcing the idea that *cbp-1* is important in improved longevity due to downregulation of insulin-signalling (M. Zhang et al., 2009). On the other hand, *cbp-1* knockdown promotes nuclear localisation of the pro-longevity transcription factor DAF-16, which might suggest that *cbp-1* knockdown ought to be beneficial for lifespan (Chiang et al., 2012). The fact that my results show that *cbp-1* downregulation using haploinsufficient mutants increases lifespan support this. But published data using *cbp-1* RNAi does not. This highlights that perturbation of *cbp-1* could result in pleiotropic effects that are also sensitive to environmental stimuli/bacterial diets, making it difficult to definitively explain how *cbp-1* regulates lifespan.

cbp-1 also directly interacts with *skn-1* to facilitate embryonic mesoderm specification in worms (Shi & Mello, 1998; A. K. Walker et al., 2000). *skn-1* encodes the *C. elegans* homolog of the mammalian Nrf transcription factors. While *skn-1* was initially identified for its role in tissue specification during embryonic development, it has since been found to have important functions in oxidative stress resistance by upregulating genes involved in phase II detoxification such as glutathione-S-transferases (An, 2003; Blackwell, Steinbaugh, Hourihan, Ewald, & Isik, 2015). The role of *skn-1* in oxidative stress resistance has also been linked to how *skn-1* mediates longevity in worms. However, recent findings showed that *skn-1* mediated resistance to oxidative stress and pro-

longevity effects occur via distinct mechanisms. The study showed *skn-1* was necessary for increased oxidative stress induced by *daf-16* overexpression but is dispensable for lifespan extension (Tullet et al., 2017). The interaction between *cbp-1* and *skn-1* has not been rigorously studied in the context of lifespan regulation. This is of further interest because like *cbp-1*, *skn-1* is also important for longevity induced by dietary restriction. *skn-1* mediates pro-longevity induced by DR particularly in the ASI chemosensory neurons and *cbp-1* RNAi causes aberrant DiI staining in amphid neurons, which includes ASI – it would be fascinating to see if these observations are acting on the same pathway in mediating longevity induced by DR (Bishop & Guarente, 2007; M. Zhang et al., 2009). Furthermore, *skn-1* can be regulated in a *daf-16* dependent (insulin signalling) and independent (p38-MAPK pathway) manner, which presents an attractive theory for integrating longevity signals mediated by *cbp-1*.

In summary, I have shown that *cbp-1* knockdown using heterozygous mutants have increased lifespan compared to wild-type animals when feeding on standard OP50 bacteria, but this lifespan extension is completely abolished when the worms are fed HT115 bacteria. Overall, these findings underscore the importance of considering environmental influences such as food source in interpreting lifespan experiments.

5.6. FUTURE WORK

Results from this chapter provide evidence to suggest both a nutrient-sensing and tissue-specific angle to explain how *cbp-1* downregulation promotes longevity. It remains to be seen whether these two mechanisms are mutually exclusive or not. The finding that lifespan extension of *cbp-1* mutants in OP50 is completely abolished in HT115 provokes several questions. Is *cbp-1* downregulation only beneficial to lifespan when knocked

down at specific tissues? This seems to be the case given that GABAergic neuronal knockdown of *cbp-1* extended lifespan but also abolished lifespan extension mediated by DR. Is lifespan extension by GABAergic *cbp-1* knockdown afforded at the cost of impaired adaptive response to changing diets or environments (for example plentiful food compared to no food)? Is the lifespan extension observed in *cbp-1* mutants dependent on specific bacterial diets?

The dependence of lifespan on bacterial diet can be further tested in *cbp-1* mutants by using a wider array of bacteria strains as food source and assessing if there is any correlation with worm survival. Furthermore, tissue-specific studies by Cai *et al*. (2017) on lifespan due to *cbp-1* downregulation have highlighted the importance of GABAergic neurons as the site of action for normal lifespan extension and DR-mediated lifespan extension by *cbp-1*. Transcriptomic profiling of GABAergic neurons after subjecting worms to *cbp-1* RNAi or empty vector controls could reveal specific gene targets that are important for lifespan or DR-specific regulation.

Furthermore, performing tissue-specific knockdown of *cbp-1* using RNAi-compatible OP50 might be equally informative in addressing the tissue-specific and nutrient-sensing aspect of *cbp-1* lifespan regulation. For instance, systemic knockdown of *cbp-1* using RNAi-compatible OP50 phenocopying the lifespan extension observed in *cbp-1* mutants feeding on OP50 would suggest an over-riding diet-specific effect on lifespan. Metabolomic profiling of the different bacterial diets would also be useful in identifying if specific metabolites are important for lifespan regulation due to *cbp-1* downregulation.

CHAPTER 6: GENERAL DISCUSSION

6.1. Summary

- Both up- and downregulation of *utx-1* promotes lifespan extension in *C. elegans.*
- Lifespan extension is observed when *utx-1* is depleted in the hypodermis, neurons and the intestine, but not in the muscle.
- Lifespan extension due to neuron- and intestine-specific knockdown is completely dependent on *daf-16*, while lifespan extension due to hypodermis-specific knockdown of *utx-1* is only partially dependent on *daf-16*.
- Intestine- and neuron-specific overexpression of *utx-1* promotes lifespan extension, while hypodermal- and muscle-specific overexpression of *utx-1* does not influence worm lifespan.
- Lifespan extension mediated by *utx-1* overexpression is independent of *daf-2*, but partially dependent on *daf-16*.
- A catalytically functional UTX-1 is crucial for lifespan extension due to *utx-1* overexpression.
- Differential gene expression analysis comparing long-lived *utx-1(tm3118)+UTX-1::GFP* and *utx-1(tm3118)+UTX-1DD::GFP* animals showed that overexpression of a catalytically functioning UTX-1 is associated with upregulation of immune response genes and downregulation of collagen genes.
- The RNA-Seq data led to the identification of *hsp-1* as key player in lifespan extension mediated by *utx-1* overexpression.
- *cbp-1* depletion causes lifespan extension when worms are fed standard OP50 bacteria, but not when fed HT115, suggesting that the lifespan phenotypes due to knockdown of *cbp-1* is sensitive to environmental factors such as food source.

6.2. Model for lifespan extension mediated by *utx-1*

Investigations in this thesis revealed that tissue-specific manipulation of the levels of *utx-1* can differentially affect lifespan and is graphically outlined below.

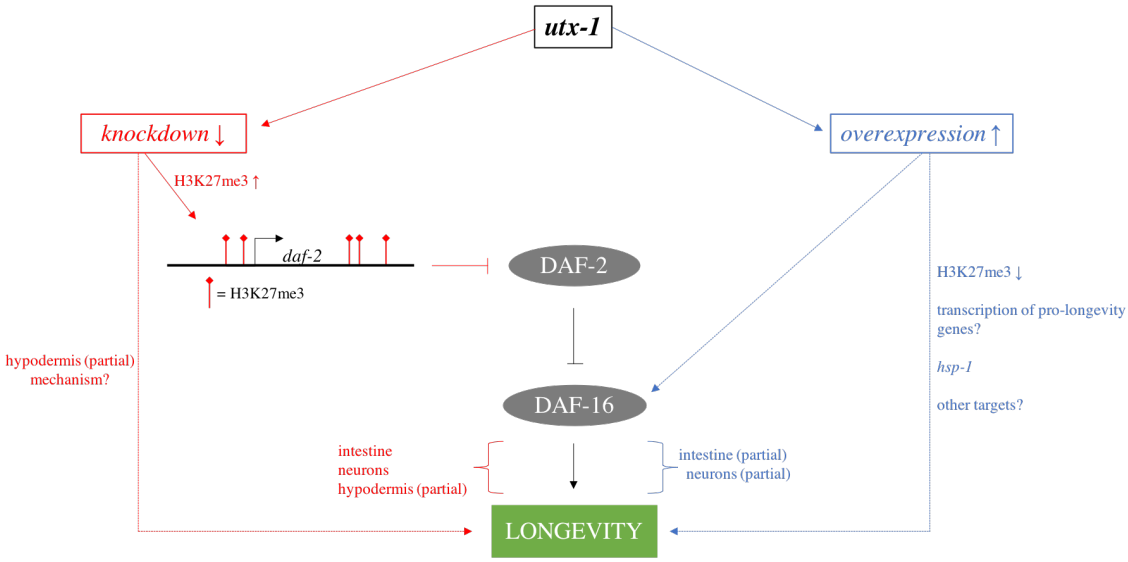


Figure 6.1: Current understanding of utx-1 mediated lifespan regulation.

I identified that knockdown of *utx-1* in the intestine and neurons enhance worm longevity in a *daf-16* dependent manner, where downregulation of *utx-1* likely promotes lifespan extension by causing retention of transcriptionally inactivating H3K27me3 marks on the *daf-2* locus. This effectively inhibits the insulin signaling pathway, thereby promoting nuclear localization of DAF-16 to facilitate transcription of pro-longevity genes. Interestingly, hypodermal knockdown of *utx-1* extends lifespan in a manner that is only partially dependent on *daf-16* and it would be interesting to investigate what other mechanisms are involved in lifespan extension due to hypodermal-specific knockdown of *utx-1*. Overexpression of some collagen genes, which are primarily expressed in the hypodermis, have been shown to promote worm longevity. I've shown in my RNA-seq study that collagen genes are largely downregulated in *utx-1* overexpressing worms. It is

therefore possible that hypodermis-specific *utx-1* downregulation could upregulate collagen expression and enhance worm lifespan, which could be a potential *daf-16* independent mechanism for lifespan extension. This could be addressed by determining if hypodermal-specific knockdown of *utx-1* enhances mRNA expression of collagen genes and determining if lifespan extension is observed in worms with collagen mutations where *utx-1* is hypodermally knocked down.

On the other hand, the neurons and intestine are key tissues for lifespan extension due to *utx-1* overexpression and in both cases are only partially dependent on *daf-16*. Furthermore, systemic overexpression of *utx-1* further enhances the long lifespan of *daf-2* mutants, suggesting that *utx-1* overexpression promotes lifespan independent of insulin signaling. I also validated findings that the UTX-1 catalytic activity is important for longevity in the context of overexpression. Demethylation of H3K27me3/2 marks is associated with transcriptional activation, therefore, I posited that overexpressing a catalytically active UTX-1 could promote transcription of genes that are associated with pro-longevity effects and extend worm lifespan. This was addressed by determining gene targets that were upregulated in the long-lived *utx-1* overexpressing strain and performing an RNAi screen to assess if knockdown of that particular gene target abolishes lifespan extension. In the 12 gene targets that were initially assessed, I found that knockdown of *hsp-1* completely abolished the lifespan extension of *utx-1* overexpressing worms, implying that *hsp-1* is required for lifespan extension mediated by overexpressing *utx-1*. Future efforts in expanding on the RNAi screen will help determine whether there are other such gene targets that are required for longevity in the context of *utx-1* overexpression.

Overall, lifespan extension due to downregulation of *utx-1* is largely dependent on the inhibition of the insulin signaling cascade – a known anti-longevity pathway. On the other hand, enhanced longevity due to *utx-1* overexpression is independent of insulin signaling but partially dependent on *daf-16*. Furthermore, lifespan extension due to *utx-1* overexpression requires the presence of *hsp-1*, which implies that *utx-1* overexpression extends lifespan partially by upregulating transcription of pro-longevity genes. It remains to be seen if there are any other gene targets that affect the lifespan of *utx-1*OE animals in this way. Nevertheless, in this thesis, I demonstrate that there are parallel mechanisms involved in lifespan regulation due to *utx-1* knockdown or overexpression, which are further complicated by tissue-specific activities of *utx-1*. We are beginning to understand how *utx-1* regulates worm lifespan but the picture is by no means complete and further studies are necessary to tease out the mechanistic nuances of *utx-1* mediated lifespan regulation.

6.3. H3K27 methylation and the heterochromatin loss theory of ageing

Chromatin is tightly regulated during development, where the interplay of chromatin modifications to switch genes on or off play an important role in facilitating proper cellular differentiation. With increasing age, the chromatin adopts a more euchromatic, transcriptionally active state. This "open" chromatin state is thought to promote transcription of key anti-longevity genes that negatively impact lifespan (Tsurumi & Li, 2012). The theory is supported by findings that downregulation of genes involved in the deposition of transcriptionally activating H3K4me3 marks promotes longevity in worms (Greer et al., 2010). Additionally, polyamine spermidine treatment, which leads to histone hypoacetylation, increases the lifespan of worms, flies and human cells (Eisenberg et al., 2009). This supports the idea that removal of transcriptionally activating

marks is beneficial to longevity and therefore agrees with the heterochromatin loss theory of ageing.

The heterochromatin loss theory of ageing also implies that globally increasing the levels of transcriptionally inactivating H3K27me3 marks will make the chromatin more heterochromatic (ie. compacted), and therefore be beneficial to lifespan. This is supported by findings that H3K27me3 levels decrease with age in worms and downregulation of the H3K27-specific lysine demethylase *utx-1* increases H3K27me3 levels with concomitant lifespan extension (Jin et al., 2011; Maures, Greer, Hauswirth, & Brunet, 2011).

However, as I have shown in this thesis, overexpression of *utx-1* which is associated with a global DECREASE in transcriptionally repressive H3K27me3 marks, still causes lifespan extension, and therefore goes against the principles of the heterochromatin loss theory of ageing. Furthermore, heterozygous mutations in the H3K27-specific methyltransferase E(Z) decrease H3K27me3 levels and increase the lifespan of male flies, suggesting that H3K27me3 is not a determinant for lifespan extension (Siebold et al., 2010).

The confounding lifespan outcomes when H3K27me3 levels is manipulated to go up or down suggests that the mechanism by which histone modifiers regulate lifespan is a lot more nuanced than simply changing the global levels of histone modifications, and that the heterochromatin loss theory of ageing is an inaccurate explanation for how chromatin changes mediate longevity. It is likely that lifespan is mediated by context-specific transcriptional changes on a subset of genes that may be involved in lifespan regulation.

This is exemplified by the discovery that upregulation of *hsp-1*, as revealed in my RNA-seq analysis, is important in promoting lifespan extension in *utx-1* overexpressing worms.

Another source of complexity in interpreting how chromatin changes mediate lifespan is that age-related changes in the levels of H3K27me3 is not consistent among different organisms or even tissues. For instance, H3K27me3 decreases with age in worms (Maures et al., 2011) and human fibroblasts of HGPS patients; but increases with age in fruit flies, killi-fish and mouse brains (reviewed in Sen:2016ct and Pal & Tyler, 2016). Therefore, it is not entirely certain if chromatin-mediated lifespan regulation is a "private" (ie. organism-specific) or "public" (evolutionarily conserved) mechanism and caution must be exercised in the extent at which findings can be extrapolated from one model organism to another.

6.4. RNA-Seq is a powerful tool for identifying lifespan regulators

Canonically, when a gene is found to affect lifespan, epistatic analysis between the gene of interest (in my case, *utx-1*) and components of a known ageing pathway (eg. those involved in insulin signalling) are conducted to infer pathways that the gene of interest might be involved in to mediate longevity. However, insights from these studies are limited to establishing if the gene of interest works on the same pathway, but does not give any more information about what other processes or mechanisms are involved to produce a lifespan phenotype. Furthermore, this approach is limiting in that we can only test the involvement of the gene of interest in pathways that are already known to affect ageing.

In this thesis, RNA-Seq was employed to probe transcriptional changes induced by *utx-1* overexpression and identify gene targets that could be important in lifespan regulation.

Transcriptomic analysis allowed us to go beyond pinpointing the involvement of known ageing pathways and identify novel gene targets that mediate longevity. In the case of *utx-1* overexpression, *hsp-1* was identified as a lifespan regulator due to the finding that it was upregulated in our long-lived mutants and subsequently chosen as part of an RNAi screen (ie. RNA-seq driven RNAi screen). Knockdown of *hsp-1* in wild-type animals does not affect lifespan, therefore it would not have been possible to identify *it* as a lifespan regulator in traditional RNAi screens. Furthermore, the RNA-Seq data identified 5 genes (*fbxa-163*, *srg-31*, *Y69A2AR.25*, *spp-9* and *T26H5.9*) to be involved in lifespan regulation, which were previously not known to impact longevity.

This validates the use of RNA-Seq as a powerful tool for identifying novel lifespan regulators. This is especially useful in understanding how chromatin regulates longevity, given that age-related chromatin changes have been known to promote transcriptional dysregulation. Now that next generation sequencing techniques are becoming easier and cheaper to do, there is more incentive to adopt a "big picture" approach using transcriptomics to identify gene regulators and processes that are involved in a biological process as complex as ageing.

6.5. Transcriptomics in multicellular organisms

Multicellular model systems are a necessary tool for studying organismal ageing. However, the presence of multiple cell types can make transcriptional analysis difficult to interpret. In *C. elegans*, RNA-Seq experiments are routinely performed on whole-worm extracts. The transcriptional data generated from these studies are therefore an average of gene expression contributions from different cell types. Thus, the context-specific resolution is automatically lost in the data set. RNA-Seq on whole-worm extracts could also mask the presence of lowly expressed genes, which could be important

mediators of lifespan. In *C. elegans*, development in techniques on harvesting RNA from specific tissues have allowed researchers to perform tissue-specific transcriptomic analysis (Kaletsky et al., 2018). In light of our tissue-specific lifespan data on *utx-1,* it is becoming more apparent that longevity can be mediated at a specific tissue. Therefore, it is important that transcriptional profiling can be accurately assessed in the tissue of interest to determine tissue-specific gene targets that could be involved in lifespan regulation.

6.6. Lifespan is sensitive to environmental factors

Previously, I described that *cbp-1* heterozygous mutants are long-lived when feeding on OP50 bacteria, but short-lived (or have no change in lifespan) when fed HT115 bacteria, suggesting that lifespan can be highly sensitive to changes in food source. *E. coli* OP50 is the standard worm diet for general worm maintenance, while *E. coli* HT115 is routinely used for RNAi feeding protocols. The fact that I saw a difference in the lifespan phenotype when *cbp-1* mutants were fed OP50 and HT115 highlights an issue that is often taken for granted when performing lifespan investigations in *C. elegans*. Lifespan experiments that have been exclusively performed on either OP50 (eg. when using mutants) or HT115 (eg. when using RNAi to knockdown the gene of interest) could give dramatically different results when food is switched from one type to another – possibly confounding interpretations about the longevity function of the gene of interest. To date, only 4 “gene-diet” pairs have been identified in worms, where lifespan is differentially affected depending on whether worms are fed OP50 or HT115 (Table 6.1).

Table 6.1: Known gene-diet pairs in C. elegans.

Gene	Lifespan in OP50 (compared to wild-type)	Lifespan in HT115 (compared to wild-type)	Reference
alh-6 (aldehyde dehydrogenase)	decreased	increased	(Pang & Curran, 2014)
nmur-1 (neuromedin U-receptor-like)	increased	no change	(Maier, Adilov, Regenass, & Alcedo, 2010)
osm-3 (kinesin motor protein)	increased	no change	(Maier et al., 2010)
flr-4 (serine/threonine kinase)	no change	increased	(Verma, Jagtap, Goyala, & Mukhopadhyay, 2018)

This is relatively rare, which is surprising given that nutrient adaptation (an extreme example being dietary restriction) can dramatically affect lifespan. It is unknown whether this rarity is due to lack of parallel lifespan investigations in different food sources in general (and therefore failure to identify diet-specific lifespan changes); or if there is genuinely only a handful of genes involved in mediating adaptive responses to dietary change. Future lifespan studies should take this into consideration and bear in mind that lifespan outcomes can be different across a range of different diets, which could also be informative about the function of the gene of interest.

6.7. Is lifespan an appropriate readout for ageing?

In ageing research, the traditional readout for determining how genetic or pharmacological interventions affect ageing is through lifespan experiments. We often look at lifespan extension or shortening as a measure of whether an intervention is pro- or anti-ageing. The ultimate goal of ageing research is to promote healthy ageing rather than extend lifespan, and lifespan extension does not automatically mean that healthspan is also improved. In that regard, it seems inappropriate to have lifespan as a common readout for ageing studies. Understandably, survival curves are universally understood in the field and can be performed across different model organisms being studied. However,

more attention should be paid to analysing the shape of survival curves, which could be more informative about health. For instance, a mutant that has a longer maximum lifespan but shorter median lifespan compared to wild-type could indicate that the mutant is longer-lived but unhealthier compared to controls. Additionally, it might be useful to officially classify species-specific measures that are more indicative of healthspan. An example of these are motility assays across different species – for example, thrashing in worms and climbing experiments in flies. It should be noted that there are caveats to this, particularly if the gene of interest is involved in animal locomotion, thereby confounding healthspan interpretations. Monitoring the development of species-specific senescent pathologies could also be a good readout for health. Overall, measuring healthspan parameters might be more informative of whether a gene of interest has anti-ageing functions or not, and could be more beneficial in interpreting ageing studies in the future.

6.8. The future of ageing research

Ageing is incredibly complex and there is a vast number of molecular mechanisms implicated in defining how an organism ages. Such mechanisms have identified genes that affect ageing on a cellular level (cellular senescence), ones that promote longevity through metabolic alterations (such as nutrient sensing and dietary restriction) or even changes in the microenvironment (eg. stem cell niche, microbiome). Given that there are many processes involved in ensuring the robustness of lifespan, is there really a "holy grail" for slowing down the rate of ageing? Or does such a "holy grail" simply not exist?

I believe the jury is still out. One of the biggest challenges in ageing research is to map the interconnectedness of the many molecular mechanisms that affect ageing and identify potential signalling nodes (or gene targets) that, when pharmacologically targeted, could slow down the rate of ageing most efficiently. An example of a drug that hits a key

signalling node that profoundly affects ageing is Rapamycin. Rapamycin inhibits mTOR, a key mediator of mitogenic and growth pathways. This drug is commonly used as an immunosuppressant to avoid organ rejection following transplant surgery in humans. However, at smaller doses, rapamycin and its chemical analogs have been found to prevent cancer, atherosclerosis and decrease obesity in mammals (including humans) (reviewed in (Blagosklonny, 2017)). This suggests that Rapamycin is a potent drug for slowing down the development of age-related diseases, and therefore ageing.

On the other hand, the Polypill – a combination of aspirin (to reduce platelet aggregation), folic acid (to lower homocysteine levels) and statins (anti-hypertensive), has failed to produce age-related benefits when tested in humans (Viera, 2017). Polypill was developed as a prophylactic intervention against cardiovascular disease in middle-aged individuals by targeting cardiovascular risk factors such as low density cholesterol, blood pressure, serum homocysteine and platelet function (Wald & Law, 2003). Folic acid was found to have no effect in reducing cardiovascular events (Lee, Hong, Chang, & Saver, 2010), while aspirin modestly reduced incidences of myocardial infarction. However, this is countered by increased incidences of gastrointestinal bleeding, thereby neutralising any possible overall benefit (Baigent et al., 2009).

Could there be other possible targetable genes that are as effective as mTOR? With the emergence of studying biological processes from a systems biology perspective, the idea that interactions between regulatory networks that affect ageing can be modelled may not be too far-fetched. This presents a new angle for studying ageing that could identify new targets where interventions could efficiently slow down the development of age-related diseases and promote better healthspan in individuals.

APPENDIX I: RNA-Seq script

Filtering and quality controls of clean reads were performed by BGI Sequencing (China). Subsequent steps analyses were performed on a standard MacBook Pro laptop.

TRANSCRIPT ABUNDANCE

I used Kallisto (version 0.43., from https://pachterlab.github.io/kallisto) for performing pseudoalignment and abundance modelling. The first step required for my analysis was to download the *C. elegans* transcriptome (genome assembly: WBcel235), using the following code (Mac terminal):

```
curl –O
http://bio.math.berkeley.edu/kallisto/transcriptomes/Caenorhabditis_elegans.WBcel235.rel79.cdna.all.fa.gz
```

File was renamed as "cele_trans.fa.gz" for ease. A transcriptome index was necessarily built based on the *C. elegans* transcriptome, which is the basis for pseudoalignment of clean reads from the RNA-Seq experiment. The index was built using the following command (in Mac terminal):

```
kallisto index –i cele_trans.kidx cele_trans.fa.gz
```

For each of our samples (6 in total: 3 x demethylase dead (DD) and 3 x nonDD), transcript abundance was analysed through pseudoalignment to the index built in Kallisto using the following code:

```
kallisto quant –i cele_trans.kidx –o output_L01_11_1 –b 100 –-
single –l 200 –s 20 CL100011312_L01_11_1.fq.gz
```

Text in grey represents the output name, average fragment length, standard deivation and input name (ie. filename of our data) respectively. For each sample, the following terms were used for the code:

	Input Name	**Average Fragment Length** [a]	**Standard Deviation** [a]	**Output Name**
1 (DD)	CL100011312_L01_14_1.fq.gz	222	22	Sample1
2 (DD)	CL100011312_L01_15_1.fq.gz	216	22	Sample2
3 (DD)	CL100011312_L01_16_1.fq.gz	261	26	Sample3
4 (nonDD)	CL100011312_L01_11_1.fq.gz	211	21	Sample4
5 (nonDD)	CL100011312_L01_12_1.fq.gz	218	22	Sample5
6 (nonDD)	CL100011312_L01_13_1.fq.gz	219	22	Sample6

[a] Fragment lengths per sample were provided by BGI Sequencing and standard deviations were assumed to be 10% as standard.

DIFFERENTIAL TRANSCRIPT EXPRESSION ANALYSIS

The following codes were inputted on RStudio; modified from code written by Dylan Dahan (Stanford University):

```
#LOADING LIBRARIES, REUSLTS AND DATAFRAME

#First download all the necessary libraries, on the bottom left
hand side,under the "Packages" tab, make sure biomaRt is
ticked.
#Download the sleuth package

library("sleuth")

#Next, assign the path to the folder where we put in our
kallisto result
#In my case, this is at ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results

base_dir <- "~/Documents/Abby/Coding Stuff/Kallisto Results"

#Then we need to add data to our samples using the following
code

sample <- list.files(base_dir)

#Now, assign conditions to our samples and how many replicates
each
#In my case, first 3 are "utx-1_DD" and the next 3 are "utx-
1_nonDD"

condition <- c(rep("DD_control",2),rep("non_DD",3))
```

```
#If you have to censor any data, remove that particular data
from your base directory.
#The number of total replicates (in my case, 6) and the files
in your base diractory should match.

#Now, combine into a dataframe:

s2c <- data.frame(sample,condition)

#Then, we assign paths to each sample:

kal_dirs <- sapply(sample,function(id) file.path(base_dir, id))

#Then add path to results to dataframe with samples and types

s2c <- dplyr::mutate(s2c, path = kal_dirs)

#Check dataframe:

s2c

#You'll get something like this:
#sample  condition
path
#1 Sample1 DD_control ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results/Sample1
#2 Sample2 DD_control ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results/Sample2
#3 Sample3 DD_control ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results/Sample3
#4 Sample4     non_DD ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results/Sample4
#5 Sample5     non_DD ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results/Sample5
#6 Sample6     non_DD ~/Desktop/RNA-
Seq/Abby/Trial/Trimmed/Results/Sample6

#LOAD GENE NAMES

mart <- biomaRt::useMart(biomart = "ENSEMBL_MART_ENSEMBL",
                         dataset = "celegans_gene_ensembl",
host="www.ensembl.org")

#Add transcript id, ensemble gene id (WormBase here) and
external gene name (e.g., asp-3)
t2g <- biomaRt::getBM(attributes = c("ensembl_transcript_id",
"ensembl_gene_id",
                                     "external_gene_name"),
mart = mart)

t2g <- dplyr::rename(t2g, target_id = ensembl_transcript_id,
                     ens_gene = ensembl_gene_id, ext_gene =
external_gene_name)
```

```
#Check our annotations, should have transcript it (target_id),
wormbase identifier (ens_gene) and external gene name
(ext_gene)

head(t2g)

#FIT MODEL, RUN WALD TEST AND VIEW RESULTS

so <- sleuth_prep(s2c, ~ condition, target_mapping = t2g)

#Then we use sleuth_fit convenience wrapper for fitting a
measurement error model.
#It uses the bootstraps from our kallisto quantification to
perform techinical variance estimation, performs biological
variance estimation, and shrinkage estimation.

so <- sleuth_fit(so)

#More info on the model sleuth uses for differential analysis
can be found here
#vignette('intro', package = 'sleuth')

#We then perform a Wald test for every transcript, where the
base is our utx-1_DD and we estimate transcript change in utx-
1_nonDD strain.

so <- sleuth_wt(so, which_beta = 'conditionnon_DD')

#We then use this convenient shiny function to view our
results. Not opening here but can do on your own machine.

sleuth_live(so)

#You can also do it one by one

plot_sample_heatmap(so)
plot_pca(so, color_by = "condition")

#And of particular interest, we can retrieve our Wald test
results

so.res <- sleuth_results(so, 'conditionnon_DD', test_type =
"wt")
head(so.res)

#You can extract your data using the following:

#Calls your results table
sor <- sleuth_results(so, "conditionnon_DD", test_type = "wt")

#Creates a subset such that the qval is <= 0.01
sorp <- subset(sor, qval <= 0.01)
```

```
#Writes a csv file with the sorted values into the path you
direct it to
write.csv(sorp, "~/Documents/Abby/Coding Stuff/Sleuth
Results/Sample 1 Censored/qval_0_01.csv")
```

GENE ONTOLOGY ENRICHMENT

Enriched processes due to differentially expressed transcripts were inputted in DAVID version 6.8 (https://david.ncifcrf.gov). GO terms highlighted in Figure 4.6 are ones that were statistically significant (adj. p-val < 0.05).

APPENDIX II: List of differentially expressed transcripts

Kallisto and Sleuth analysis can resolve abundance on a transcript (rather than gene) level. For ease of classification based on gene ontology, transcripts were condensed to gene names of results.

Target ID	Gene ID	b-value ("log_2FC")	q-value (adj. p value)
C24G7.2	acd-1	2.601934973	4.74E-42
T21E8.3	pgp-8	2.587096383	2.60E-38
F08G2.8	F08G2.8	1.725383011	4.26E-32
Y46D2A.2	Y46D2A.2	-2.631156246	4.69E-32
T13F3.6	T13F3.6	-1.511385538	1.05E-24
M60.2.1	M60.2	6.955123565	1.48E-21
F11H8.3	col-8	-1.120302114	3.01E-18
T04H1.9	tbb-6	1.363965414	3.01E-18
F08F3.7	cyp-14A5	1.216598619	6.32E-18
Y68A4A.13	Y68A4A.13	1.320595396	3.77E-17
C50F7.5	C50F7.5	1.016199637	4.03E-15
Y46C8AL.4	clec-71	1.207892748	3.70E-14
Y77E11A.15	col-106	-0.972143603	7.96E-14
C06B3.3	cyp-35C1	-0.930818784	1.12E-13
C08E3.13	C08E3.13	1.212532299	7.94E-13
F15A2.1b.1	col-184	-0.997794774	1.65E-12
C24G7.1	delm-2	2.812483673	2.19E-12
F49C5.12	F49C5.12	1.271073485	3.48E-12
T24E12.5	T24E12.5	1.073381163	8.16E-12
F22F7.8	F22F7.8	-0.857439099	9.15E-12
T26H5.9a	T26H5.9	1.177802834	9.15E-12
F08G2.5	F08G2.5	2.080775669	1.19E-11
C33A12.19a	C33A12.19	-0.978621916	5.21E-11
W10G11.3	W10G11.3	-1.264860711	1.88E-10
C49G7.10	C49G7.10	0.955462213	2.59E-10
F49E12.2	dod-23	-0.857356163	3.03E-10
K09D9.2	cyp-35A3	-1.114535191	5.37E-10
T11F9.9	col-157	-0.755403093	8.47E-10
W03G11.1a	col-181	-1.253139478	1.00E-09
F11G11.11	col-20	-1.027256231	1.51E-09

Y69A2AR.12	Y69A2AR.12	1.025824447	1.74E-09
Y73B6BL.24	acp-6	-0.770602832	3.01E-09
F15B9.6	F15B9.6	1.01450518	6.25E-09
Y69A2AR.25	Y69A2AR.25	1.341720512	6.25E-09
Y39B6A.21	Y39B6A.21	-0.897528561	7.01E-09
Y46C8AL.5	clec-72	0.790114603	8.14E-09
T05A1.2	col-122	-1.166183977	9.73E-09
ZK896.1	ZK896.1	1.11619907	1.10E-08
C29F4.2	best-7	-0.881670966	1.29E-08
C09H5.2a	catp-3	0.706745346	1.35E-08
R03D7.2	R03D7.2	0.76082596	3.11E-08
F21H11.2a	sax-2	0.930612752	4.01E-08
F57G4.8	fbxa-192	0.780220016	4.20E-08
F43C11.3	decr-1.1	-0.782211307	5.71E-08
ZK1193.1	col-19	-1.129381509	6.99E-08
K10B2.2a	ctsa-1	-0.671283658	7.17E-08
F56D6.2	clec-67	0.707254251	7.17E-08
F58B3.1	lys-4	-0.996433445	7.35E-08
W05B2.5	col-93	-0.852726163	7.67E-08
C15A11.5	col-7	-0.965512149	1.34E-07
ZK1055.7	ZK1055.7	0.682257007	1.61E-07
F32A11.7	mltn-1	-0.750604262	1.96E-07
B0507.10	B0507.10	-1.107795241	2.87E-07
F35E12.5	F35E12.5	1.069890281	3.61E-07
M01G12.9	M01G12.9	0.668773787	5.62E-07
B0336.2.2	arf-1.2	-6.299883939	6.04E-07
C35D10.14	clec-5	0.621195587	1.36E-06
Y43D4A.5a	Y43D4A.5	-5.439407688	1.38E-06
T28D6.3	T28D6.3	-1.029510901	1.39E-06
ZK678.5	wrt-4	-0.614196613	1.43E-06
F43G6.11	hda-5	0.759403171	1.43E-06
K11H12.11	K11H12.11	1.114790958	1.73E-06
Y53G8AM.5	Y53G8AM.5	-1.688825942	2.85E-06
C34F6.2	col-178	-0.772289135	2.85E-06
C04G6.2	C04G6.2	-1.045235078	8.86E-06
Y54G9A.4	Y54G9A.4	-0.59222147	9.17E-06
C33C12.8	gba-2	-1.099790665	1.00E-05
F35E12.6.1	F35E12.6	0.646432161	1.12E-05
T02G5.4	T02G5.4	0.612044833	1.36E-05
F20G2.5	F20G2.5	1.063429847	1.40E-05

F41F3.4	col-139	-0.713677887	1.60E-05
H25K10.1	H25K10.1	1.399422971	1.60E-05
C10C5.3	C10C5.3	-0.778342437	1.73E-05
Y22D7AL.14	cht-4	0.657567831	1.78E-05
ZK666.6	clec-60	1.017198876	2.19E-05
M18.1a	col-129	-0.721841276	2.46E-05
F08H9.5	clec-227	-0.808559972	2.78E-05
F22F4.4a.1	F22F4.4	-0.566713162	2.78E-05
F57G4.4	fbxa-191	1.086246028	2.92E-05
Y39E4B.6a.2	Y39E4B.6	-0.773595776	3.29E-05
C14A6.1	clec-48	0.652091397	3.67E-05
F47H4.2b.2	F47H4.2	0.926385112	4.02E-05
B0286.3	B0286.3	0.545042962	4.53E-05
T20D4.7	T20D4.7	0.986962606	4.73E-05
T07H8.5	srg-31	1.094673156	5.04E-05
F42A8.1	F42A8.1	-0.541786689	5.70E-05
C35D10.15	clec-6	1.600520583	6.01E-05
F36A2.3	F36A2.3	-0.544841308	6.98E-05
K06A4.5	haao-1	-0.584768458	7.49E-05
T19H12.9	ugt-12	-0.551206804	8.58E-05
F35C5.5a	clec-62	0.610050267	8.58E-05
C49F5.7	C49F5.7	-0.876679575	8.79E-05
F46B6.8	lipl-2	0.622743481	9.43E-05
B0462.5	B0462.5	1.268503515	9.71E-05
C08F11.7	C08F11.7	0.901419451	9.77E-05
F47H4.2b.1	F47H4.2	0.885989186	0.000103228
C05C10.1	pho-10	-1.124557955	0.000114784
C36C5.14	C36C5.14	0.954215075	0.000114784
C36C5.12	C36C5.12	0.903504278	0.000115201
C41C4.7a	ctns-1	-0.533312556	0.000137838
C52E4.1	cpr-1	-0.701560893	0.000152403
E03H4.8	E03H4.8	0.677125284	0.000152403
W07G1.3.2	zip-3	-0.572037511	0.000165783
C03G6.15	cyp-35A2	-0.515178133	0.000168503
F37B1.1	gst-24	1.031353053	0.000171411
T10D4.6b	T10D4.6	0.661774838	0.000178939
T25C12.2	spp-9	1.468244423	0.000202946
C53B4.5	col-119	-1.098417806	0.000225687
ZK1058.6	nit-1	0.886407985	0.00026624
F38A3.1	col-81	-0.656731018	0.000282326
C05E11.4	amt-1	0.870213589	0.000327738
Y9C9A.6	str-168	0.856815539	0.000344075

D1086.3	D1086.3	-0.749130008	0.000348606
F46C5.10	F46C5.10	-0.626128265	0.000404087
K05C4.6a.1	hmp-2	-5.416269897	0.000431775
F10F2.2	F10F2.2	0.493087089	0.00044409
Y70C5A.3	Y70C5A.3	0.869966499	0.000495791
ZC443.6	ugt-16	-0.505815217	0.000509515
F58G6.7	F58G6.7	-0.501988882	0.000509839
T03D8.1e	num-1	-0.596328206	0.000533195
C36A4.14a	C36A4.14	4.346116282	0.000539053
W05E7.1	grd-3	-0.487161288	0.000543826
K10D3.6	K10D3.6	-0.66449996	0.000874875
F41C3.2	F41C3.2	0.516546969	0.000898714
F21F8.4.1	asp-12	-1.334273764	0.00092985
F38B6.4	F38B6.4	0.480284462	0.001048931
AC3.7	ugt-1	-0.496516967	0.001214203
H06O01.3a	ctg-1	0.784661111	0.001214203
Y116A8C.34 a.2	cyn-13	-5.962608296	0.001226841
R08E5.3	R08E5.3	-0.545850045	0.001276199
M02H5.8	NA	-0.46729041	0.001336728
C24F3.6.2	col-124	-1.156528862	0.001339778
B0024.2	col-150	-0.488095411	0.001482893
C34H4.3a	tag-244	0.568970814	0.001485063
Y46C8AL.3	clec-70	0.963635914	0.001608314
C44C1.5a.1	abhd-3.2	-0.468843956	0.001624194
Y73B6BL.34	bli-6	-0.527868701	0.001734148
K11G9.3	K11G9.3	0.815384534	0.001734148
Y43D4A.5b	Y43D4A.5	-4.58352087	0.002031596
K09F5.2	vit-1	-1.000327328	0.002051415
K09A11.4	cyp-14A3	-1.822994009	0.002128103
R12A1.4	ges-1	0.464976383	0.00231697
F16H6.10	F16H6.10	0.574058408	0.002705264
C15H9.1	nnt-1	0.662665277	0.002705264
C09G5.3	col-79	-0.499785903	0.002729519
T28D6.2	tba-7	0.524643786	0.002729519
F22F4.4b	F22F4.4	-1.065362517	0.00273421
K01D12.14	hrg-2	-0.887092589	0.00284462
C54D1.1	pgp-10	0.473373092	0.003029212
F35E12.7a	dct-17	1.50739595	0.003031201
F09E10.3	dhs-25	-0.607149988	0.003224687
W07B8.5	cpr-5	-0.560807106	0.003228705
ZK909.3.2	ZK909.3	0.490019467	0.003423205

T05A10.5	NA	-0.505182228	0.003440187
T27F2.4	zip-10	0.57030852	0.003656662
K11H12.4	K11H12.4	0.463975855	0.003823769
Y53F4B.33	gst-39	0.496755584	0.003828086
T01C3.9	T01C3.9	-0.438969286	0.003836055
F49E2.5b.2	F49E2.5	0.584270925	0.00414661
C09G5.6	bli-1	-0.459698136	0.004400985
C09C7.1	zig-4	0.715567766	0.004400985
H23L24.5	parg-2	1.744633642	0.004400985
C07D8.6	C07D8.6	-0.4614925	0.004402962
C08E3.6	fbxa-163	2.391403236	0.004584633
F17E9.4	F17E9.4	-0.460516346	0.004676189
K08D8.4a	K08D8.4	0.853353114	0.004676189
C31C9.1a	tag-10	-0.456437259	0.004922268
F57H12.6	F57H12.6	-0.767686327	0.005384948
F57F5.1	F57F5.1	-0.480687575	0.005384948
F14F9.4	F14F9.4	0.576831895	0.005594517
Y47G6A.33	Y47G6A.33	-0.713451723	0.005629534
F08H9.12	srz-97	3.659655242	0.005629534
T03E6.7a	cpl-1	-0.51554028	0.005878959
R02D3.1a	R02D3.1	-0.455112048	0.005934882
F36G9.12	oac-20	-0.437399808	0.006066675
Y69H2.3e	Y69H2.3	0.561070949	0.006208889
F35E12.8a	cld-9	0.446343637	0.006243314
C31A11.5	oac-6	0.534849673	0.006276474
H23N18.1	ugt-13	-0.483252898	0.006378418
F21C10.11a	F21C10.11	1.676821474	0.006378418
T25B2.2a	T25B2.2	4.419230229	0.006378418
T15B7.4	col-142	-0.586997482	0.006640731
Y41D4B.16	hpo-6	0.422350878	0.006671494
W04B5.3c	W04B5.3	0.618156762	0.006677354
R04A9.9.2	NA	-0.765024047	0.006957939
F21A3.11	F21A3.11	0.514032425	0.007062003
Y54G2A.3a	Y54G2A.3	-0.44762468	0.007122912
T01C3.8b	mut-15	-0.85285489	0.007738205
C24F3.6.1	col-124	-0.739477573	0.007898937
D1086.7	D1086.7	-0.417046301	0.007948443
M28.8	M28.8	0.431096487	0.008041398
C07A9.4	ncx-6	-0.559029641	0.008240784
F28D1.5	thn-2	0.419886257	0.008284918
F26D10.3.2	hsp-1	3.627986917	0.008476218
T01C3.8a	mut-15	-0.56155538	0.00880897

B0365.3.2	eat-6	0.521562625	0.008870407
C18H7.11	C18H7.11	0.449377175	0.009084617
Y18D10A.23	Y18D10A.23	-0.471799908	0.009162209
W06D4.6	rad-54	-0.421169779	0.009162209
F47E1.2	F47E1.2	0.458514336	0.009162209
F49E12.9a	drd-1	-0.505011247	0.009373995
F09B9.1	NA	0.711341289	0.009598901

APPENDIX III: Complete list of lifespan data

Knockdown of *utx-1* promotes lifespan extension.

Repeat #	Strain	RNAi condition	N (# of animals)	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	p-value (log-rank test)
1 (as shown in Fig. 3.5)	wild-type	*EV*	47	14.5	14	22	p = 0.0003 (***) vs wild-type in EV
	wild-type	*utx-1 RNAi*	52	17.1	16	28	
2 (as shown in Fig. 3.9)	wild-type	*EV*	53	14.4	15	20	p = 0.0058 (**) vs wild-type in EV
	wild-type	*utx-1 RNAi*	58	16.4	15	27	
3	wild-type	*EV*	45	14.5	14	21	p < 0.0001 (****) vs wild-type in EV
	wild-type	*utx-1 RNAi*	50	17.8	18	23	

Overexpression of *utx-1* promotes lifespan extension.

Repeat #	Strain	Food source	N (# of animals)	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	p-value (log-rank test)
1 (as shown in Fig. 3.5 and of Fig. 3.14B)	wild-type	*HT115 - EV*	57	16.2	15	22	p < 0.0001 (****) vs wild-type in EV
	ZR802 = wild-type + utx-1::gfp	*HT115 - EV*	54	26.9	27	36	
2	wild-type	*OP50*	47	15.7	17	23	p < 0.0001 (****) vs wild-type
	ZR802 = wild-type + utx-1::gfp	*OP50*	49	20.4	21	35	
3 (as shown in Fig. 4.1 – Karolina's data)	wild-type	*OP50*	55	18.3	18	30	p < 0.0001 (****) vs wild-type
	ZR802 = wild-type + utx-1::gfp	*OP50*	49	23.1	22	38	

Lifespan due to tissue-specific knockdown of *utx-1*.

Repeat #	Strain	RNAi condition	N (# of animals)	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	p-value (log-rank test)
1 (as shown in Fig 3.9)	TU3401 (neuron RNAi)	*EV*	56	16.1	16	21	p < 0.0001 (****) vs TU3401 in EV
	TU3401 (neuron RNAi)	*utx-1 RNAi*	58	22.8	24	30	
	VP303 (intestine RNAi)	*EV*	42	13.7	14	24	p = 0.0079 (**) vs VP303 in EV
	VP303 (intestine RNAi)	*utx-1 RNAi*	42	16.2	16	24	
	NR222 (hypodermis RNAi)	*EV*	44	14.4	14	24	p < 0.0001 (****) vs NR222 in EV
	NR222 (hypodermis RNAi)	*utx-1 RNAi*	43	19.3	20	28	
	NR350 (muscle RNAi)	*EV*	47	13.6	14	22	

	NR350 (muscle RNAi)	*utx-1 RNAi*	48	13.3	14	20	p = 0.5386 (ns) vs NR350 in EV
2	TU3401 (neuron RNAi)	*EV*	48	15.1	14	20	p < 0.0001 (****) vs TU3401 in EV
	TU3401 (neuron RNAi)	*utx-1 RNAi*	48	21.8	22	29	
	VP303 (intestine RNAi)	*EV*	51	14.7	14	24	p = 0.0094 (**) vs VP303 in EV
	VP303 (intestine RNAi)	*utx-1 RNAi*	56	16.6	16	24	
	NR222 (hypodermis RNAi)	*EV*	55	15.8	16	25	p < 0.0001 (****) vs NR222 in EV
	NR222 (hypodermis RNAi)	*utx-1 RNAi*	59	20.2	21	27	
	NR350 (muscle RNAi)	*EV*	37	13.7	14	20	p = 0.9635 (ns) vs NR350 in EV
	NR350 (muscle RNAi)	*utx-1 RNAi*	43	13.7	14	18	

Lifespan due to tissue-specific overexpression of *utx-1*.

Repeat #	**Strain**	**Food source**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 3.10 – Karolina's data)	wild-type	*OP50*	59	16.5	16	24	
	ZR694 (muscle utx-1OE)	*OP50*	53	15.1	14	30	p = 0.1374 (ns) vs wild-type
	ZR695 (hypodermis utx-1OE)	*OP50*	42	17.1	16	26	p = 0.2704 (ns) vs wild-type
	ZR698 (neuron utx-1OE)	*OP50*	55	22.8	22	34	p < 0.0001 (****) vs wild-type
	ZR699 (intestine utx-1OE)	*OP50*	58	21.7	22	34	p < 0.0001 (****) vs wild-type
2	wild-type	*HT115 - EV*	56	16.3	16	29	
	ZR698 (neuron utx-1OE)	*HT115 - EV*	56	20.7	18	35	p = 0.0045 (**) vs wild-type
	ZR699 (intestine utx-1OE)	*HT115 - EV*	58	22.4	23	40	p < 0.0001 (****) vs wild-type
	ZR254 = utx-1(tm3118)+utx-1::gfp	*EV*	53	29.9	31	47	p < 0.0001 (****) vs wild-type
3	wild-type	*HT115 - EV*	57	16.2	18	22	
	ZR698 (neuron utx-1OE)	*HT115 - EV*	53	18.7	19	37	p = 0.0014 (**) vs wild-type
	ZR699 (intestine utx-1OE)	*HT115 - EV*	54	17.9	15	36	p = 0.0002 (***) vs wild-type

Lifespan due to tissue-specific knockdown of *daf-2*.

Repeat #	Strain	RNAi condition	N (# of animals)	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	p-value (log-rank test)
1 (as shown in Fig 3.11)	wild-type	*EV*	56	15.7	15	24	p < 0.0001 (****) vs wild-type in EV
	wild-type	*daf-2 RNAi*	55	22.3	20	44	
	NR222 (hypodermis RNAi)	*EV*	57	18.2	18	26	p < 0.0001 (****) vs NR222 in EV
	NR222 (hypodermis RNAi)	*daf-2 RNAi*	58	24.4	22	44	
	VP303 (intestine RNAi)	*EV*	58	16.9	18	28	p < 0.0001 (****) vs VP303 in EV
	VP303 (intestine RNAi)	*daf-2 RNAi*	59	24.3	23	39	
	TU3401 (neuron RNAi)	*EV*	59	17.2	18	26	p < 0.0001 (****) vs TU3401 in EV
	TU3401 (neuron RNAi)	*daf-2 RNAi*	58	33.7	35	47	
	NR350 (muscle RNAi)	*EV*	54	14.9	14	24	p = 0.8217 (ns) vs NR350 in EV
	NR350 (muscle RNAi)	*daf-2 RNAi*	58	14.9	14	19	

daf-16 dependence of lifespan in *utx-1* knockdown animals.

Repeat #	Strain	RNAi condition	N (# of animals)	Mean Lifespan (days)	Median Lifespan (days)	Maximum Lifespan (days)	p-value (log-rank test)
1 (as shown in Fig 3.12)	*daf-16(mu86)I*	*EV*	56	14.5	14	18	p = 0.3842 (ns) vs daf-16(mu86) in EV
	daf-16(mu86)I	*utx-1 RNAi*	54	14.7	14	18	
	daf-16(mu86)I; intestine RNAi	*EV*	56	12.5	12	16	p = 0.7605 (ns) vs daf-16(mu86); intestine RNAi in EV
	daf-16(mu86)I; intestine RNAi	*utx-1 RNAi*	55	12.4	11	16	
	daf-16(mu86)I; neuron RNAi	*EV*	57	12.8	14	17	p = 0.7890 (ns) vs daf-16(mu86); neuron RNAi in EV
	daf-16(mu86)I; neuron RNAi	*utx-1 RNAi*	58	13.0	14	16	
	daf-16(mu86)I; hypodermis RNAi	*EV*	57	16.3	16	22	p = 0.0001 (***) vs daf-16(mu86); hypodermis RNAi in EV
	daf-16(mu86)I; hypodermis RNAi	*utx-1 RNAi*	58	18.3	18	23	
2	*daf-16(mu86)I*	*EV*	56	14.3	14	17	p = 0.2708 (ns) vs daf-16(mu86) in EV
	daf-16(mu86)I	*utx-1 RNAi*	57	14.6	14	17	
	daf-16(mu86)I; intestine RNAi	*EV*	59	11.6	11	16	p = 0.0669 (ns) vs daf-16(mu86); intestine RNAi in EV
	daf-16(mu86)I; intestine RNAi	*utx-1 RNAi*	54	11.9	11	16	
	daf-16(mu86)I; neuron RNAi	*EV*	60	13.3	13.5	19	p = 0.5741 (ns) vs daf-16(mu86); neuron RNAi in EV
	daf-16(mu86)I; neuron RNAi	*utx-1 RNAi*	57	13.0	13	19	

	daf-16(mu86)I; hypodermis RNAi	*EV*	59	14.3	14	19	p = 0.0190 (*) vs daf-16(mu86); hypodermis RNAi in EV
	daf-16(mu86)I; hypodermis RNAi	*utx-1 RNAi*	60	14.8	15	21	

daf-2 dependence of lifespan in *utx-1* overexpressing animals.

Repeat #	**Strain**	**Food source**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 3.13)	*daf-2(e1370)III*	*OP50*	59	33.0	32	48	p < 0.0001 (****) vs *daf-2(e1370)III*
	daf-2(e1370)III; utx-1(tm3118)X + utx-1::gfp	*OP50*	52	42.2	42	65	

daf-16 dependence of lifespan in *utx-1* overexpressing animals.

Repeat #	**Strain**	**RNAi condition**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig 3.14)	wild-type	*daf-16 RNAi*	56	13.3	14	17	
	ZR254 = utx-1(tm3118)+utx-1::gfp	*daf-16 RNAi*	53	14.1	15	19	p = 0.0005 (***) vs wild-type in daf-16 RNAi
	ZR698 (neuron utx-1OE)	*daf-16 RNAi*	56	14.5	14	21	p = 0.0002 (***) vs wild-type in daf-16 RNAi
	ZR699 (intestine utx-1OE)	*daf-16 RNAi*	58	14.5	16	20	p = 0.0001 (***) vs wild-type in daf-16 RNAi
2 (as shown in Fig. 3.14)	wild-type	*daf-16 RNAi*	59	14.8	13	18	
	ZR802 = wild-type + utx-1::gfp	*daf-16 RNAi*	60	15.4	15	22	p = 0.0291 (*) vs wild-type in daf-16 RNAi
	ZR698 (neuron utx-1OE)	*daf-16 RNAi*	57	15.5	15	22	p = 0.0022 (**) vs wild-type in daf-16 RNAi
	ZR699 (intestine utx-1OE)	*daf-16 RNAi*	57	15.5	13	26	p = 0.0343 (*) vs wild-type in daf-16 RNAi

Lifespan data showing that the catalytic activity of UTX-1 is important for lifespan extension due to *utx-1* overexpression.

Repeat #	**Strain**	**Food source**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig 4.3)	wild-type	*OP50*	47	15.6	17	23	
	ZR254 = utx-1(tm3118)+utx-1::gfp	*OP50*	46	20.8	20	34	p < 0.0001 (****) vs wild-type; p = 0.0117 (*) vs ZR256

	ZR256 = utx-1(tm3118)+utx-1DD::gfp	*OP50*	54	18.9	18	30	p < 0.0001 (****) vs wild-type
2	wild-type	*OP50*	56	14.7	14	24	
	ZR254 = utx-1(tm3118)+utx-1::gfp	*OP50*	46	21.3	18	38	p < 0.0001 (****) vs wild-type; p = 0.0041 (**) vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp	*OP50*	54	17.8	18	30	p < 0.0001 (****) vs wild-type
3 (as shown in Fig. 4.2)	wild-type	*OP50*	48	14.9	14	22	
	ZR254 = utx-1(tm3118)+utx-1::gfp	*OP50*	44	29.6	30	50	p < 0.0001 (****) vs wild-type; p < 0.0001 vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp	*OP50*	44	20.0	19	34	p < 0.0001 (****) vs wild-type

Stress survival assays on *utx-1* overexpressing animals

Repeat #	**Strain**	**Treatment**	**N (# of animals)**	**Mean Lifespan (hours)**	**Median Lifespan (hours)**	**Maximum Lifespan (hours)**	**p-value (log-rank test)**
1 (as shown on Fig. 4.8A)	wild-type	10mM paraquat	47	49.0	52	72	
	ZR254 = utx-1(tm3118)+utx-1::gfp		49	60.5	59	95	p < 0.0001 (****) vs wild-type; p < 0.0001 (****) vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp		49	37.9	42	64	p < 0.0001 (****) vs wild-type
	wild-type + *utx-1* RNAi		49	58.9	62	86	p < 0.0001 (****) vs wild-type
2	wild-type	5mM paraquat	44	65.7	72	95	
	ZR254 = utx-1(tm3118)+utx-1::gfp		58	92.2	81	168	p < 0.0001 (****) vs wild-type; p < 0.0001 (****) vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp		57	52.6	52	78	p < 0.0001 (****) vs wild-type
Repeat #	**Strain**	**Treatment**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown on Fig 4.8B)	wild-type	35 deg C	40	6.9	7.5	12	
	ZR254 = utx-1(tm3118)+utx-1::gfp		45	18.0	20	30	p < 0.0001 (****) vs wild-type; p < 0.0001 (****) vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp		40	12.5	13	20	p < 0.0001 (****) vs wild-type

1 (as shown in Fig 4.8C)	wild-type	1000 J/m² UVC	40	4.6	5	9	
	ZR254 = utx-1(tm3118)+utx-1::gfp		45	6.5	7	11	p < 0.0001 (****) vs wild-type; p < = 0.0177 (*) vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp		39	5.4	5	9	p = 0.0274 (*) vs wild-type
2	wild-type	1000 J/m² UVC	55	3.9	4	7	
	ZR254 = utx-1(tm3118)+utx-1::gfp		54	5.4	6	10	p < 0.0001 (****) vs wild-type; p < = 0.0021 (**) vs ZR256
	ZR256 = utx-1(tm3118)+utx-1DD::gfp		55	4.7	5	7	p = 0.0042 (**) vs wild-type

RNAi screen

Repeat #	**Strain**	**RNAi condition**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 4.9)	wild-type	*EV*	53	14.4	15	20	
	wild-type	*clec-71 RNAi*	57	13.8	13	18	p = 0.1731 (ns) vs wild-type in EV
	wild-type	*parg-2 RNAi*	58	14.2	13	20	p = 0.3321 (ns) vs wild-type in EV
	wild-type	*K11H12.11 RNAi*	55	13.9	13	20	p - 0.3632 (ns) vs wild-type in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*EV*	46	23.1	20	48	
	ZR254 = utx-1(tm3118)+utx-1::gfp	*clec-71 RNAi*	49	22.7	22	41	p = 0.9173 (ns) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*parg-2 RNAi*	50	26.6	27	36	p = 0.1216 (ns) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*K11H12.11 RNAi*	47	26.6	27	39	p = 0.0826 (ns) va ZR254 in EV
Repeat #	**Strain**	**RNAi condition**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 4.10 – 4.12	wild-type	*EV*	47	13.4	13	19	
	wild-type	*fbxa-163 RNAi*	50	15.0	14	25	p = 0.0220 (*) vs wild-type in EV
	wild-type	*srg-31 RNAi*	49	15.5	15	25	p = 0.0009 (***) vs wild-type in EV
	wild-type	*Y69A2AR.25 RNAi*	48	17.5	17	25	p < 0.0001 (****) vs wild-type in EV
	wild-type	*spp-9 RNAi*	39	16.4	17	25	p < 0.0001 (****) vs wild-type in EV

	wild-type	*T26H5.9 RNAi*	50	14.6	14	20	p = 0.0396 (*) vs wild-type in EV
	wild-type	*delm-2 RNAi*	48	14.4	13	25	p = 0.1361 (ns) vs wild-type in EV
	wild-type	*tbb-6 RNAi*	48	13.9	13	19	p = 0.3011 (ns) vs wild-type inEV
	wild-type	*Y69A2AR.12 RNAi*	44	13.8	13	30	p = 0.4877 (ns) vs wild-type in EV
	wild-type	*hsp-1 RNAi (I)*	47	13.2	13	19	p = 0.4343 (ns) vs wild-type in EV
	wild-type	*hsp-1 RNAi (II)*	48	14.0	15	25	p = 0.3923 (ns) vs wild-type in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*EV*	48	18.4	17	27	
	ZR254 = utx-1(tm3118)+utx-1::gfp	*fbxa-163 RNAi*	47	25.0	25	35	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*srg-31 RNAi*	48	27.1	27	37	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*Y69A2AR.25 RNAi*	49	26.9	27	37	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*spp-9 RNAi*	51	27.3	27	37	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*T26H5.9 RNAi*	47	27.5	28.5	37	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*delm-2 RNAi*	50	23.6	25	37	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*tbb-6 RNAi*	47	24.4	25	35	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*Y69A2AR.12 RNAi*	48	29.8	30	37	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*hsp-1 RNAi (I)*	48	13.5	13	19	p < 0.0001 (****) vs ZR254 in EV
	ZR254 = utx-1(tm3118)+utx-1::gfp	*hsp-1 RNAi (II)*	50	13.6	13	19	p < 0.0001 (****) vs ZR254 in EV

Heterozygous *cbp-1* mutants are longer-lived than controls when fed OP50 bacteria.

Repeat #	**Strain**	**Food source**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 5.2B)	*wild-type - No FuDR*	*OP50*	51	18.2	18	26	
	+/hT2 - No FuDR	*OP50*	58	18.2	18	23	p = 0.0532 (ns) vs wild-type
	cbp-1(ok1491)/hT2 - No FuDR	*OP50*	58	20.8	21	30	p = 0.0040 (**) vs wild-type; p < 0.0001 (****) vs +/hT2

2	*+/hT2 - No FuDR*	*OP50*	59	15.4	16	23	
	cbp-1(ok1491)/hT2 - No FuDR	*OP50*	53	16.5	17	24	p = 0.0184 (*) vs +/hT2
1 (as shown in Fig. 5.2C)	*+/hT2*	*OP50*	60	15.2	15	28	
	cbp-1(ok1491)/hT2	*OP50*	60	18.4	18	34	p < 0.0001 (****) vs +/hT2
2	*+/hT2*	*OP50*	54	15.3	16	22	
	cbp-1(ok1491)/hT2	*OP50*	45	17.3	17	22	p = 0.0001 (***) vs +/hT2
1 (as shown in Fig. 5.2C)	*+/hT2*	*OP50*	35	14.2	15	28	
	cbp-1 (bm1)/hT2	*OP50*	31	18.1	18	23	p = 0.0120 (*) vs +/hT2
	cbp-1(bm2)/hT2	*OP50*	46	19.0	18	28	p = 0.0008 (***) vs +/hT2
2	*+/hT2*	*OP50*	47	17.7	19	29	
	cbp-1 (bm1)/hT2	*OP50*	48	19.9	19	30	p = 0.0357 (*) vs +/hT2
	cbp-1(bm2)/hT2	*OP50*	46	22.6	23	30	p < 0.0001 (****) vs +/hT2

Oxidative stress survival assay of heterozygous *cbp-1* mutants.

Repeat #	**Strain**	**Treatment**	**N (# of animals)**	**Mean Lifespan (hours)**	**Median Lifespan (hours)**	**Maximum Lifespan (hours)**	**p-value (log-rank test)**
1 (as shown in Fig. 5.3C)	*+/hT2 - 5mM paraquat*	5mM paraquat	53	98.2	101	141	p = 0.1725 vs +/hT2
	cbp-1(ok1491)/hT2 - 5mM paraquat	5mM paraquat	52	87.2	95	144	

Lifespan analysis of *cbp-1(ku258)gf* mutants.

Repeat #	**Strain**	**Food source**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 5.4C)	*wild-type - No FuDR*	*OP50*	54	16.1	17	23	p < 0.0001 (****) vs wild-type
	cbp-1(ku258) - No FuDR	*OP50*	69	4.7	4	14	
	wild-type	*OP50*	40	14.4	14	22	p < 0.0753 (ns) vs wild-type
	cbp-1(ku258)	*OP50*	48	12.6	13	23	

Extended lifespan of heterozygous *cbp-1* mutants feeding on OP50 is abolished when fed HT115 bacteria.

Repeat #	**Strain**	**Food source**	**N (# of animals)**	**Mean Lifespan (days)**	**Median Lifespan (days)**	**Maximum Lifespan (days)**	**p-value (log-rank test)**
1 (as shown in Fig. 5.5A)	*+/hT2*	*HT115 - EV*	60	16.0	17	24	p = 0.2709 (ns) vs +/hT2
	cbp-1(ok1491)/hT2	*HT115 - EV*	52	17.1	17	30	

2	*+/hT2*	*HT115 - EV*	59	16.3	16	21	p = 0.8017 (ns) vs +/hT2
	cbp-1(ok1491)/hT2	*HT115 - EV*	62	16.1	16	21	
1 (as shown in Fig. 5.5B)	*+/hT2*	*HT115 - EV*	50	20.1	23	27	
	cbp-1 (bm1)/hT2	*HT115 - EV*	58	16.1	17	23	p < 0.0001 (****) vs +/hT2
	cbp-1(bm2)/hT2	*HT115 - EV*	54	16.4	17	21	p < 0.0001 (****) vs +/hT2

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