The roles of *ccf-1* and *pal-1* genes in aging and stress resistance of *Caenorhabditis elegans*

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ABSTRACT

The CCR4-NOT complex is a critical regulator of gene expression involved in multiple cellular processes, including transcriptional regulation, RNA metabolism, and stress responses. In this thesis, I investigated the role of *ccf-1*, a component of the CCR4-NOT complex, in stress responses and its necessity for normal lifespan and its implications in various pro-longevity signaling pathways in *C. elegans*. Through my experiments using environmental stressors such as cadmium and acrylamide, I measured several stress-responsive gene expression levels and observed that *ccf-1* is required for stress-induced transcriptional changes in the model organism *Caenorhabditis elegans*. Additionally, I found that the *ccf-1* gene plays an important role in the lifespan of several long-lived mutants, highlighting its significance in the aging process. I also showed that other components of the CCR4-NOT complex are also involved in promoting proper transcriptional response to environmental stressors. Furthermore, my research led to the discovery of the PAL-1 protein as a novel transcriptional factor involved in stress response with pleiotropic effects, demonstrating additive impacts when interacting with the *ccf-1* gene. These findings contribute to a better understanding of the molecular mechanisms governing stress responses and aging in *C. elegans and* may have broader implications in unraveling similar processes in higher organisms, including humans.

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LIST OF ABBREVIATIONS

AD	Activating domain
ADH2	Alcohol dehydrogenase 2
AID	Auxin-inducible degron
AKT-1	AKT serine/threonine kinase 1
AKT-2	AKT serine/threonine kinase 2
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
C. elegans	*Caenorhabditis elegans*
CAF- 1	4-CCR4-associated factor
CAT	Catalase
CCF-1	Carbon catabolite repression associated factor
CCR4-NOT	Carbon Catabolite Repression—Negative On TATA-less
cdc-42	Cell division cycle 42
cDNA	Complimentary DNA
cdr-1	Cadmium responsive 1
cdr-4	Cadmium responsive 4
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Cycle threshold
daf-16	Dauer formation 16
daf-16	Dauer formation protein 16
daf-2	Dauer formation protein 2
DBD	DNA binding domain
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
dNTPs	deoxynucleotide triphosphates
dsRNA	Double stranded RNA
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid

ETOH	Ethyl alcohol (ethanol)
EV	Empty vector
gcs-1	Gamma glutamyl-cysteine synthetase 1
GPx	Glutathione peroxidase
gst	Glutathione S-transferase
gst-12	Glutathione S-transferase 12
gst-14	Glutathione S-transferase 14
gst-30	Glutathione S-transferase 30
gst-33	Glutathione S-transferase 33
gst-38	Glutathione S-transferase 38
gst-4	Glutathione S-transferase 4
gst-5	Glutathione S-transferase 5
H_2O_2	Hydrogen peroxide
hsp-16.41	Heat shock protein 16.41
hsp-16.49	Heat shock protein 16.49
hsp70	Heat shock protein 70
IIS	Insulin/insulin-like signaling
IPTG	Isopropylthio-β-galactoside
LB	Lysogeny broth
LET-711	Lethal-711
Msn-2	multicopy suppressor of SNF1 mutation protein 2
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NaOH	Sodium hydroxide
NGM	Nematode growth media
Nrf2	Nuclear factor erythroid 2-related factor 2
NTL-2	Negative on TATA-less- like 2
NTL-3	Negative on TATA-less- like 3
numr-1	Nuclear localized metal responsive 1
p-value	Probability value

PAL-1	Posterior Alae
PCR	Polymerase chain reaction
PDK-1	3-Phosphoinositide-dependent kinase 1
Pol II	RNA polymerase II
qPCR	Quantitative polymerase chain reaction
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RNAi	RNA interference
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
rpl-2	Ribosomal protein, large subunit 2
Rpm	Revolutions per minute
RRF-3	RNA-dependent RNA polymerase family
SKN-1	Skinhead-1
sod-2	Superoxide dismutase 2
TAF	TBP-associated factors
TBP	TATA binding protein
TF	Transcription factor
TIR1	Transport inhibitor response 1
TOR	Target of rapamycin
TOR	Target of rapamycin
tRNA	Transfer ribonucleic acid
Y2H	Yeast 2-hybrid

1. INTRODUCTION

1.1 Biology of aging

The process of aging is broadly defined as the deterioration over time of physiological functions necessary for survival that results from a progressive accumulation of molecular and cellular damage that ultimately leads to death (1–3). Human lifespans have increased significantly in the last century as new drugs, vaccines, and nutritional and hygiene standards were discovered, developed, and implemented. Despite scientific efforts to extend the human lifespan, age-related diseases still pose a challenge to humans. Aging is the leading risk factor for many human diseases such as Alzheimer's, Parkinson's, and different types of cancer (2). Consequently, there has been a growing interest in aging research to overcome aging related diseases. The main goal of aging research is to gain a better understanding of age-related diseases by characterizing the genes, mechanisms, and biological processes involved. Numerous studies showed that genes that increase lifespan can also be beneficial for postponing neurodegenerative diseases, which is one of the important goals of aging research (4). To study aging, shorter-lived model organisms such as worms and flies are commonly used due to the difficulty of ethically collecting tissues of humans and mammals. Model organisms such as worms and flies have been studied for decades, and researchers have discovered genes, nutritional influences, and environmental factors that regulate their lifespans (4–6).

In the 1990s, the *daf-2* (Dauer Formation) gene was discovered as the first gene that can regulate lifespan in the roundworm *Caenorhabditis elegans. C. elegans* mutant worms with loss of function to the *daf-2* gene lived twice as long as wildtype worms (7). After this discovery, scientists began to realize how easily and drastically a single gene can influence the lifespan of a whole organism. This finding sparked a new field of aging research that relies on model organisms for which manipulation of different genes can be studied for aging purposes. In the past few decades, several other genes involved in regulating lifespan have also been found (8). More recently, aging signaling and mechanistic pathways were gradually mapped. Initially, by discovery of *daf-2* gene and then other longevity regulating genes, researchers found that they can influence aging via altering a variety of cell processes such as metabolism, stress responses, growth, and fertility (9,10).

1.2 Theories of aging:

There are several theories of aging; however, there is no consensus on a comprehensive theory. Although some theories have been disproven, others remain credible or have gained support. There are two major theories of aging: stochastic and programmed (11). According to the stochastic theory, aging is caused by external factors that lead to mutations, oxidative stress damage, or metabolic rate changes (12). On the other hand, the theory of programmed aging claim genetic composition plays a major role in aging, meaning aging is governed internally by genes effects (13,14). Other theories such as the antagonistic pleiotropic theory by Williams in 1957 proposed that an evolutionary origin of aging that states aging is a side effect of a group of certain genes that are beneficial for the organisms in their early life (i.e. enhanced fertility), but can become detrimental later in life by causing long-term deterioration (15). This is also known as the disposable soma theory that states genes that were beneficial during the early stages of life turn detrimental later, eventually causing death (15,16).

On the other hand, there are several non-evolutionary theories of aging. Raymond Pearl, proposed "the rate of living" theory, that organisms with higher metabolic rates live shorter than those with lower metabolic rates (17). Several studies supported this theory, which included studies on animals with lower metabolic rates during hibernation that tend to be long-lived (18–20). Further, the mitochondrial theory suggests that the progressive mitochondrial dysfunction over time would result in the death of the organism by mitochondrial fatigue as it provides energy for cells (21).

Another commonly accepted theory is the "free radical theory of aging" that states aging is caused by the accumulation of damages from the progressive accumulation of reactive oxygen species (ROS) during cell physiological processes that lead to cellular damage (22). This theory is linked to the mitochondria theory as ROS mostly accumulates in the mitochondria where free radicals are produced frequently. These free radicals serve as mutagens and are reactive towards biological molecules such as proteins, lipids, and DNA and destroy the molecules and subsequently the organelles (22). The mitochondrial and free radical theories are based on the rate of living theory which again states that the rate of energy consumption is the main cause of aging, meaning if the metabolic rate is higher in an organism, its lifespan will likely be shorter (23). These theories for which damage to cellular components is the primary driver of aging have led to the

identification of intrinsic cellular stress defense mechanisms that are important in maintaining longevity such as the immune system and the antioxidant defense. In this thesis, I will explore the role of stress defense mechanisms in regulating aging in the *C. elegans* model.

1.3 Pathways implicated in aging

Studies have mapped several age-related genetic signaling pathways. Studying these pathways is potentially beneficial for identifying therapeutic targets to slow aging and prevent aging-related diseases in humans. Many of these pathways are evolutionarily conserved in model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster* (fruit flies), and human. Among these pathways, a number of genes have been extensively studied that include the mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK), and insulin/insulin-like signaling (IIS) pathway receptor (24–26).

1.3.1 The insulin/insulin-like growth factor signaling (IIS) pathway

When the *daf-2* gene, which encodes the *C. elegans* insulin receptor, was demonstrated to be an important aging regulator, the IIS pathway had just begun to be mapped. At the moment when scientists discovered the importance of the *daf-2* gene as a crucial controller of aging, they were in the early stages of figuring out and mapping the insulin-like growth factor signaling (IIS) pathway. This evolutionarily conserved pathway plays a key role in various physiological processes, including growth, cell integrity, gametogenesis, cell differentiation, nutrient level sensing, and aging (27). As the IIS pathway acts primarily on the downstream transcription factor DAF-16/FOXO, it serves as a nutrient sensing system for development and behaviour response (28). In normal growth conditions, DAF-2 is activated and serves to inhibit DAF-16/FOXO (by activating PDK-1, AKT-1, and AKT-2; Figure 1.1) (29). However, under stressed or unfavorable conditions, inhibition of IIS results in the activation of DAF-16/FOXO that in *C. elegans* promotes larval arrest and decreases larval development to enhance long-term survival. For instance, when exposed to elevated temperatures, overcrowding, or limited food supply, the signaling of the *daf-2* pathway diminishes, leading to a developmental arrest of animals at the dauer larval stage, and the metabolism changes to promote both survival and longevity. In optimal environmental

circumstances where nutrient is not a limiting factor, the *daf-2* signaling pathway becomes active, prompting rapid metabolism resulting in a relatively short lifespan (30–32). Beyond promoting larval survival, *daf-2* mutants exhibited activation of the DAF-16 transcription factor, which enhances *C. elegans* resistance to stressful conditions such as heat stress, heavy metal stress, hypoxia, radiation, and oxidative stress (28,29).

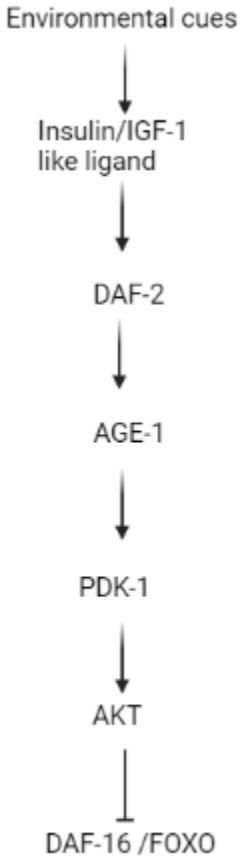

Figure 1.1 Schematic of conserved insulin and IGF-1-like signaling pathway in nematodes (Pathways were adapted from Sutphin and Kaeberlin, 2011).

1.3.2 TOR signaling pathway

The discovery of an inhibitory metabolite of mTOR protein kinase known as rapamycin was a crucial starting point in the discovery of the mTOR protein, as it was found to have the ability to inhibit cell division in mammalian cells and be involved in cell growth, protein synthesis,

apoptosis, and autophagy through two protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (33,34). Later, it was discovered that inhibiting mTOR extended the lifespan of various organisms primarily by reducing protein synthesis and activating dietary/nutrient restriction signals (34,35). It is interesting to note that inhibition of mTOR has also been shown to enhance the stress response (36). Notably, the mTOR signaling pathway actively interacts with other aging-related pathways such as IIS, and stress defense mechanisms including oxidative stress, heat shock, and hypoxic responses.

1.4 Aging and stress response

Aging has been linked to several factors such as epigenetic modifications, metabolism, stress response, and inflammation (37). Generally speaking, there is a negative relationship between aging and stress response. As we age, our resistance against environmental stressors will be diminished compared to when we are young (38). Several studies have indicated that mutations or overexpression of some genes that increase resistance to stressors such as starvation, heat stress, or oxidative stress can also extend lifespan, supporting a positive correlation between stress resistance and longevity. It should be noted, however, that this is not always the case, meaning that an increase in lifespan does not guarantee an increase in stress resistance, and vice versa (39,40). In *C. elegans*, various transcriptional pathways that regulate environmental stress response have also promoted longevity (41,42). Consistently, impaired stress response has been associated with aging in *C. elegans* and various human diseases (41,43). There are also studies that show counter results meaning impaired stress response is not linked to a decrease in aging or human diseases. For example, studies on mice and *C. elegans* showed that mutation in the *sod-2* (superoxide dismutase-2) gene decreases stress resistance, but does not lead to premature aging in mice (44,45). Other studies focusing on different aging-related genes and pathways have also shown counter outcomes. (46). For example, loss of function to the *gpdh-1/2* (Glycerol-3-phosphate dehydrogenase) gene in *C. elegans* decreases the stress resistance of the *daf-2* mutants without affecting lifespan. This means that these mutations somehow impair the organism's stress response without influencing its overall lifespan (47). Additionally, other conflicting studies suggest an increase in stress resistance might lead to a reduction in lifespan. For instance, the constitutive activation of the mitochondrial unfolded protein response transcription factor ATFS-1 in *Drosophila melanogaster* increases stress resistance but decreases overall lifespan (5,48). The

complex relationship between stress resistance and lifespan emphasizes the intricate nature of stress response and aging.

1.4.1 Transcription factors and stress response

Gene expression involves converting genetic information into functional products, beginning with the transcription of DNA into RNA and subsequently translation into proteins. Several factors affect the rate at which transcription occurs to prevent inappropriate gene expression and can stimulate or suppress gene synthesis based on metabolic conditions. RNA polymerase II (Pol II) transcribes most protein-coding genes during transcription from DNA to RNA; however, RNA polymerases function together with transcription factors that recognize specific DNA sequences and recruit Pol II to carry out transcription (49,50). Depending on the cellular condition, these transcriptional factors, along with other regulatory factors may be involved in all transcription stages, including initiation, elongation, and termination. A transcription factor acts as a trans-regulatory element on distant genes and can have an overall influence on gene expression. These factors are essential in the development and growth of all cells, especially in stress conditions. Since a single mutation in the regulatory regions of transcription factors or cofactors can cause various diseases, including cancer, neurological disorders, developmental syndromes, and diabetes, their roles in development and cell growth cannot be negligible.

Several signaling pathways regulate transcriptional factors and thus are important in stress response, aging, lifespan, and several diseases (51–53). For example, the main lifespan regulatory factor in the IIS pathway is the forkhead box protein O (FOXO)/DAF-16 transcription factor. In various organisms, IIS pathway mediates the majority of longevity-related characteristics through FOXO/DAF-16 transcription factor (54–56). In another illustrative instance, the mTOR signaling pathway controls hundreds of downstream targets, many of which encode transcription factors that play a crucial role in fostering anabolic processes, such as protein synthesis, and inhibiting catabolic processes such as autophagy (57,58).

1.4.2 Common types of environmental stress

There are several types of environmental stressors and their relevant response assays allow us to determine how exposure to these stressors affects organismal physiology. Some common

stress factors include oxidative stress, heavy metal stress, temperature (heat and cold) stress, and oxygen (hypoxia and hyperoxia) stress. Examining the organismal response to these external stressors can offer valuable insights into how environmental factors influence biological physiology.

1.4.2.1 Heavy metal stress

Heavy metal is harmful to humans as it is capable of causing cellular stress by altering the structure and function of proteins and enzymes. The accumulation of heavy metals in humans can result in neurodegenerative disorders, impaired cognition, gastrointestinal tract malfunction, and renal dysfunction (59–61). Body responses to heavy metals are mainly initiated by metallothionein proteins, which chelate the metals in cells and prevent harmful effects from occurring. Using model organisms such as *C. elegans*, a metal resistance survival assay can provide insight into the mechanisms involved in the interaction between other protective molecules and heavy metals and is of translational relevance toward understanding metal-induced human diseases since many of these protective molecules are well conserved within species (62,63). In this thesis, I will explore how *C. elegans* respond to cadmium as an effective well-known inducer of heavy metal toxicity to uncover new regulatory mechanisms of heavy metal protective responses.

1.4.2.2 Oxidative stress

Reactive oxygen species (ROS) are byproducts of natural cellular processes that can cause lipid, DNA, and protein damages. The result can be significant cell damage, negatively impacting development, homeostasis, immunity, and health (64,65). Glutathione is the most important molecule when oxidative stress is induced. To mitigate the harmful impacts of ROS, organisms employ an antioxidant defense mechanism to neutralize ROS and prevent its downstream reactions from further damage (39,65). Detoxification genes specialized in oxidative stress conditions will be upregulated to induce the production of related enzymes such as superoxide dismutase (SOD), glutathione S-transferase (GST), catalase (CAT), and glutathione peroxidase (GPx) (66,67). Conducting survival assays under oxidative stress conditions will give insights into the essential biological functions in response to oxidative stress. In this thesis, I will explore how *C. elegans* respond to acrylamide as a well-recognized inducer of oxidative stress to uncover new regulatory mechanisms of the oxidative stress responses.

1.5 CCR4-NOT complex

As RNA polymerase II initiates transcription of protein-coding genes, several transcription factors guide the polymerase to position on promoters. TATA-binding protein (TBP) recruitment is the first step in this process. All three types of polymerases (I, II, and III) used for transcribing mRNA share a TATA-binding protein factor that makes it more possible as a binding target of all different types of polymerases to initiate the transcription (68). Several factors such as TBP-associated factors (TAFs), are physically associated with TBP and are essential for RNA transcriptions (69).

Another important factor in this regard is the CCR4-NOT complex. The CCR4-NOT complex is an evolutionarily conserved multi-protein complex named after the catalytic subunits, carbon catabolite repressor protein (CCR) CCR4-associated factor (CAF) 1 complex (CCR4 and CAF1), and the structural subunits, CCR4-negative on TATA-less (NOT) (70). At first, it was recognized to function in transcription elongation as CCR4-NOT mutant strains were susceptible to the transcription elongation inhibitor 6-azauracil and mycophenolic acid. It was first discovered that CCR4-NOT regulates the transcription of the alcohol dehydrogenase 2 (ADH2) gene, which converts alcohol into acetaldehyde. Still, subsequent studies have revealed that it is also involve in DNA repair, translation regulation, mRNA translatability, and cell wall integrity (71,72). That being said, the complex has a series of roles in different physiological processes within the cell including cell proliferation, control of metabolism, apoptosis, and embryogenesis. In humans, this complex consists of eight subunits. CCR4 and CAF1, as two subunits of the complex, were further recognized as regulators of deadenylation of mRNA, the first catalytic reaction, which shortens the poly(A) tail from mRNA and, consequently, degrades it leading to mRNA translation repression (71). The NOT subunit is generally involved in the second catalytic reaction, ubiquitination, which is the degradation of mRNA by binding ubiquitin(s) to target proteins. So far, researchers have shown several roles of CCR4-NOT in the cell cycle that involve transcription, mRNA, and protein degradation, as well as several other studies that brought this complex to attention as a key gene expression regulator. When transcription occurs, two consequent events stabilize the mRNA; first adding a poly-(A) tail and then the 5′cap (73). The poly-(A) tail protects the mRNA from degradation by nuclease. It is hypothesized that one of the significant events during mRNA decay is removal of the poly-(A) tail during deadenylation, which is usually catalyzed by the CCR4-NOT complex within the cells.

1.5.1 CCR4-NOT complex roles in cellular function and stress response

Stress activates genes associated with stress response through specific signaling pathways and as a result, these genes protect the cells from damage. The CCR4-NOT complex was initially identified as a eukaryotic deadenylase that post-transcriptionally regulates mRNA stability through poly-A tail removal (74,75). Subsequent studies in yeast have also revealed a role for the CCR4-NOT in transcriptional initiation through interactions with RNA polymerase II, implicating a broad regulatory function of this complex in mRNA synthesis and decay as well as transcription elongation (75). In yeast, transcription initiation is also controlled indirectly by the CCR4-NOT complex through the Msn-2 transcription factor. Besides its role in transcription, the CCR4–NOT complex subunits also represent a major role as cytoplasmic mRNA deadenylase in many species. A direct role of the CCR4-NOT complex in stress regulation has also been recently reported in yeast (76). Evidence shows that the CCR4–NOT complex plays a role in resistance to UV radiation and DNA damage or replication stress-inducing chemicals. The DNA damage induced by stressors increases the level of dNTPs essential for DNA replication and repair. The CCR4-NOT complex mediates this process recruited by ribonucleotide reductase (RNR) genes, which elevates the stress response. Skn-7 is another transcription factor that regulates oxidative and osmotic stress responses in yeast, which is controlled by the CCR4-NOT complex. However, beyond the yeast model, the role of CCR4-NOT in stress response regulations remains poorly understood. Here, in this thesis, I will explore the role of the CCR4-NOT deadenylase subunit *ccf-1* (CCR4-associated factor) in stress-induced transcriptional programming in *C. elegans* in response to cadmium and acrylamide.

1.6 Organism models for aging studies

Research on aging in humans is challenged by obstacles such as the long lifespan of humans, the inability to recapitulate whole organism aging by studying individual tissues, and the challenge of manipulating human genes for study purposes. As such, the use of short-lived simple model organisms would be preferable and provide numerous advantages (77–79). The first organism model for aging studies was yeast, a unicellular eukaryote (80). It emerged as a valuable model for aging research due to its short lifespan, genetic similarities to humans, adaptability to different growth conditions, small genome, and ease of creating mutant cells. The studies on yeast have identified multiple molecular pathways that affect lifespan.

Furthermore, environmental factors have been shown to influence longevity by interacting with genes. However, it is important to note that not all yeast pathways apply to complex multicellular organisms. Even so, yeast is an important research tool because of its short lifespan and simple genome (81,82). Fruit flies, or *Drosophila melanogaster*, are also commonly used in aging research. A short lifespan and physiological similarities to mammals, such as similarities between the neurons and a muscular and nervous system in *Drosophila melanogaster* and mammals, make them useful for studying aging (83,84). In contrast to mice or humans, all somatic cells in fruit flies are postmitotic and do not multiply which should be considered when applying findings from fruit flies to humans (85). *Drosophila* studies have identified several signaling pathways involved in aging, including the insulin signaling pathway. *Drosophila* can also be exploited for various stress responses to stressors, such as metabolic changes and oxidative stress (86).

C. elegans is a popular model for studying aging because of its short lifespan, and its genetic tractability allows us to learn about individual gene's effect on longevity and lifespan (87). Almost two-thirds of the human disease-related genes have a homolog in *C. elegans*. One study showed that 108 out of 143 essential genes are present in the human genome and considered orthologs. This study also showed that 97 of these genes were associated with 1,218 human diseases (88). In the subsequent section, I will provide a more in-depth introduction to the *C. elegans* model system.

Rodents, such as rats and mice, are also used in aging studies due to their shorter lifespan than humans. The diet of these animals can easily be controlled and modified, allowing scientists to study the effects of food and chemicals on lifespan (89). Using rodents, researchers can determine whether genes that affect longevity in simpler organisms, such as yeast, also affect mammals. In addition, rodents are susceptible to age-related diseases similar to humans (90).

1.7 Studying aging in *C. elegans*

C. elegans, a small nematode, is a preferred genetic model organism due to its practical characteristics such as its small size, simple anatomy, genetic flexibility, transparency, ease of cultivation, and rapid reproductive period with a large number of progenies in its lifetime. From embryonic to four larval stages, including L1-L4, leading to a mature adult stage takes about three days (Figure 1.2), and since 99.9% of *C. elegans* are naturally hermaphrodites, it is an easy model to repopulate in the laboratory (91). Worms are approximately 1 mm long as adults. Because of its rapid developmental rates and predictable short lifespan, *C. elegans* is ideal for measuring and

revealing developmental and aging related changes. During *C. elegans* lifespan, many genes known to be responsible for aging will change their expression. As the worms grow older, the genes that initially contribute to extended lifespan undergo a decrease in expression (92). *C. elegans* can be grown in both solid-phase and liquid-phase media. Petri dishes filled with a special agar called nematode growth media (NGM) are used in the laboratory to cultivate worms that feed on *E. coli* bacteria as a primary food source for worms. With a platinum wire pick, they can be easily handled for experimental purposes. When there is a crisis such as food deficiency or high temperature, or overcrowded population, they enter the stage called dauer as a defense mechanism to the harsh conditions. They can survive for up to six months without food (93,94). *C. elegans* is divided into two sexes, self-fertilizing hermaphrodites with a frequency of 99.9% and males (0.1%). In liquid nitrogen, worms can also be frozen indefinitely and then revived and repopulated. The wild-type strain is called the N2 strain, which can live for 2-3 weeks at 20 degrees Celsius (7). The normal reproductive period is within the first week when the progeny of an adult *C. elegans* hermaphrodite ranges from 100 to 300 (95,96); after which they do not produce progenies as sperm depletes.

Many of the key genes in *C. elegans* are highly conserved across species. For example, genes such as FOXO (*daf-16*) transcription factors or *daf-2* in the insulin signaling pathway show significant similarities to human genes, allowing researchers to study these genes in the context of behavior, metabolism, and aging (5,97,98). *C. elegans* are also affected by many of the same genes involved in lifespan in mammals, indicating that these genes are highly conserved. Hence, studying genes and signaling pathways in *C. elegans* can give us a better understanding of human lifespan and healthspan (99,100). *C. elegans* was the first animal in which genes were discovered to significantly influence lifespan. Two independent research groups discovered that a single mutation in either the *age-1* or *daf-2* genes can double the lifespan of worms. As a result of this breakthrough, numerous additional genes and pathways that affect lifespan have been identified (7,101).

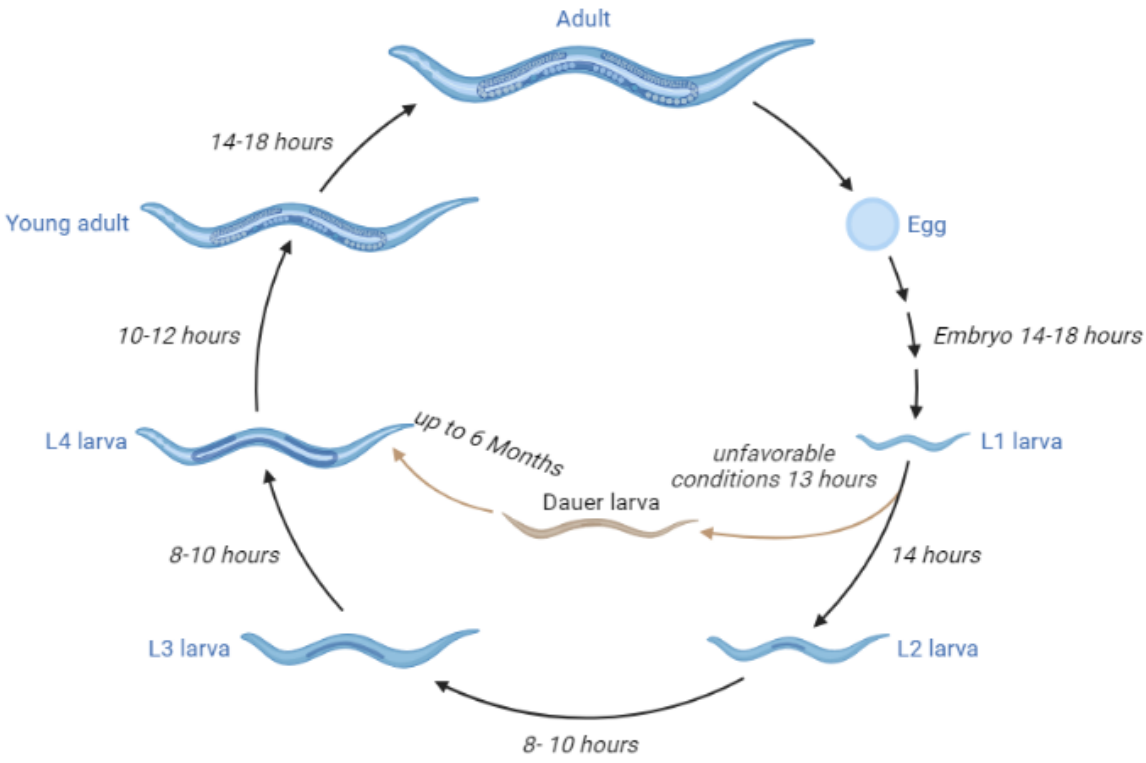

Figure 1.2: *C. elegans* life cycles take place over 3 days at 20°C. *C. elegans* eggs hatch into larvae, which go through three larval stages before becoming adults. During the L1 stage, environmental stress may cause larvae to enter the dauer stage (which allows them to survive for a long time). They will exit it once conditions return to normal, resuming at the L4 stage (by skipping the L3 stage). Figure created with BioRender.

1.8 RNA interference (RNAi)

There are three major delivery methods for knocking down the genes in *C. elegans* using RNA interference. These are injection (102), soaking (103), and feeding (104). RNAi feeding technique is the most common and is a post-transcriptional gene silencing method. This technique is used for knocking down a specific gene. This method is accomplished by consuming the bacteria expressing dsRNA that are antisense to the target gene. These dsRNA molecules can induce the inactivation of that target gene in the worms (105). Mechanistically, RNA interference inhibits

gene expression by the presence of an anti-sense RNA molecule that can complementarily bind and degrade the targeted mRNA molecules. It means that we can deactivate the expression and function of a specific gene of interest by using RNAi.

Although the efficacy rate in RNAi by feeding is slightly less than the two other methods, it is the most inexpensive and least intensive method (106). Another advantage of this method is treating a large number of worms at once. When a gene is knocked down, the worms' progenies will also be affected by the knockdown. It should be noted that targeting more than one gene by RNAi could reduce the efficiency when we compare the results with targeting them separately. RNAi technique has some downsides, including the fact that some cells, such as neuron cells, are incompliant with this method as they tend to exhibit RNAi resistance (107). Furthermore, RNAi studies target only one gene at a time, but as a result of its broad applicability, effectiveness, and cost-effectiveness, RNAi has become the preferred method for large-scale genome-wide functional screens.

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

I hypothesized that CCR4-NOT is required for the *C. elegans* stress response and that loss of the catalytic subunit gene *ccf-1* will decrease *C. elegans* resistance to various environmental stressors and accelerate aging. I also hypothesized that using the Y2H assay would confirm novel binding partners of CCF-1 to reveal the role of CCF-1 subunit and consequently CCR4-NOT in transcriptional regulation under stress.

2.2 Objectives

A major interest in the field of aging research is to understand the underlying cellular mechanisms that influence stress response pathways, as defects in stress response have been associated with accelerated aging. Although the roles of transcriptional factors in stress response are well recognized, there is a lack of information regarding the molecular functions of regulators and co-partners of transcription factors. As my main research objective, I will investigate the functions of *ccf-1* in regulating cellular responses in normal as well as stress conditions. Further, using the yeast 2-hybrid (Y2H) technique, I will confirm a co-partner of CCF-1 protein in regulating stress response. Additionally, I aim to explore the signaling pathways through which these genes operate. Uncovering novel regulators of transcriptional co-regulators involved in stress responses can provide new insights into genes that are crucial in protecting cells against stress and aging.

3. MATERIALS AND METHODS

3.1 Maintenance of *C. elegans* worm strains and RNA interference

The *C. elegans* strains were grown on NGM agar plates using standard techniques (108). *E. coli* OP50 was used as the food source for the worms. All experiments were conducted at 20°C except for *daf-2* and *rrf-3* mutants. These 2 strains were grown at 16°C during development and then moved to 20°C on day 1 of adulthood to prevent dauer formation. RNAi bacteria (described previously) were fed to worms using a methodology similar to that described by Mello and Conte in 2004 (106) to understand how silencing certain genes affect worms. Depending on the assay, RNAi plates were prepared and left overnight to dry before being used with worms in their L1 or L4 stage. Bacterial RNAi strains were added into liquid LB broth with carbenicillin and grown overnight before being spotted onto RNAi plates. All RNAi experiments were conducted on NGM plates supplemented with carbenicillin 50 g/mL and IPTG 1 mM (to induce bacterial dsRNA expression) and seeded with about 500µL of appropriate *E. coli* HT115 liquid RNAi bacteria. L1 or L4 worms were then transferred manually via washing with M9 buffer or with a platinum worm pick onto pre-seeded RNAi plates that had dried overnight, allowing gene knockdown effects to be investigated. After knockdown was placed, the plates were moved to an incubator at 20°C.

3.2 *C. elegans* culturing and synchronization

As *C. elegans* progresses through its life cycle, it experiences an embryonic stage, four larval stages, and finally, adulthood, which takes about 2-4 days, depending on conditions such as temperature. During each stage, they undergo different changes and exhibit distinct characteristics. An advantage of *C. elegans* is the possibility of age synchronizing populations of worms through a relatively simple protocol, making it an ideal model to study aging. There are several techniques for age synchronization in *C. elegans* such as alkaline hypochlorite (bleaching) and worm plating (110). The bleaching method is based on the gravid worm's sensitivity to bleach, whereas the eggshell protects embryos. As a result of treatment with alkaline hypochlorite solution (or other solutions), eggs inside the worms survive, and then are incubated in NGM plates without food, allowing hatching but preventing further development. The gravid worms with eggs inside are washed with M9 buffer, then after 2-3 washes, 1330 µL of bleach and 330 µL of NaOH are added, allowing them to lyse the adult tissues for 5-8 minutes depending on the number of worms. To stop the reaction, I add sterilized ddH_2O and immediately centrifuge the mix at 2500 *x g* for 2

minutes to pellet the eggs and neutralize the bleaching solution. I discard the supernatant and wash the eggs three more times to ensure no bleach is left. After adding 1 mL of M9 buffer to the washed pellet, the eggs are moved to an unseeded NGM plate and incubated for 24 hours at the desired temperature to allow egg hatching. After hatching, worms are synchronized at the L1 stage and will only continue to develop once transferred to a food plate.

Table 3.1 List of worm strains used in this thesis

Strain Name	**Genotype**	**Purpose**	**Source**
N2 Bristol	Wild-type	Control strain	*Caenorhabditis* Genetics Centre (University of Minnesota, Minneapolis, MN)
AGD710	*uthIs235 [sur-5p:: hsf-1:: unc-54 3'UTR; myo-2p:: tdTomato::unc-54 3' UTR]*	A long-lived mutant to assess the effects of *ccf-1* gene knockdown in heat shock proteins signaling pathway	*Caenorhabditis* Genetic Centre (University of Minnesota, Minneapolis, MN)
QV212	*skn-1(k1023)*	A long-lived to assess the effects of *ccf-1* gene knockdown mutant in oxidative stress response pathway	Dr. Keith P. Choe lab, University of Florida.
CB1370	*daf-2(e1370) III*	A long-lived mutant to assess the function of reduced Insulin/IGF-1 receptor signaling pathway	*Caenorhabditis* Genetic Centre (University of Minnesota, Minneapolis, MN)
NL2099	*rrf-3(pk1426) II*,	A RNAi hypersensitive strain to investigate the effects of *pal-1* gene knockdown	*Caenorhabditis* Genetic Centre (University of Minnesota, Minneapolis, MN)
MWU206	*pal-1(syb6872) [pal-1::AID*::3XFLAG::wrmScarlet];wrdSi23 [eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2*	An auxin inducible degradation of PAL-1 protein strain to investigate	CRISPR strain created by SUNY Biotech

	3'UTR] (I:-5.32); wrdSi3 [sun-1p::TIR1::F2A::mTagBFP 2::AID::NLS::tbb-2 3'UTR] (II:0.77)*	knockdown of PAL-1 at the protein level.	

Table 3.2 List of reagents used in this thesis

Reagent	**Source**	**Catalog Number**
6X DNA loading dye	Thermo Fisher Scientific	FERR0611
96-well plates	Falcon	08-772-2C
Agar	Thermo Fisher Scientific	DF0479-17-3
Agarose	Thermo Fisher Scientific	BP160-500
Bactone peptone	Thermo Fisher Scientific	DF0118-17-0
β-mercaptoethanol	Thermo Fisher Scientific	PI35602
Cadmium chloride	Thermo Fisher Scientific	AC315270050
Calcium chloride	Thermo Fisher Scientific	C79-3
Carbenicillin	Thermo Fisher Scientific	BP26485
Cholesterol	Thermo Fisher Scientific	MP21013803
Conical centrifuge tubes	Falcon	Varies
DNAse enzyme	Thermo Fisher Scientific	FEREN0521
DreamTaq	Thermo Fisher Scientific	FEREP0702
EDTA	Thermo Fisher Scientific	AC327345000
Ethanol	Greenfield Global	N/A
Glycerol	Thermo Fisher Scientific	PI17904

Hydrochloric acid	Thermo Fisher Scientific	SA48-500
Individually sealed pipettors	Thermo Fisher Scientific	Varies
Isopropyl ß-D-1-thiogalactopyranoside (IPTG)	Thermo Fisher Scientific	15529019
Lysogeny broth	Thermo Fisher Scientific	DF0446-17-3
Magnesium chloride	Thermo Fisher Scientific	M33-500
Magnesium sulphate	Thermo Fisher Scientific	M65-3
Microcentrifuge tube	Corning	Varies
Microscope cover slips	Thermo Fisher Scientific	12-545-87
Microscope slides	Thermo Fisher Scientific	12-550-A3
MultiScribe reverse transcriptase	Thermo Fisher Scientific	4311235
NA22 bacteria	CGC	N/A
OP50 bacteria	CGC	N/A
PCR grade water	Thermo Fisher Scientific	10977015
Petri dishes	Corning	Varies
Pipette tips	Thermo Fisher Scientific	Varies
Platinum wire, 0.25mm	Alfa Aesar	AA45093BY
Potassium chloride	Thermo Fisher Scientific	P217-3
Potassium phosphate	Thermo Fisher Scientific	P285-3
PowerUp SYBR green master mix	Thermo Fisher Scientific	A25778
Proteinase K	Thermo Fisher Scientific	25530031
qPCR primers	Eurofin Genomics	Varies
RNA purelink micro kit	Thermo Fisher Scientific	K310250

RNAi bacteria library	(54, 55)	N/A
Sodium azide	Thermo Fisher Scientific	BP922I-500
Sodium chloride	Thermo Fisher Scientific	18606413
Sodium phospate	Thermo Fisher Scientific	S374-1
SYBR I nucleic acid gel stain	Thermo Fisher Scientific	S7563
TAE	Thermo Fisher Scientific	FERB49
Tris	Thermo Fisher Scientific	BP152-500
Tween-20	Thermo Fisher Scientific	AAJ20605AP

Table 3.3 Recipe of **c**ulture media or molecular reagents used in this thesis

Media	**Recipe source**	**Recipe**
M9 Buffer	[56]	1 L H2O 5.0 g NaCl 3.0 g KH2PO4 6.0 g Na2HPO4 Autoclave 1 mL $MgSO_4$ (1M)
NGM Buffer	[56]	1 L H20 3.0 g NaCl Autoclave 1 mL Cholesterol (1M) 1 mL $CaCl_2$ (1M) 1 mL $MgSO_4$ (1M) 25 mL KH_2PO_4 (1M, pH 6)
NGM Plates	[56]	1 L H20 3 g NaCl 2.5 g Peptone 25 g Agar Autoclave 1 mL Cholesterol (1M) 1 mL $CaCl_2$ (1M) 1 mL $MgSO_4$ (1M) 25 mL KH_2PO_4 (1M, pH 6)

NA22 Plates	[56]	1 L H_2O 1.2 g NaCl 20 g Peptone 25 g Agar Autoclave 1 mL Cholesterol (1M) 1 mL $CaCl_2$ (1M) 1 mL $MgSO_4$ (1M) 25 mL KH_2PO_4 (1M, pH 6)
Lysis Buffer	[57]	3.73 g KCl 0.508 g MgCl2 10 mL 1M Tris-HCl, pH 8.8 4.5 mL NP-40 4.5 mL Tween-20
100x SYBR Green	Thermo Fisher	2 uL 10,000x SYBR Green Dye 198 uL 1x TAE buffer
SYBR Green + Loading Dye	Thermo Fisher	100 uL 100x SYBR Green 200 uL 6x Loading Dye
DNA Ladder	Thermo Fisher	30 uL SYBR Green + Loading Dye 90 uL H_2O 10 uL DNA Ladder
Primer Mix	Eurofin Genomics	10 uL forward primer (100 μM) 10 uL reverse primer (100 μM) 180 uL RNAse free water

3.3 Lifespan assays

All assays were performed at 20°C using methods previously described (87,111) except for *daf-2(e1370)* and *rrf-3(pk1426)* strains which were grown at 16°C during development as they are temperature sensitive and need to stay in lower temperature until past the dauer arrest decision point, and then moved to higher temperatures. For lifespan assays, the embryos of gravid adults were released by bleaching as described above to synchronize worm growth. Bleached embryos were hatched overnight on unseeded NGM plates, and synchronized L1 larvae were grown to adulthood. One-day-old adult worms were moved to new plates and continuously transferred via daily picking to a new seeded NGM plate during the reproductive window to accomplish progeny separation and also avoid depletion of food sources on the agar plates. At least 100 hermaphrodite

worms were selected for each condition per trial tested with 3 independent trials performed for each assay. Each independent trial used a fresh batch of plates and a new worm population to ensure that experimental results were reproducible. When a worm crawled off the plate or died from the expulsion of internal organs, it was recorded as a censor for the experiment and was taken into account in the Kaplan-Meier survival analysis. Worms that were not moving were prodded three times using a worm pick, and those that did not respond were considered dead. Additionally, a control group of wild-type worms was monitored alongside each assay.

3.4 Stress resistance assay (cadmium and acrylamide survival assays)

For survival assays, the protocol was similar to the lifespan assays. Synchronized L1 worms from bleaching were first grown on control NGM plates seeded with appropriate RNAi bacteria until the first day of adulthood, followed by transfer to NGM plates containing either 100 μM of cadmium chloride or 5 mM of acrylamide seeded with the corresponding RNAi bacteria along with control groups seeded with the same conditions without any stressors. It is important to take into account that the worms exposed to these stressors do not have the same lifespan as the control group and require less frequent transfer to new plates since they produce fewer offspring and live shorter. For both lifespan and survival assays, the first day of adulthood was considered one day old, and worms were scored every 1–2 days for death by gentle prodding with a flame-sterilized metal pick. All assays were conducted in three separate trials, and the number of animals scored in each trial was recorded.

3.5 RNA extraction and gene expression level assays using qPCR

The Purelink RNA mini kit (12183020; ThermoFisher) was used to isolate total RNA with a Qsonica Q55 sonicator used for tissue lysis. Synchronized L1 N2 or *rrf-3(pk1426)* worms were grown on NGM agar plates seeded with EV or corresponding RNAi until day 1 of adulthood. thus, the worms were transferred to either control agar plates, agar plates containing 100 μM of cadmium chloride, or agar plates containing 5 mM of acrylamide, each seeded with the corresponding RNAi bacteria for an additional 24 hours. Next, worms were washed off by M9 buffer and transferred to microcentrifuge tubes followed by RNA extraction. As RNA reflects the current state of gene expression, measuring mRNA levels can determine which genes are currently being expressed and at what levels, which is why the RNA is extracted in such experiments. The extraction process

involved adding 300 µL of lysis buffer containing 1% β-mercaptoethanol to each sample, followed by sonication for at least 10 seconds for a total of three cycles. Between each cycle, the sample was chilled on ice for one minute to prevent heat denaturation of the sample. The concentration of total RNA was measured using Nanodrop Lite (A260/A280 ratio) and 1µg of the RNA was used for further steps. For each condition, N = 4 biological RNA replicates were prepared with each replicate containing ~500 worms. To prepare for qPCR, the RNA samples were first treated with DNaseI (EN0521; ThermoFisher) at 37°C for 30 minutes. Then, 1 µL of EDTA was added to each sample and they were incubated at 65°C for an additional 10 minutes and cDNA synthesis was performed using an Applied Biosystems ProFlex thermocycler. 5 µL of the product was added to the tubes along with buffer, dNTP mix, random primers, Invitrogen Multiscribe™ reverse transcriptase (4311235; ThermoFisher), and RNAse-free water. The tubes were then centrifuged, loaded into a ProFlex PCR System, and incubated at 25°C for 10 minutes, followed by 37°C for 120 minutes and finally at 85°C for 5 minutes. After incubation, 90 µL of RNAse-free water was added to each tube to dilute the cDNA. Real-time PCR (qPCR) was performed with the PowerUp™ SYBR™ Green Master Mix (#A25741) and forward and reverse primers pairs in a QuantStudio3 system. Relative gene expression was normalized to the housekeeping gene *rpl-2* (ribosomal protein large subunit) and *cdc-42* (cell division cycle related), primers used for this study are shown in Table 3.4.

Table 3.4 List of primers used in this study

Gene of interest	**Forward primer sequence (5' – 3')**	**Reverse primer sequence (5' – 3')**
cdc42	CTTCTGAGTATGTGCCGACAGTCT	GGCTCGCCACCGATCAT
cdr-1	ATTACTGCTGCGCTGTTTGG	GGGGACAAGTTCGGACAGTT
cdr-4	GCGCTTCTGACTCGCTTTAC	ATCTCCGATTGCTCCAACAC
daf-16	CATCGGCTCTTCCAATTGAT	ATGAATATGCTGCCCTCCAG
F57B9.3	TCTTTCGACGACATGGAGCT	AACTGCGCTCTTTTGGATGG
gcs-1	AATCGATTCCTTTGGAGACC	ATGTTTGCCTCGACAATGTT
gst-12	ATTTCATCTTGCCGGAGTTCC	CAGGCATTTGACCCATTGG
gst-14	GGAGTTCCGTTTGAGGATGA	CTGGAGTTTTTCCAGCGAAC

gst-30	GATTCCACAGGCTGATTGGC	GGCGAGCAAGAAAACGAGAA
gst-33	GCTGGACAAACTCCGGTAGA	TTCTCCTTAGCAGGGTCCAA
gst-38	TCCAATGCTCGAGGTAGATGGCAA	ACGAGCCTCCGCGTAATAGTCTTT
gst-4	TCCGTCAATTCACTTCTTCCG	AAGAAATCATCACGGGCTGG
gst-5	TATGCCGGACAACAATACGA	GAGCCAAGAAACGAGCAATC
hsp-16.41	CCGTATTGGAGAAATGCTGATC	GAGAGACATCGAGTTGAACCGA
hsp-16.49	TGAGTTGTGATCAGCATTTCTC	GGATGAAATCACTGGATCTGTT
hsp-70	TTGGAATCGACTTGGGTACTACGT	GGCCGTAGTCTTGTTCCCTTCG
numr-1	AGACGTCACTGTTTTGGTGGA	CCGAATCCTCCAGTTGGACC
rpl-2	CTTTCCGCGACCCATACAA	CACGATGTTTCCGATTTGGAT
skn-1	GGACGTCAACAGCAGACTCA	GAGAGCACGTTGATGACGAA

3.6 Agarose gel electrophoresis

After sample normalization and before DNAse treatment in each experiment, gel electrophoresis was performed to evaluate the RNA quality and to check for any signs of degradation. To prepare the samples for gel electrophoresis, 5 μL of each normalized RNA sample was mixed with 5 μL of RNAse-free water and 3 μL of SYBR™ loading dye. The mixture was loaded onto a 1.5% agarose gel, which was then subjected to 120 V and 400 mA for 25 minutes, allowing the RNA molecules to migrate through the gel based on their charge and size. The gel was then imaged to visualize the RNA bands and confirm their quantity and quality based on their band pattern.

3.7 Yeast 2-Hybrid

A Y2H screen was conducted using the ProQuest two-hybrid system which is a reliable and affordable approach for finding possible protein-protein interactions. I used this platform to identify interactors of *C. elegans* CCF-1. The full-length *ccf-1* cDNA clone was generated through PCR using the Q5® High-Fidelity DNA polymerase (M0491L; New England BioLabs) and cloned into the pDEST32 vector containing the GAL4 DNA binding domain (DBD) through Gateway cloning (11789020 and 12538120; ThermoFisher) and used as the Y2H bait construct. A Y2H prey cDNA library of *C. elegans* genes was cloned into the pDEST22 vector containing the GAL4 activating domain (AD) using the CloneMiner™ cDNA library construction kit (A11180; ThermoFisher). A forward Y2H library screen was performed using the ProQuest Two-Hybrid

System (PQ1000101; ThermoFisher) to identify protein interactors of the *C. elegans* CCF-1 protein (112). Yeast colonies that grew on Trp−/Leu−/His− + 25 mM 3-Amino- 1,2,4- triazole (3AT) selection plates were extracted using the Zymoprep Yeast Plasmid Miniprep II (Zymo Research, D2004) followed by Sanger sequencing for gene identification. Three independent colonies from each bait-interacting prey construct were further tested for its interaction with CCF-1 by evaluating for (1) growth on Trp−/Leu−/Ura− selection plate, (2) growth on Trp−/Leu−/His− + 25 mM AT selection plate, and (3) positive phenotype from the X-gal assay. Yeast cells were normalized to OD600 = 0.5 for the 1^0 concentration followed by serial dilution on the growth assay. Yeast cells were imaged on a Bio-Rad Gel Doc EQ system.

3.8 Statistical analysis

Graphical data and statistical analysis were performed using Graphpad Prism software (V7.04). Student's t-test was used for the comparison of two groups with the Holm-Sidak multiple test correction applied when the test was repeated for multiple genes, one-way ANOVA with Dunnett's test was used for comparison of more than two groups, two-way ANOVA with Holm-Sidak test was used for comparison of two factors, and categorical data were analyzed using the Chi-square test. Lifespan and survival data were analyzed with the log-rank test using the OASIS2 software (https:// sbi.poste ch.ac.kr/oasis 2/histo ry/). For RNA sequencing data, false discovery rate (FDR) correction was applied to determine the statistical significance. The following designations were used for all statistical tests to indicate the p-value, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. It should be noted that all error bars in the figures are indicators of standard deviation.

4. RESULTS

4.1 Lifespan and stress resistance is reduced by *ccf-1* knockdown

The lifespan assay is a commonly employed technique to explore how modifying the activity of a particular gene affects the aging process. This modification can involve various alterations, such as gene knockdown, gain of function, overexpression, and other activity changes. As mentioned in the previous chapter, conducting a lifespan assay involves ensuring that the worms used in each trial are of the same generation and age. To prevent them from mixing with their offspring, it is necessary to transfer the worms to fresh plates several times until the worms stop reproducing. However, it is essential to ensure that the plates contain enough food for the rest of their lives and that each plate remains free from bacterial contamination that can negatively influence worm lifespan. The plates were checked every other day to document the number of dead worms and the final age of each worm within a population was then used to calculate the average lifespan of that population. The findings showed that knockdown of *ccf-1* has a major impact on reducing lifespan by approximately 40% (p-value < 0.001), suggesting its involvement in aging-associated pathways and indicated that *ccf-1* is essential for maintaining normal lifespan. It is important to note that the specific percentage may vary in each experiment (Figure 4.1A); however, the overall trend observed was consistent.

Similarly, the stress resistance of a population of worms can be examined by introducing stressors to the agar plate. In our experiments, I examined metal stress resistance assay using 100µM cadmium in plates as a heavy metal stress inducer. In this assay, the worms first undergo normal development in the absence of stress until reaching adulthood under standard conditions. They are then provided with RNAi and transferred to identical RNAi plates containing a sub-lethal amount of the stressor (cadmium) for the remainder of their lifespan to induce gene knockdown.

The results demonstrate that the knocking down of the *ccf-1* gene significantly diminishes resistance against a metal stressor and its absence exacerbates the negative effect of stressor in survival, resulting in a significant decline of roughly 38% (p-value < 0.001) in the survival of a *C. elegans* population exposed to cadmium stress (Figure 4.1B). Overall, these findings indicate that *ccf-1* is essential for both normal lifespan and protection against environmental stresses, including cadmium which can affect stress response (Table 4.1).

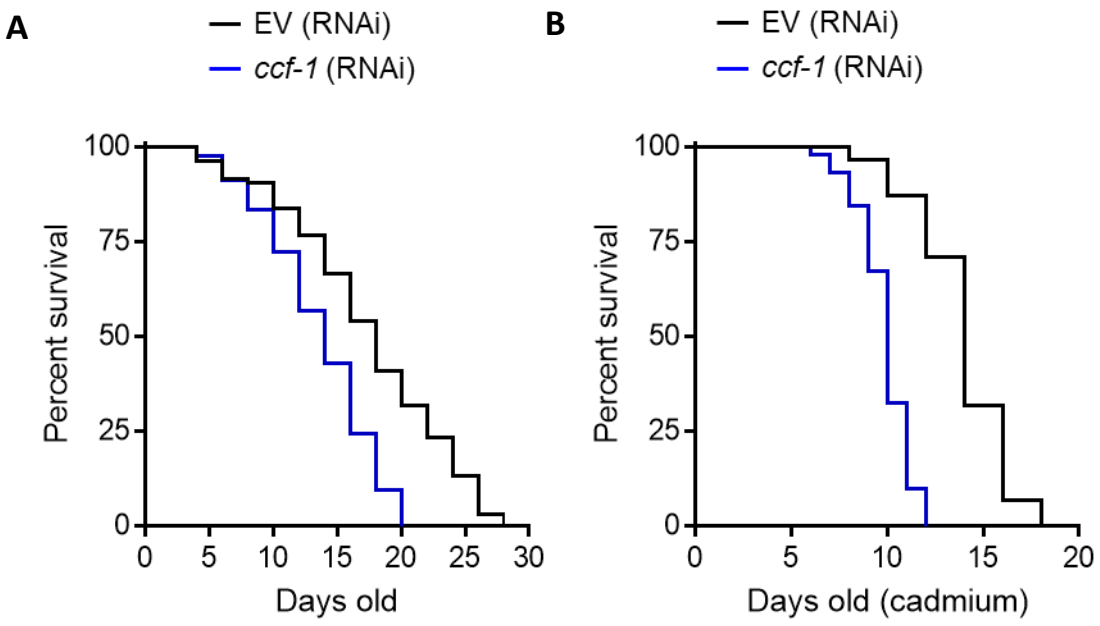

Figure 4.1: The lifespan and survival (cadmium exposure) experiments in wildtype worms involved *ccf-1* gene knockdown using RNA interference (RNAi). The worms were transferred to plates containing either empty vector (EV) or *ccf-1* RNAi. For the survival assay, the worms were exposed to solid media containing 100µM cadmium. The number of deceased worms was recorded regularly, and data is represented on a graph using Prism 8.0 software. **A)** The knockdown of *ccf-1* gene through RNAi reduced the lifespan of wildtype worms compared to the control group (*p*-value < 0.001). **B)** The RNAi knockdown of *ccf-1* gene resulted in decreased resistance to cadmium exposure. The experiments were conducted three times independently (*p*-value < 0.001).

Table 4.1: Mean lifespan of N2 worms in EV and *ccf-1* RNAi conditions in normal lifespan and also cadmium survival assay. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator.

Groups	Number of subjects	Mean lifespan (Days)
N2 EV	83	17.51
N2 *ccf-1*	107	11.18
N2 EV + cadmium	103	12.89
N2 *ccf-1* + cadmium	95	9.48

4.2 *ccf-1* is required for survival in oxidative stress

In many experiments, such as studies on aging, acrylamide is commonly used to induce oxidative stress. Antioxidant genes play a crucial role in defending against this type of stress. These genes are also involved in the detoxification process within the cells which have been extensively studied. Understanding the influence of *ccf-1* on these antioxidant genes can provide insights into shared pathways that *ccf-1* works through.

To investigate this further, I first conducted survival assays with wildtype worms exposed to acrylamide, simulating oxidative stress conditions, and to see how oxidative stress changes their survival time. I compared the survival times of worms with and without the *ccf-1* gene under these conditions to understand the impact of the *ccf-1* gene. The worms were placed on plates containing 5 mM acrylamide with the respective RNAi conditions (either EV control or *ccf-1*). Our results showed that acrylamide significantly reduced the survival time of worms lacking the *ccf-1* gene by 28% (p-value < 0.01) compared to their normal lifespan (Figure 4.2; Table 4.2).

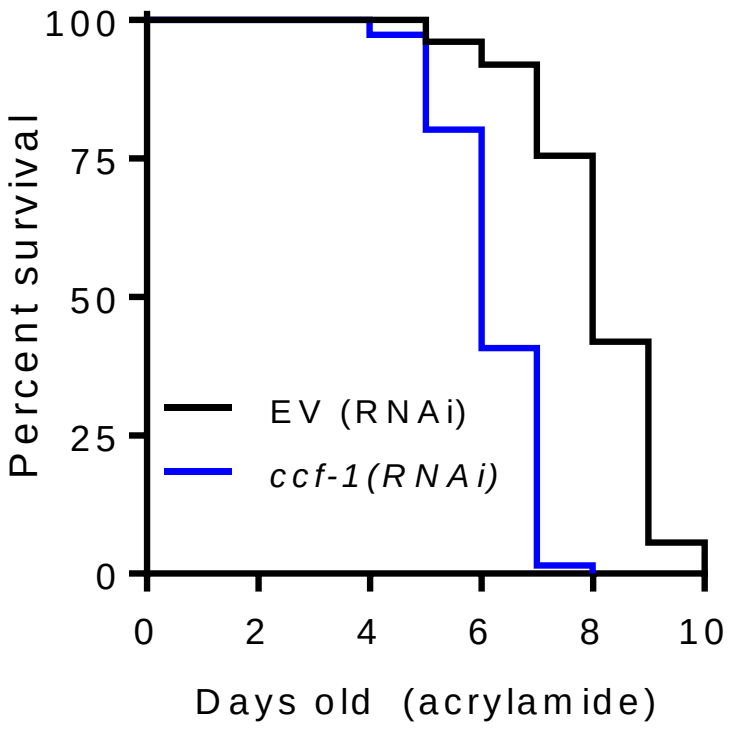

Figure 4.2: The survival (acrylamide exposure) experiments in wildtype worms involved *ccf-1* gene knockdown using RNA interference (RNAi). The worms were transferred to plates containing either empty vector (EV) or *ccf-1* RNAi. For the acrylamide survival assay, the worms were exposed to solid media containing 5 mM acrylamide. The number of deceased worms, as well as censored worms, was documented frequently. Being exposed to 5 mM acrylamide significantly decreased survival time for the *ccf-1* knockdown group compared to N2 worms (p-value < 0.01). The experiments were conducted three times independently. Data is represented on a graph using Prism 8.0 software. All lifespan trials were independently performed three times.

Table 4.2: Mean lifespan of N2 worms in EV and *ccf-1* conditions in acrylamide survival assay. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator.

Groups	Number of subjects	Mean lifespan (Days)
N2 EV + acrylamide	316	10.81
N2 *ccf-1* + acrylamide	243	7.91

Based on these findings, I decided to examine the changes in gene expression levels when *ccf-1* was knocked down in the presence of acrylamide. The worms were initially grown on plates with either EV control or *ccf-1* RNAi for 48 hours. Subsequently, they were transferred to plates containing 5 mM acrylamide, and seeded with the appropriate RNAi bacteria. Next, the worms were exposed to acrylamide for 24 hours and then collected for RNA extraction. I measured their mRNA expression levels using qPCR. Through qPCR analysis, I observed that the knockdown of *ccf-1* resulted in reduced activation of glutathione-related antioxidant genes, including *gst-12, gst-30, gst-38, gst-14* (p-value < 0.001), and *gcs-1* (p-value < 0.01), following exposure to acrylamide (Figure 4.3). For a thorough interpretation of the data for each tested gene in the figure, it is essential to conduct individual comparisons between all four groups. This approach will enhance our comprehension of the results.

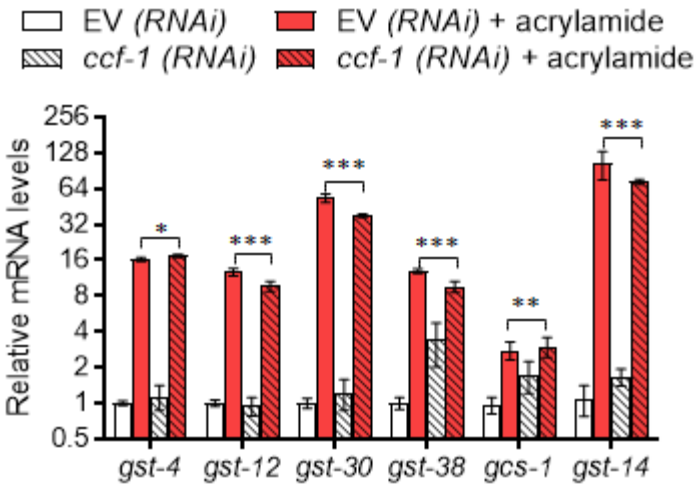

Figure 4.3: Results of RNA extraction and mRNA expression levels for N2 worms fed with *ccf-1* RNAi and EV RNAi exposed to acrylamide. RNA was extracted from the worms in each group, and the results were assessed by qPCR. Several *gst* family genes including *gst-4, gst-12, gst-30, gst-38, gcs-1,* and *gst-14* showed a significant decrease in *ccf-1* groups when exposed to acrylamide. Lack of the *ccf-1* gene decreases stress response genes in oxidative stress exposure. Error bars are indicating the standard deviation, shown for each condition. Each condition is based on N = 4 samples. Statistical significance was determined using two-way ANOVA with Holm-Sidak post hoc tests. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences between EV (RNAi) and *ccf-1* (RNAi) groups exposed to acrylamide. Four replicates were conducted, with each replicate comprising a sample size of 200 to 300 worms.

4.3 Long-lived mutants longevity influenced by *ccf-1*

To investigate the role of *ccf-1* in the lifespan of long-lived worm mutants, I conducted tests to determine its requirement in several core aging-related genes which are known for promoting longevity compared to wildtype worms. Specifically, I focused on three mutants associated with different aging signaling pathways: full-length *hsf-1(FL)* overexpression strain, *daf-2 (e1370)* loss of function mutant, and *skn-1 (k1023)* gain of function (*gof*) mutant. To assess the involvement of *ccf-1* in these long-lived mutants and its potential interactions with established longevity pathways, I conducted lifespan experiments comparing them to wildtype N2 worms as a control.

At first, I used the *daf-2(e1370)* mutant and N2 worms. Under EV RNAi conditions, the *daf-2(e1370)* mutant showed an average lifespan of 41.19 days, while N2 worms had an average lifespan of 17.83 days. I also used *ccf-1* RNAi conditions. In these two conditions (N2 *ccf-1* (RNAi) and *daf-2(e1370)* mutant *ccf-1* (RNAi)), their lifespans were reduced significantly to 21.06 and 10.7 days, respectively (Figure 4.4A). This indicates that the *daf-2(e1370)* mutant extended its median lifespan by approximately 131.03% compared to N2 worms in normal control conditions. However, in the presence of *ccf-1* RNAi, this extension decreased to around 93%, suggesting that *ccf-1* reduced the median life extension by approximately 37% (p-value < 0.001) (Table 4.3).

The *skn-1(k1023)* mutant, which promotes longevity through constitutive activation of the oxidative stress response pathway, exhibited an average lifespan of 20.67 days under EV RNAi, while N2 worms lived for 18.5 days in the same condition. When they were subjected to c*cf-1* RNAi, *skn-1 (k1023)* mutant lived for 12.48 days compared to N2 worms with an average lifespan of 12.16 days (Table 4.4) which is a significant decrease in both groups (p-value < 0.001). Compared to the wildtype, the *skn-1(k1023)* mutant extended its lifespan by approximately 11.72% (p-value < 0.05). However, when fed with *ccf-1* RNAi, the lifespan extension decreased to approximately 2.63% (p-value > 0.05) (Figure 4.4B).

Lastly, I examined the full-length *hsf-1(FL)* overexpression strain and N2 worms in both EV and *ccf-1* conditions. In normal conditions, *hsf-1(FL)* overexpression strain had an average lifespan of 22.13 days, while N2 worms lived for about 17.49 days, but in *ccf-1* RNAi exposure, *hsf-1(FL)* overexpression strain lived for 13.1 days compared to N2 worms with an average lifespan of 12 days (Table 4.5). Therefore, *hsf-1(FL)* overexpression strain exhibited approximately 26.52% (p-

value < 0.01) longer lifespan in the EV RNAi condition and a 9.17% (*p*-value < 0.05) longer lifespan in the *ccf-1* RNAi condition compared to N2 worms. It means that *hsf-1(FL)* overexpression strain decreased the lifespan extension by about 17% (*p*-value < 0.01) when the *ccf-1* was knocked down which means it partially lost its ability to extend the lifespan in *ccf-1* knockdown condition (Figure 4.4C). Overall, these findings indicate that *ccf-1* is essential for promoting the full longevity effects of the *daf-2(e1370)*, *skn-1(k1023)*, and *hsf-1(FL)* mutants.

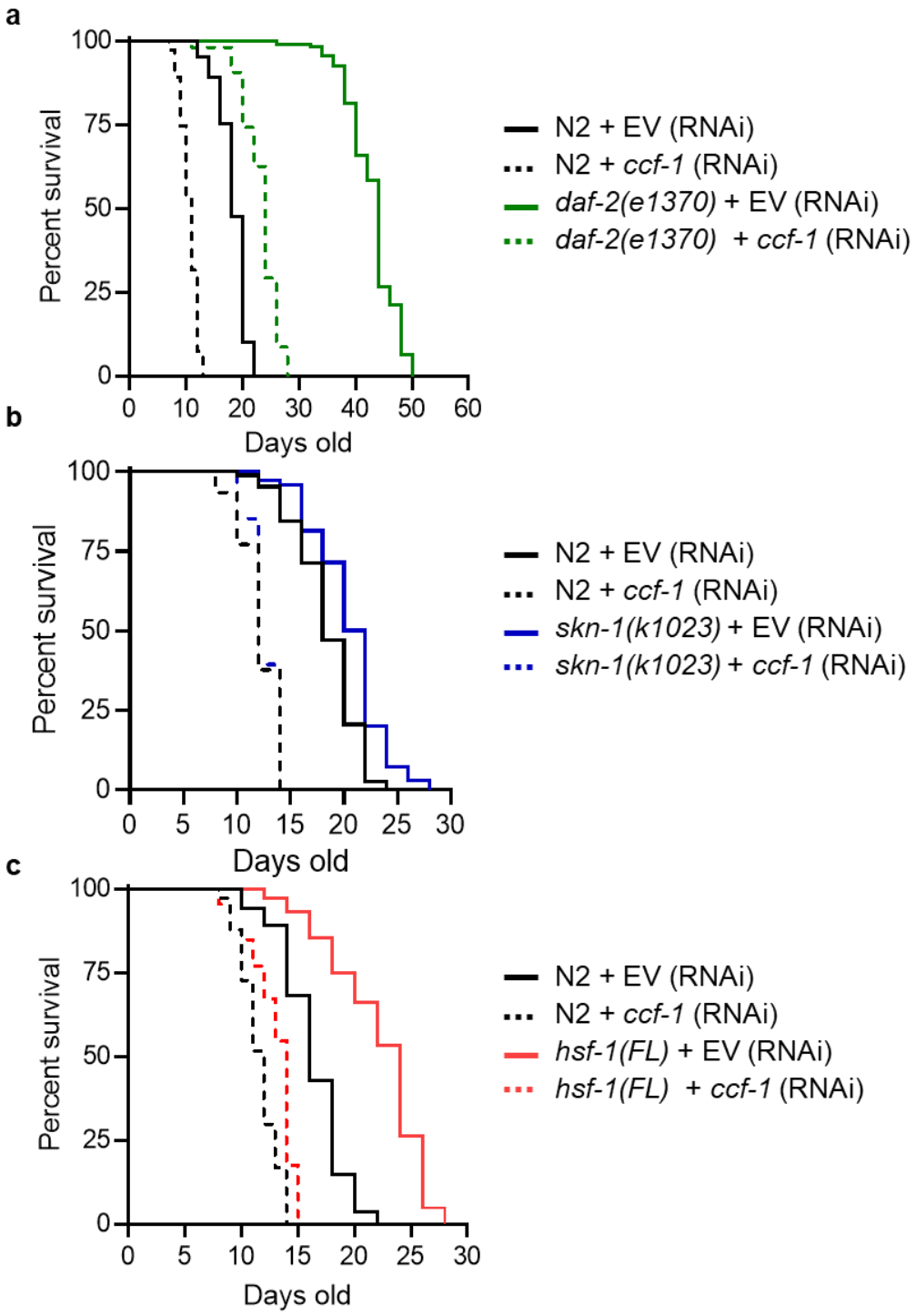

Figure 4.4: The lifespan assays in long-lived mutants worms when *ccf-1* gene is knocked down by RNA interference (RNAi) method. The worms were transferred to plates containing either empty vector (EV) or *ccf-1* RNAi. Lifespan of N2 wildtype worms and **A)** *daf-2(e1370)* mutant,

B) *skn-1(k1023)* gain of function (*gof*) strain, and **C)** full-length *hsf-1(FL)* overexpression fed with EV or *ccf-1* RNA. All lifespan trials were independently performed three times.

Table 4.3: Mean lifespan comparison of N2 worms and *daf-2 (e1370)* mutants in both EV and *ccf-1* conditions. The figure also illustrates the extent of lifespan increment following *ccf-1* knockdown. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator

Condition	Mean lifespan (Days) *daf-2(e1370)*	Mean lifespan (Days) N2	Percentage Increase
EV RNAi	41.19	17.83	131.032%
ccf-1 RNAi	21.06	10.7	93%

Table 4.4: Mean lifespan of N2 worms and *skn-1 (k1023)* mutants under two different conditions: empty vector (EV) and *ccf-1* knockdown. Furthermore, it demonstrates the magnitude of lifespan improvement achieved after *ccf-1* knockdown. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator

Condition	Mean lifespan (Days) *skn-1(k1023)*	Mean lifespan (Days) N2	Percentage Increase
EV RNAi	20.67	18.5	11.72%
ccf-1 RNAi	12.48	12.16	2.63%

Table 4.5: Comparison of the mean lifespan between N2 worms and *hsf-1 (FL)* mutants under EV and *ccf-1* conditions. Additionally, the increase in lifespan before and after *ccf-1* knockdown is shown. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator

Condition	Mean lifespan (Days) *hsf-1(FL)*	Mean lifespan (Days) N2	Percentage Increase
EV RNAi	22.13	17.49	26.52%
ccf-1 RNAi	13.1	12	9.17%

4.4 Other components of CCR4/NOT complex are also required for lifespan

The CCR4-NOT complex consists of several units whose roles and functions have not been fully elucidated. While the roles of only a few proteins in this complex have been studied, there may be other important components that participate in various biological functions (74,113). In *C. elegans*, some of the proteins from this complex such as *ntl-2, ntl-3,* and *let-711* are believed to play roles. While NTL-2 and NTL-3 units are contributing to the functional integrity and activity of the complex (114), the LET-711 (the ortholog of the human CNOT7 protein) has been implicated in mRNA deadenylation and degradation, which are crucial steps in gene expression regulation (115).

To gain insights into their effects on longevity, I conducted a lifespan assay. However, I skipped the lifespan assay for the *let-711* gene because its knockdown caused larval arrest that prevented worms from developing into adults. Worms introduced to *ntl-2* and *ntl-3* RNAi conditions develop normally to adulthood but had notably shorter lifespans, with average durations of 8.8 (p-value < 0.001) and 15.31 (p-value < 0.05) days, respectively (Table 4.6). These results indicate that both genes significantly impact the lifespan of N2 worms, with *ntl-2* exerting a stronger effect than *ntl-3* (as shown in Figure 4.5).

Next, I performed mRNA expression analysis for these three genes under cadmium-induced stress conditions, comparing them to the control (EV) condition. The worms were placed onto EV, *ntl-2, ntl-3,* and *let-711* RNAi plates for 48 hours, followed by 24 hours of cadmium exposure. The RNA extraction was carried out, and the expression levels of several stress-responsive genes were measured. Our findings revealed that knockdown of other CCR4-NOT subunits, including *ntl-2, ntl-3,* and *let-711*, also attenuated the activation of cadmium-induced genes. The genes that showed significant decrease were *gcs-1, gst-4, gst-30, gst-38, hsp70, hsp-16.41, hsp-16.49, cdr-1, F57B9.3,* and *numr-1 (*$*p < 0.05, **p < 0.01, and ***p < 0.001). This finding is in line with *ccf-1* effects on cadmium exposure which means not only *ccf-1* has important role in stress response, but also the entire CCR4-NOT complex is involved in the transcriptional stress response (Figure 4.6).

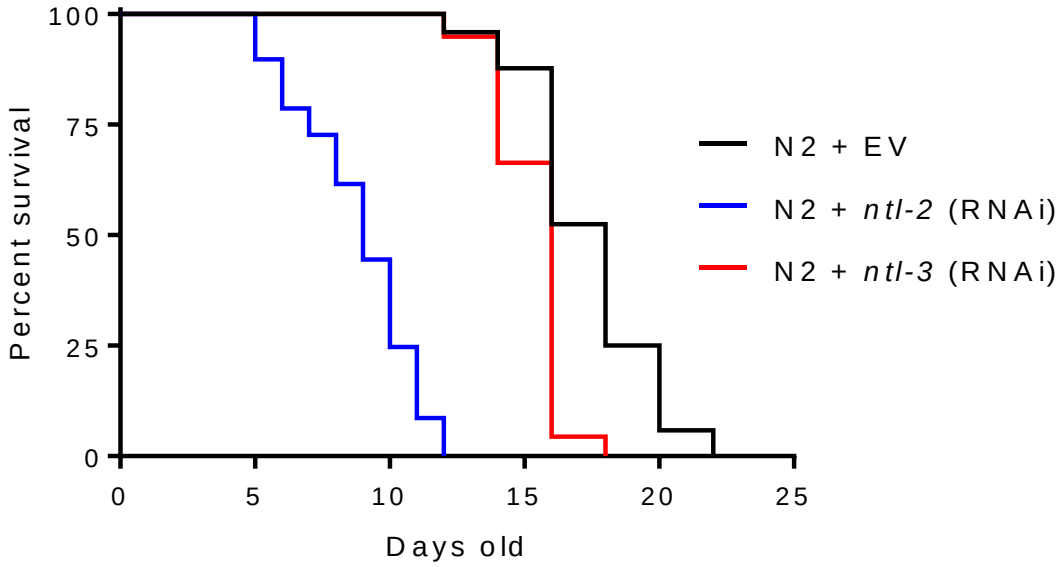

Figure 4.5: Lifespan assays in N2 worms with EV (RNAi) and *ntl-2* (RNAi) and *ntl-3* (RNAi). Loss of *ntl-2* (*p*-value < 0.001) drastically decreases the lifespan in N2 worms compared to N2 EV**.** Knocking down of *ntl-3* also decreased the lifespan when compared to N2 EV (RNAi) (*p*-value < 0.05). All lifespan trials were independently performed two times.

Table 4.6: Mean lifespan comparison of N2 worms in three different RNAi conditions; *ev (RNAi), ntl-2 (RNAi),* and *ntl-3 (RNAi*). Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator

Condition	Number of subjects	Mean lifespan (Days)
EV RNAi	98	17.34
ntl-2 RNAi	117	8.8
ntl-3 RNAi	101	15.31

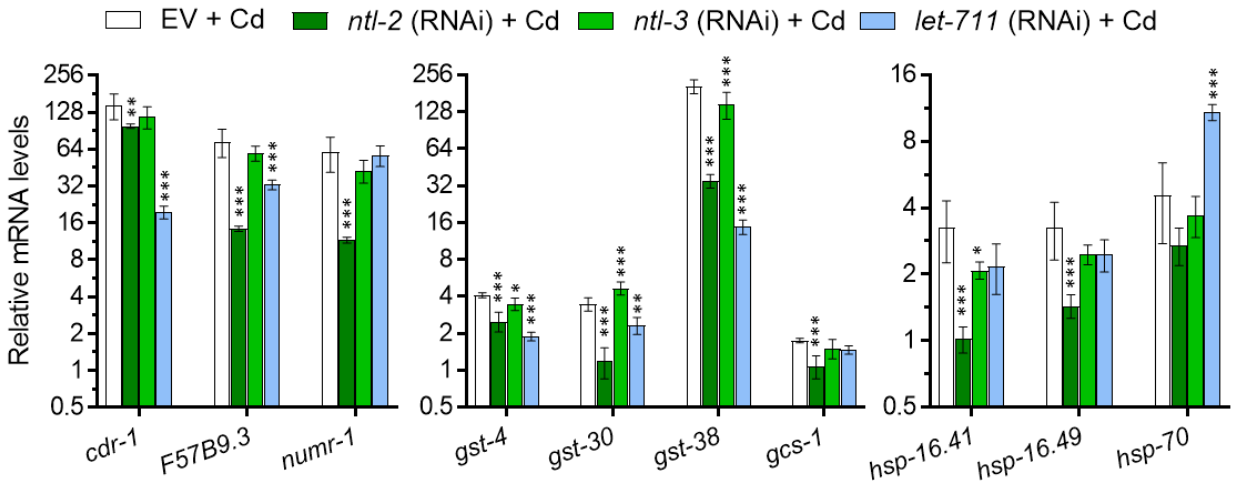

Figure 4.6: mRNA expression analysis under cadmium-induced stress conditions in *ntl-2* (RNAi), *ntl-3* (RNAi), *let-711* (RNAi), and control (EV) conditions. Several genes, including *gcs-1, gst-4, gst-30, gst-38, hsp70, hsp-16.41, hsp-16.49, cdr-1, F57B9.3, and numr-1*, showed significant attenuation when each of the aforementioned genes was knocked down. Error bars represent the standard deviation for each condition, with each condition based on N = 4 samples. Statistical significance was assessed using two-way ANOVA with Holm-Sidak post hoc tests. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate significant differences between the EV (RNAi) *ntl-2* (RNAi), *ntl-3* (RNAi), *let-711* (RNAi). Four replicates were performed, each with a sample size ranging from 200 to 300 worms.

4.5 CCF-1 protein physically interacts with PAL-1 protein

The PAL-1 (Posterior Alae) is an important protein in the nematode *Caenorhabditis elegans*. PAL-1 plays a crucial role in the development especially embryonic development. This protein has a role in determining cell fate and morphogenesis as well as patterning along the anterior-posterior axis. PAL-1 protein in *C. elegans* is the orthologue of the human CDX1 transcription factor which encodes a transcription factor that belongs to the Caudal-related homeobox (CDX) family. This factor is highly conserved across species. The CDX family genes are involved in various developmental processes, including posterior patterning and organogenesis (116–119). Los of function in the *pal-1* gene can result in severe defects in posterior development such as abnormalities in tail development in *C. elegans*. The interaction between CCF-1 and PAL-1 can led us to find a new role for PAL-1 in co-regulating stress responses alongside CCF-1, as well as being necessary for having a normal lifespan.

Following the forward Y2H screening that previously performed by Wu's lab, I used the Y2H system (120) to confirm CCF-1 and PAL-1 protein physically interact. The proteins of interest are typically referred to as the "bait" and the "prey." I inserted the *ccf-1* gene into the bait construct and *pal-1* into the prey construct. In the bait construct, a DNA binding domain (DBD) allows it to bind to specific DNA sequences in the yeast genome called promoter sequences. On the other hand, PAL-1 plays a role as the "prey" that is going to interact with the bait protein. It is fused to the activation domain (AD), which activates the transcription of downstream genes. If the bait and prey proteins interact, the AD and DBD domains will be brought closer, allowing the assembly of a functional transcription factor. The yeast used in the Y2H system has a special characteristic which is a mutation in histidine biosynthesis. This mutation does not allow the yeast to grow in lack of this amino acid, but if the two proteins of interest interact, the reconstituted transcription factor can activate the HIS3 gene. This interaction between the two proteins allows yeast cells to grow in histidine-deficient media as it is now able to produce histidine on its own. This growth in selective media indicates protein-protein interaction. The same mechanism exists for other amino acids tests such as Leucine and Tryptophan.

Three independent colonies from the prey construct that involved PAL-1 were tested for their interaction with CCF-1. There were two different media to assess the interaction quality: (1) $Trp^{-}/Leu^{-}/His^{-}$ + 25 mM AT selection plate and (2) positive phenotype from the X-gal assay.

Induction of the lacZ gene results in a blue color when assayed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto pyranoside). Yeast cells were normalized to OD600 = 0.5 for the 1^0 concentration followed by serial dilution on the growth assay. In this scenario, the three prey colonies exhibited growth under histidine deficient media and displayed a blue color in the X-Gal assay (Figure 4.7). This indicates positive interactions between the PAL-1 and CCF-1 proteins. Their growth levels also reflect how many yeast cells were introduced as determined by the serial dilution.

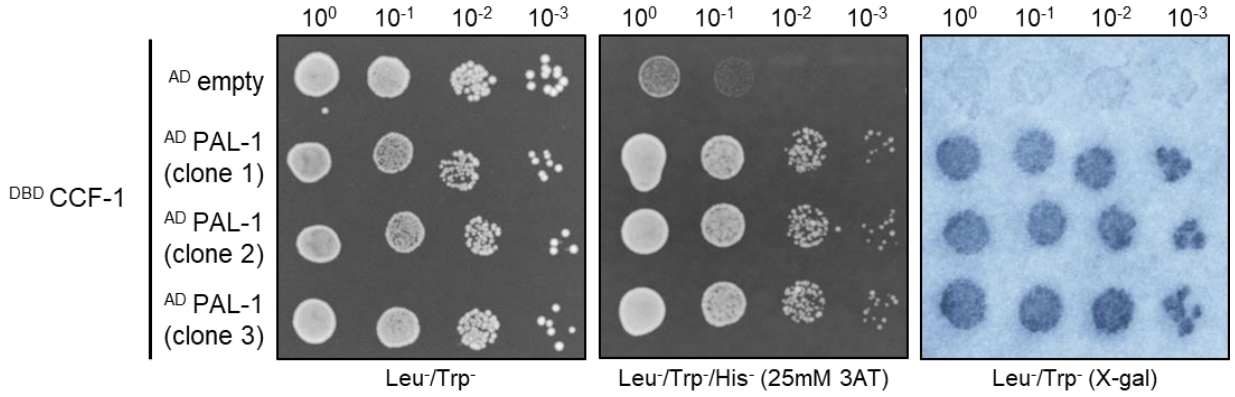

Figure 4.7: Yeast two-hybrid interactions between CCF-1 (bait) and three independent clones of PAL-1 (prey) across a dilution gradient on the control Leu^-/Trp^- plate, $Leu^-/Trp^-/His^-$/25 mM 3AT selection plate, and Leu^-/Trp^- X-gal assay. The color in the X-gal image is artificially shaded blue.

4.6 The *pal-1* gene is required for lifespan and survival by reducing stress resistance

From the Y2H system I observed a direct and strong physical interaction between the PAL-1 and CCF-1 proteins, suggesting a potential collaboration between them in stress response and lifespan regulation. To investigate this hypothesis, I first performed a lifespan assay using wildtype worms in *pal-1* RNAi condition to figure out whether *pal-1* also has an impact on normal lifespan. However, no significant differences were observed compared to the control EV RNAi condition in this regard (data not shown). This lack of effect on wildtype could be attributed to the insensitivity of wildtype worms to *pal-1* RNAi bacteria. To address this concern, I explored an alternative approach by utilizing the *rrf-3(pk1426)* strain, which is known for its enhanced sensitivity to RNAi (121). RNAi hypersensitive mutants in *C. elegans* are characterized by their increased responsiveness to RNAi when compared to wildtype worms. This strain allows for increased efficient knockdown of target genes using RNAi methodology. The lifespan assay for *pal-1* gene followed a similar methodology as I previously described, but instead of using wildtype worms, I utilized the *rrf-3(pk1426)* strain. In contrast to our previous findings regarding the impact of *pal-1* knockdown on the lifespan of N2 wildtype worms, the *rrf-3(pk1426)* strain illustrated a significant reduction in worm lifespan by 16.3% (p-value < 0.01), demonstrating the significant role of the *pal-1* gene in lifespan regulation (Figure 4.8).

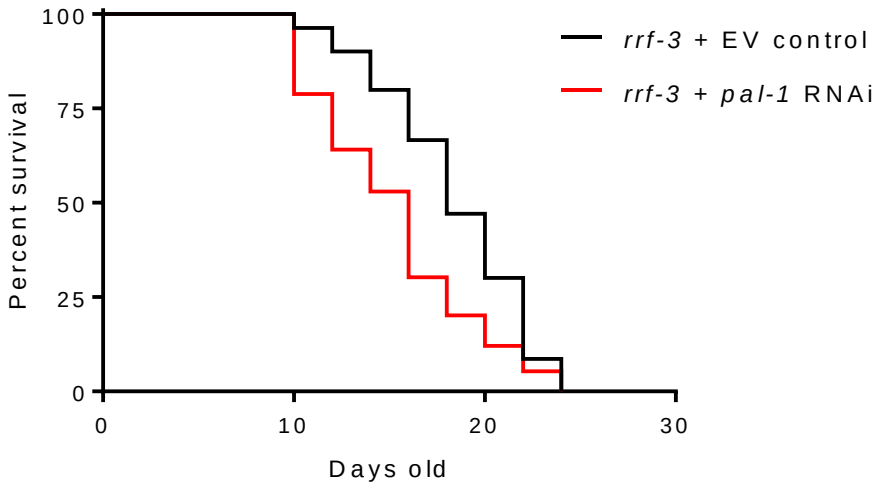

Figure 4.8: Lifespan assay in *rrf-3(pk1426)* strain worms with EV or *pal-1* knockdown effect. Loss of *pal-1* shortens the lifespan of *rrf-3(pk1426)* mutant compared to EV (p-value < 0.01). The *rrf-3(pk1426)* strain worms were moved to EV (RNAi) and *pal-1* (RNAi) for lifespan and the number of dead worms was recorded every day.

Table 4.7: Comparison of the mean lifespan between EV and *pal*-1 conditions using *rrf-3(pk1426)* strain worms. The knockdown of *pal-1* in *rrf-3(pk1426)* worms led to a significant decrease in lifespan compared to the EV control group. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator

Groups	Number of subjects	Mean lifespan (Days)
rrf-3(pk1426) EV	140	18.37
rrf-3(pk1426) pal-1	113	15.28

Next, I conducted survival assays by exposing worms to 100 µM cadmium and 5 mM acrylamide. In line with the results from the *pal-1* lifespan assay, the finding of the survival assays revealed substantial reductions in survival time upon *pal-1* knockdown under both acrylamide (p-value < 0.01) and cadmium exposure (p-value < 0.05) (Figure 4.9A-B; Table 4.8). These results indicate that the absence of *pal-1* alters worm survival to different stressors, indicating a novel role for *pal-1* in stress response pathways.

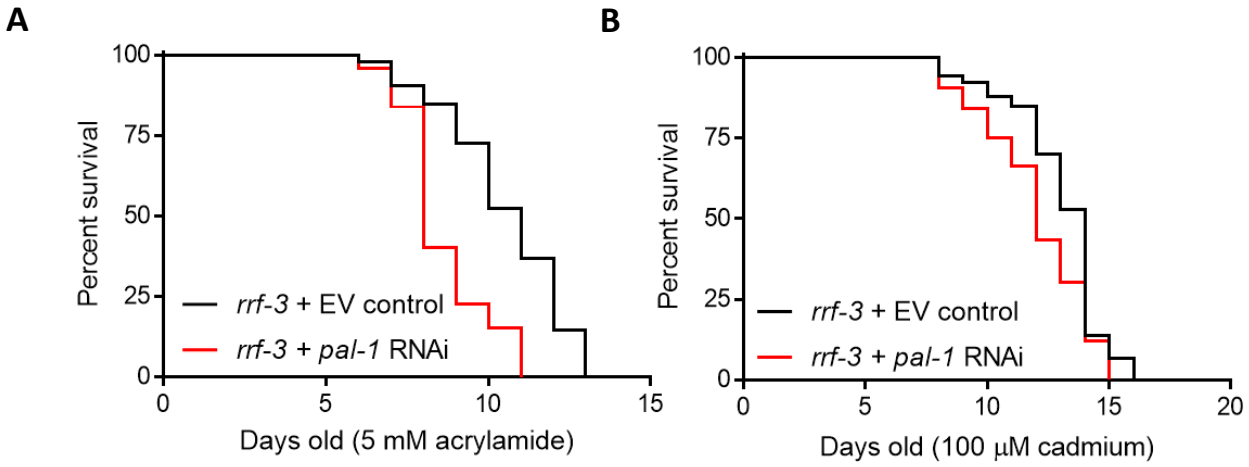

Figure 4.9: Survival assay in *rrf-3(pk1426)* strain comparing EV (RNAi) and *pal-1* (RNAi) conditions exposed to **A)** 5mM acrylamide (p-value < 0.01) and **B)** 100 µM cadmium (p-value < 0.05). The survival time of *pal-1* knockdown worms exposed to either cadmium or acrylamide is

significantly decreased compared to EV (RNAi). Each trial was independently repeated three times. The worms were transferred to plates containing either empty vector (EV) or *pal-1* RNAi. The number of deceased worms, as well as censored worms, was frequently documented.

Table 4.8: Mean lifespan of *rrf-3(pk1426)* strain worms in EV and *pal-1* conditions in normal lifespan and also survival assays including 100 µM cadmium and 5mM acrylamide exposure. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator.

Groups	Number of subjects	Mean lifespan (Days)
EV cadmium	105	12.52
pal-1 cadmium	120	11.15
EV acrylamide	138	10.26
pal-1 acrylamide	134	9.2

4.7 *pal-1* knockdown with acrylamide decreased *gst* family genes expression

Based on our previous findings that the PAL-1 protein physically interacts with CCF-1 and knockdown of *pal-1* reduces lifespan in worms and its potential collaboration with *ccf-1* in stress response, I aimed to further investigate whether *pal-1* gene co-regulates *ccf-1*-dependent stress response genes as well. To conduct this investigation, I employed *rrf-3(pk1426)* worms and fed them with EV and *pal-1* RNAi bacteria for 48 hours when they become young adults. Subsequently, the worms were transferred to plates containing cadmium and acrylamide, along with the corresponding RNAi, for an additional 24 hours. Following this exposure, I performed qPCR analysis to measure the mRNA levels of the genes of interest, which were the same as those regulated by *ccf-1*, to determine if *pal-1* had similar effects on these genes. Our findings revealed that the knockdown of *pal-1* primarily affected *gst* family genes when exposed to acrylamide (Figure 4.10). Notably, *pal-1* knockdown attenuated the activation of glutathione family genes, including *gcs-1*, *gst-4*, *gst-5*, *gst-12*, *gst-14*, *gst-30*, and *gst-38* (p-value < 0.001). As mentioned before, *gst* family of genes are involved in antioxidant defense, free radical neutralization, and detoxification processes. These results further support the crucial role of *pal-1* in protecting against various stressors.

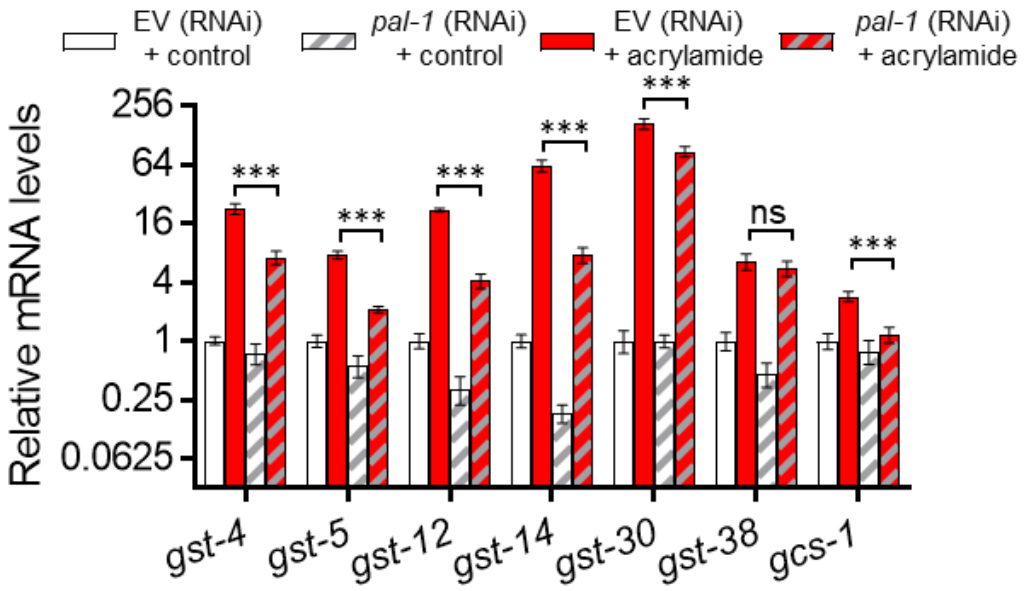

Figure 4.10: mRNA expression level assay using qPCR for *rrf-3(pk1426)* EV and *pal-1* RNAi group exposed to acrylamide. Knock down of *pal-1* significantly decreases the level of *gst-4, gst-5, gst-12, gst-14, gst-30, gst-38,* and *gcs-1* in *pal-1* (RNAi) exposed to 5 mM acrylamide using two-way ANOVA test with *post hoc* sidak. ****P* < 0.001. N = 4 replicates of 200-300 worms

Further, when *rrf-3(pk1426)* worms with *pal-1* knockdown (RNAi) were exposed to cadmium, a noticeable decrease in a variety of genes such as *cdr-1, F57B9.3, numr-1, gst-12, gst-4, gst-30, gst-33* and *gst-38* was observed (*p < 0.05, **P < 0.01, ***P < 0.001). These findings align with the earlier experiment involving *ccf-1* knockdown and changes in the expression levels of defense genes (as shown in Figure 4.11). Thus, it can be concluded that *pal-1* co-regulates genes in stress conditions, particularly during exposure to cadmium and acrylamide, and likely collaborate with *ccf-1* in this regard.

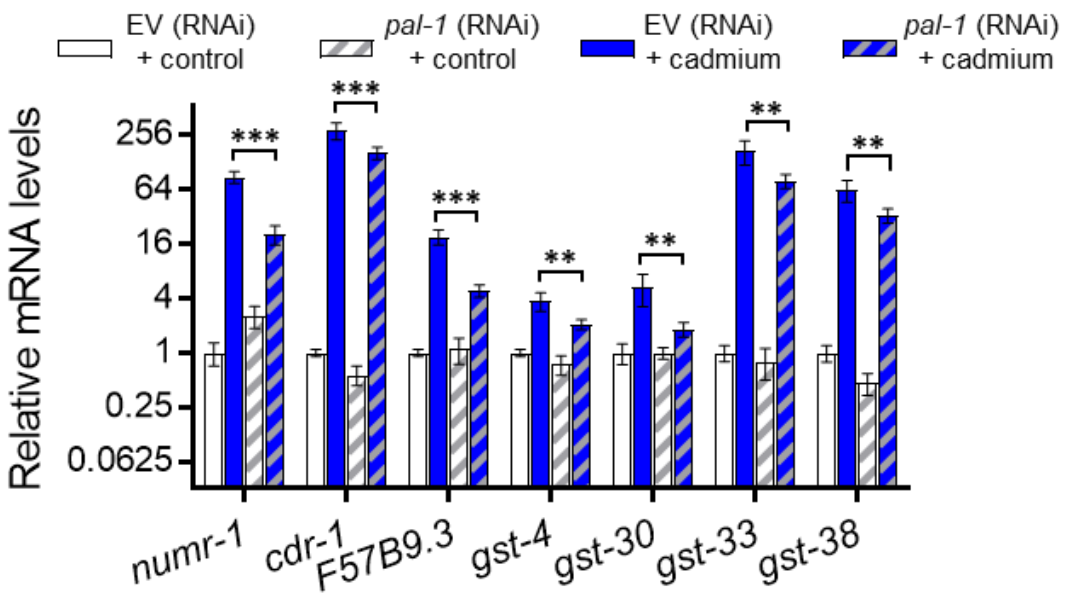

Figure 4.11: mRNA expression level assay using qPCR for *rrf-3(pk1426)* EV and *pal-1* RNAi group exposed to cadmium. The expression level of *cdr-1, F57B9.3, numr-1, gst-14, gst-30, gst-33,* and *gst-38* showed a noticeable change using two-way ANOVA test with *post hoc* sidak. *p < 0.05, **$P < 0.01$, ***$P < 0.001$. N = 4 replicates of at least 200-300 worms.

4.8 Additive interaction between CCF-1 and PAL-1

Based on my previous findings, it is determined that *ccf-1* and *pal-1* have similar effects on lifespan, survival assays, and stress response to various stressors. Additionally, their physical interaction suggests using the yeast 2-hybrid screen that these two genes are connected and function together within the same pathway to respond to stress. To further investigate the potential interactive effects of these genes and determine if they work downstream of each other, my next step was to simultaneously knockdown both *ccf-1* and *pal-1*. This approach would allow us to examine if there is a possible additive or synergistic effect, where the combined knockdown of both genes has a greater impact than knocking down each gene individually. It would also help us determine if there is a dominant effect of one gene on the other or if their simultaneous knockdown negatively affects the stress response. By studying the combined knockdown of *ccf-1* and *pal-1*, I aimed to get a better understanding of the relationship between these genes and their coordinated role in stress response pathways. Given that a viable loss of functional mutant is not available for

either *ccf-1* and *pal-1* as both are essential genes, and that double RNAi knockdown is not routinely used in the field due to the inability to control the exact ratio of RNAi feeding clones that can disproportionally knockdown one gene versus another, I employed the auxin-inducible degron (AID) system. A *C. elegans* strain with the AID epitope was tagged to the endogenous *pal-1* gene via CRISPR followed by transgenic ubiquitous expression of the *Arabidopsis* TIR1 F-box protein. In this system, exposure to the pheromone auxin results in the activation of TIR1 that recognizes the AID tagged PAL-1 to induce polyubiquitination followed by proteasome mediated protein degradation.

In this assay, I used knocked-down *ccf-1* via RNAi and PAL-1 via auxin to determine their potential additive or synergistic effects on lifespan and stress survival. The pheromone auxin is dissolved in 100% ethanol and a corresponding ethanol only agar plate is used as a vehicle control. In the ethanol vehicle control seeded with (EV) RNAi, worms had an average lifespan of 19.47 days (Figure 4.12A). When exposed to cadmium, the EV cadmium group had an average lifespan of 14.13 days (Figure 4.12B), while the EV acrylamide group, exposed to acrylamide, had an average lifespan of 11.6 days (Figure 4.12C) meaning that acrylamide has stronger effects on lifespan as I have shown in previous experiments. The ethanol vehicle *ccf-1* RNAi group, where the *ccf-1* gene was knocked down, showed an average lifespan of 12.34 days (Figure 4.12A). In the presence of cadmium, the *ccf-1* cadmium group had an average lifespan of 11.49 days (Figure 4.12B). Similarly, the *ccf-1* acrylamide group, with *ccf-1* gene knockdown and exposure to acrylamide, exhibited an average lifespan of 9.52 days (Figure 4.12C). With auxin treatment, the EV auxin group which are *pal-1* knockdown group, had an average lifespan of 15.45 days (Figure 4.12A), while the EV cadmium auxin group had an average lifespan of 13.29 days (Figure 4.12B). The use of the AID technique to knock down the PAL-1 protein resulted in more pronounced effects on lifespan when compared to using hypersensitive worms and was therefore stronger than the RNAi method. The EV acrylamide auxin group showed an average lifespan of 9.5 days (Figure 4.12C).

In the double knockdown *ccf-1* RNAi with auxin group, the worms had an average lifespan of 12.14 days (Figure 4.12A). This is similar to the single knockdown of *ccf-1* lifespan of 12.34 days, suggesting that knockdown of PAL-1 did not further reduce lifespan of *ccf-1* depleted worms. However, under cadmium, the double knockdown *ccf-1* RNAi with auxin degradation of

PAL-1 had an average lifespan of 10.39 days (Figure 4.12B), which is shorter than the single knockdown of *ccf-1* (11.49 days) or PAL-1 (13.29 days) alone. This suggests an additive effect on cadmium resistance when both factors are knocked down. Similarly, under acrylamide, the double knockdown *ccf-1* RNAi with auxin degradation of PAL-1 had an average lifespan of 7.62 days (Figure 4.12C), which is shorter than the single knockdown of *ccf-1* (9.52 days) or PAL-1 (9.5 days) alone. Overall, the data here showed that loss of *ccf-1* and PAL-1 under stress results in an additive effect that further decreases worm survival.

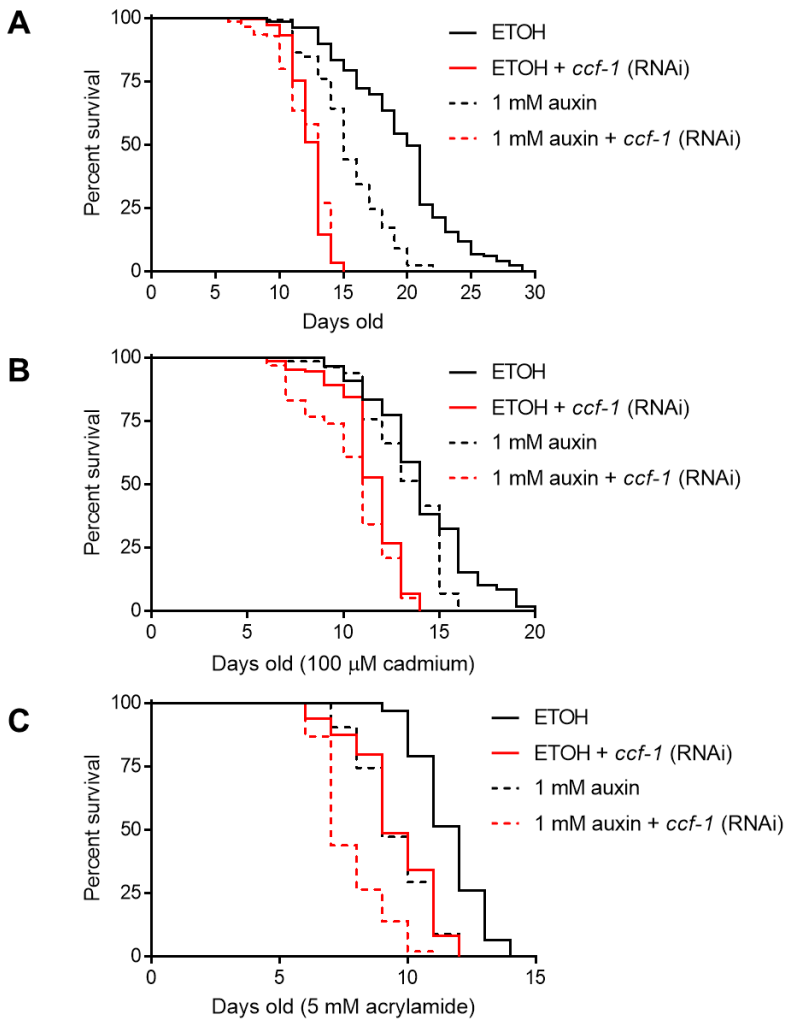

Figure 4.12: Effects of *ccf-1* and PAL-1 double knockdown on **A)** normal lifespan, **B)** cadmium survival, and **C)** acrylamide survival. Ethanol (ETOH) was used as a vehicle control for the auxin condition. Each trial was performed three times. The number of deceased worms, as well as censored worms, was frequently documented.

Table 4.9: Mean lifespan and survival times of worms in simultaneous knockdown of *ccf-1* and PAL-1 conditions. Data was analyzed by the Online Application for Survival Analysis 2 (OASIS 2) using the Kaplan-Meier estimator

Groups	Number of subjects	Mean lifespan (Days)
Ethanol + EV	134	19.47
Ethanol + EV cadmium	145	14.13
Ethanol + EV acrylamide	125	11.6
Ethanol + *ccf-1*	196	12.34
Ethanol + *ccf-1* cadmium	138	11.49
Ethanol + *ccf-1* acrylamide	133	9.52
Auxin + EV	132	15.45
Auxin + EV cadmium	199	13.29
Auxin + EV acrylamide	126	9.5
Auxin + *ccf-1*	235	12.14
Auxin + *ccf-1* cadmium	136	10.39
Auxin + *ccf-1* acrylamide	178	7.62

5. DISCUSSION

Transcription is a fundamental process in which genetic information encoded in DNA is transcribed into RNA molecules. It plays a crucial role in gene expression and regulation, allowing cells to synthesize the proteins necessary for various biological processes. Transcription is essential for normal development, growth, and function of organisms. Without proper transcription, these essential RNA molecules would not be synthesized, leading to severe cellular dysfunction and ultimately compromising the organism's viability. Transcriptional factors are proteins that bind to specific DNA sequences, known as transcription factor binding sites, and regulate gene expression by either promoting or inhibiting transcription. These factors are critical for the precise control of gene expression, as they can activate or repress the transcription of specific genes in response to various signals or environmental cues. Cells constantly encounter various environmental stresses, such as changes in temperature, nutrient availability, microbes, or exposure to toxins. These stresses can disrupt normal cellular functions and threaten the cell's survival (122–124). To counteract these challenges, cells need to respond appropriately. When a cell experiences stress, it sends distress signals that trigger the recruitment of transcription factors to the promoters of target genes. The transcription factors act as molecular switches, turning on the transcription process, which leads to the synthesis of messenger RNA (mRNA) molecules from the genes. These mRNA molecules then serve as templates for protein synthesis in a subsequent step known as translation (124,125).

By activating specific genes, the cell can produce proteins that play critical roles in managing the stress response. Different transcription factors recognize distinct DNA sequences in gene promoters, and they can act together to amplify the transcription of a target gene. This cooperative action leads to a more robust and efficient cellular response to stress. Different stress conditions require different sets of genes to be activated. By selectively turning on specific genes, cells can tailor their responses to the type and intensity of stress they encounter (126,127). These responses involve a series of complex signaling pathways that regulate gene expression and cellular functions to adapt and ensure survival. Some well-known stress response pathways include the heat shock response, unfolded protein response, DNA damage response, oxidative stress response, nutrient sensing pathways, inflammatory response, hypoxia-inducible factor pathway, and apoptosis pathway (128–130). Understanding how cells respond to stress allows us to comprehend how they

maintain their integrity and functionality under adverse conditions. Insights into these pathways can help develop strategies to enhance cellular resilience and protect against damage. Also, the relationship between stress response and aging is an active research area. An improper or dysregulated stress response is believed to contribute to the aging process and the development of aging-related diseases (131). Chronic or excessive stress can lead to cellular damage, genomic instability, and accelerated aging. It can disrupt normal cellular processes, including transcriptional regulation, and promote the accumulation of damaged molecules within cells. Over time, this can contribute to the onset of age-related diseases, such as neurodegenerative disorders, cardiovascular diseases, and certain types of cancer (37,131,132). Despite significant progress, the precise mechanisms underlying stress response, aging, and aging-related diseases are not yet fully understood. The interplay between genetic, environmental, and epigenetic factors in these processes is complex and multifaceted. Researchers continue to explore the intricate molecular pathways involved in stress response and aging, aiming to uncover new therapeutic targets and interventions that can modulate these processes and potentially mitigate the impact of aging-related diseases.

The CCR4-NOT complex is a conserved multi-subunit protein complex involved in transcriptional regulation, RNA metabolism, and mRNA decay (133,134). The CCR4-NOT complex interacts with various transcriptional factors, coactivators, and chromatin remodeling factors, thereby influencing gene expression and transcriptional regulation. The complex plays a crucial role in cellular responses to environmental changes and stress conditions by modulating mRNA stability, translation, and transcription. Dysregulation of the CCR4-NOT complex has been linked to cancer and other diseases, making it an interesting target for further research and potential therapeutic interventions (75,135). The CCR4-NOT complex interacts with various signaling pathways, allowing it to integrate different cellular responses and regulate gene expression in response to diverse environmental elements. As mentioned previously, the CCR4-NOT complex has been found to interact with components of the insulin signaling pathway as the first and also most well-known aging signaling pathway, which plays a crucial role in glucose metabolism and energy homeostasis. It is believed that the CCR4-NOT complex may modulate the expression of certain genes involved in insulin signaling, thereby influencing cellular responses to changes in insulin levels and glucose availability (136). It has been shown that Not1 gene knockdown as a subunit of the CCR4-NOT complex subunit in *Drosophila* increases the insulin-dependent

phosphorylation of a translation initiation factor (eIF) subunit 4E-binding protein (4E-BP) gene (137). Moreover, the CCR4-NOT complex interacts with the heat shock response (138). Heat shock proteins (HSPs) are crucial components of this response, and the CCR4-NOT complex has been shown to regulate the expression of HSP genes, influencing the cell's ability to cope with thermal stress. Several subunits of the CCR4-NOT complex namely NOT3 have been predicted to be involved in transcriptional response to heat shock (139). The CCR4-NOT complex's deadenylation process also potentially contributes to heat-shock sensitivity. This is because the MPT5 protein binds to specific mRNAs' 3'-untranslated regions, which are involved in essential functions like cell wall integrity, chronological lifespan, and chromatin modification (140). Other studies also show CCR4-NOT complex actively interacts with other stress signaling pathways. These examples along with several others illustrate how the CCR4-NOT complex is interconnected with other signaling pathways, allowing it to act as a versatile regulator of gene expression and cellular responses. Its ability to integrate signals from various pathways emphasizes its importance in coordinating cellular functions and maintaining cellular homeostasis in response to different environmental stimuli and stresses.

5.1 *ccf-1 as* a key regulator in stress resistance during aging across various signaling pathways

In this thesis, my focus was to unravel the impact of CCF-1, a subunit of the *C. elegans* CCR4-NOT complex, on transcriptional regulation and stress responses to various stressors. To achieve this, I commenced with lifespan and survival assays. The results from these assays provided compelling evidence that the knockdown of the *ccf-1* gene in *C. elegans* significantly influences both the lifespan and survival of the worms. These findings sparked an intriguing hypothesis that this gene may play crucial roles in age-related changes observed in worms which can act similarly to other important genes in the aging process. However further experiments are required to support and verify this hypothesis. While many studies have explored the effects of various genes on aging, research on the *ccf-1* gene and other subunits of the CCR4-NOT complex is relatively limited. It might be that the CCR4-NOT complex may influence mitochondrial metabolism, promoting their upregulation, and subsequently impacting cell survival, differentiation, and overall cellular homeostasis. The absence of the CCR4-NOT complex could lead to cellular damage and accelerated aging, resulting in a shortened lifespan. Additionally, the CCR4-NOT complex plays a crucial role in protein degradation through the ubiquitin-proteasome system (UPS). This system

involves the tagging of proteins with multiple ubiquitin molecules, a process called ubiquitination, marking them for degradation. Research findings indicate that CCR4-NOT mutants disrupt ubiquitin homeostasis, resulting in increased protein degradation and reduced resistance to stressors.

The absence of the *ccf-1* gene, a crucial subunit of this complex, leads to two significant consequences: 1) the impairment of the UPS system, disrupting cellular homeostasis and causing various side effects, such as heightened vulnerability to harmful agents, abnormal cell cycle progression, compromised DNA repair, protein accumulation, altered protein turnover, susceptibility to neurodegenerative diseases, and accelerated aging, and 2) in the absence of *ccf-1* gene, the entire CCR4-NOT complex becomes subject to regulation through ubiquitin-mediated processes. It might be possible that lack of *ccf-1* influences the UPS system and leads the the entire cell to premature aging. Some studies have suggested that specific subunits of the CCR4-NOT complex can undergo ubiquitination, influencing the complex's stability and function, which ultimately leads to reduced organism survival. Notably, I conducted mRNA level expression analysis on other subunits of the CCR4-NOT complex, including *ntl-2, ntl-3,* and *let-711*. This investigation revealed the possibility that not only *ccf-1* but also other subunits of this complex may play significant roles in the organism's responses. Knocking down each subunit individually could potentially impact the entire complex. Consequently, it is plausible that *ccf-1* knockdown alone could elicit all the outcomes mentioned above.

5.2 Multiple subunits of the CCR4-NOT complex positively promote the transcription of stress-inducible genes in *C. elegans*

The cellular RNA synthesis rate requires highly coordinated control. Three polymerases are involved in transcribing rRNA, mRNA, and small RNAs. The TATA Binding Protein (TBP) is the only shared factor among these polymerases making it a likely target for regulation. When a gene needs to be transcribed, the cells must first identify the starting point on the DNA where transcription should begin, marked by a specific DNA sequence known as the TATA box and all transcription factors probably target this site of DNA to regulate the transcription. Several factors have been identified that physically associate with TBP and stabilize its binding to DNA and are required for optimal TBP function at a subset of promoters. These factors include TAFs (TBP Associated factors), SAGA (Spt Ada Gcn5 Acetyltransferase), and CCR4-NOT complex. The

main role of these factors is to bind with TBP and is thought to stabilize its binding to DNA. The CCR4–NOT complex is a versatile group of proteins that regulates various important processes in eukaryotic cells (141). Its roles involves transcriptional regulation and protein modification, deadenylation of mRNA, transcription initiation and elongation, and degradation of the mRNA (by shortening of the poly(A) tails of mRNA) (75,142,143). In yeast, the central CCR4-NOT complex is composed of CCR4p, CAF1p, five NOT proteins (NOT1p to NOT5p), CAF40p, and CAF130p (144). Additional research has shown that CCR4p and CAF1p are the primary enzymes responsible for deadenylating cytoplasmic mRNA in yeast (145). Some of these genes in *C. elegans* (and their human orthologs) are *ccf-1* (CAF1), *ccr-4* (CCR4), *let-711* (CNOT1), *ntl-2* (CNOT2), *ntl-3* (CNOT3), *ntl-4* (CNOT4), *ntl-9* (CNOT9), *ntl-11* (CNOT11), and *tag-153* (CNOT2). There are other subunits such as CNOT10 that are exclusive to humans and are not found in *C. elegans*. The majority of NOT subunits, like NOT2, NOT3, and NOT5, function as co-factors in the deadenylation process and may play a role in interacting with the ribosome as well as stabilization of the complex and serve as RNA substrate recruitment (146,147). However, they can have other roles as well. For example, Not4 is responsible for the E3 ligase-mediated ubiquitylation (148). Several studies show these proteins work together for a variety of cellular functions. For example, *ntl-2* and *ntl-3* work in actinomycin regulation and are involved in cellular polarity processes (114).

In my study, I showed that multiple subunits of the CCR4-NOT complex positively promote the transcription of stress-inducible genes in *C. elegans*. Lack of *ntl-2*, *ntl-3*, and *let-711* had a significant impact in stress responsive genes induction, however, the extent of stress responsive genes expression varied among the knockdown of each subunit indicates that the significance of stress response extends beyond just the *ccf-1* gene, as other components of the complex also play crucial roles, but the extent of gene expression varied among the knockdown of each subunit suggesting that each subunit has a distinct role in controlling the stress response. The variations in mRNA expression level in each condition might be influenced by the varying effectiveness of RNA interference (RNAi) method for the different dsRNA clones used in the study. The data indicate that various subunits of the CCR4-NOT complex are necessary for the transcriptional response of *C. elegans* to different classes of stress resistance genes, especially cadmium-induced stress genes. This strengthens the idea that the entire CCR4-NOT complex is involved in the transcriptional stress response and functions as a whole in the transcriptional stress response.

5.3 The *ccf-1* gene mediates lifespan in long-lived mutants

Our findings revealed that *ccf-1* plays a crucial role in regulating various stress response genes involved in diverse stress signaling pathways. Notably, in several long-lived mutants, compelling evidence supports the notion that these mutants activate specific stress response genes, including those related to heat shocks (149), oxidative responsive (150), heavy metal responsive genes (151), UV-related genes (152), and more. In my experiments, I observed that *ccf-1* has comparable effects on those genes, indicating that it may operate through the same pathways as long-lived mutants. Lifespan assays on these mutants further confirmed the necessity of *ccf-1* for their longevity, although the extent of dependency on the *ccf-1* gene varied among the mutants. One possible hypothesis is that the *ccf-1* gene and its complex collaborate with other well-known longevity pathways that involve genes with extensive research about them in the field of longevity. Previous studies have demonstrated an active interaction between the CCR4-NOT complex and the mTOR (mechanistic target of rapamycin) signaling pathway, which is widely known for its role in regulating lifespan. Disruption of the CCR4-NOT complex leads to a decrease in global mTORC1 signaling. Additionally, the CCR4-NOT complex positively influences the activity of the vacuole V-ATPase which promotes mTORC1 activation under nutrient-rich conditions. However, loss of the CCR4-NOT complex compromises V-ATPase function, resulting in the inhibition of mTORC1 signaling. These findings suggest that *ccf-1* as well as CCR4-NOT complex play a significant role in pathways associated with longevity, such as the mTOR signaling pathway. The interactions between these components may contribute to the regulation of stress response genes and longevity in the organism. Further investigation into these pathways and their interplay is vital for a comprehensive understanding of the mechanisms that influence lifespan and stress responses. (153).

The necessity of *ccf-1* for stress resistance in wildtype worms may be attributed to its involvement in the insulin signaling pathways. Our findings indicate that knockdown of *ccf-1* can reduce the lifespan of *daf-2(e1370)* mutants by 37%. Research has shown that the insulin signaling pathway can regulate resistance against stressors, including heavy metals. It is essential to note that theories regarding stress resistance rely on the pleiotropic nature of genes, meaning multiple genes collectively contribute to various stress response events rather than a single gene is responsible for all occurrences. Moreover, the insulin pathway is known to activate many superoxide dismutase family genes that regulate redox balance. Thus, if there is a direct

relationship between *ccf-1* and *daf-2*, representing this pathway, it can be inferred that *ccf-1* may also impact stressors related to heavy metals through the insulin pathway (154).

5.4 Discovering a novel transcriptional factor: *ccf-1* and *pal-1* genes demonstrate pleiotropic effects with additive impacts

Genetic interactions occur when the combined effects of two or more genes are different from what would be expected based on the individual effects of each gene. These interactions can be classified into different types, such as Synergistic interaction, antagonistic interaction, and combined interaction. According to the SynergyAge (155), the above-mentioned genetic interventions were among the most promising in terms of lifespan alteration most of these interventions were studied on *daf-2* mutant, but there was no mention of either *ccf-1* or *pal-1* intervention.

The two genes may have overlapping or redundant functions in the context of aging and regulation of stress responses. When either the PAL-1 protein or the *ccf-1* gene is individually knocked down, the other gene can partially compensate for its loss, leading to a milder effect on lifespan. In this study, I observed that the knockdown of *ccf-1* decreases the lifespan to 12.34 days, which represents a 36.62% decrease compared to the control condition (EV RNAi). Conversely, when PAL-1 is knocked down, the lifespan decreases by 20.65% (15.45 days). However, when both *ccf-1* and PAL-1 are knocked down simultaneously, the lifespan decreases by 37.65%, which is similar to when *ccf-1* is deactivated. This suggests that when both genes are knocked down, the effects of *ccf-1* dominate and mask the effects of PAL-1. Additionally, under cadmium exposure, *ccf-1* knockdown leads to an average lifespan of 11.49 days, representing an 18.68% decrease compared to cadmium-exposed worms in the control condition (EV RNAi); on the other hand, PAL-1 knockdown decreases survival by 5.94%. However, when both genes are knocked down, the decrease in survival is 26.47%, indicating that both *ccf-1* and PAL-1 have additive effects on survival. Finally, when exposed to acrylamide, the *ccf-1* knockdown group showed a 17.93% decrease in survival compared to worms exposed to the same stressor under normal conditions. The *pal-1* knockdown group showed an 18.10% decrease under the same condition. However, when both genes are knocked down, the survival time decreased by 34.31%, suggesting that both genes have their individual effects on survival.

In the survival assay, the study indicates that both *ccf-1* and PAL-1 show additive effects on survival under stress conditions. When both PAL-1 and *ccf-1* are knocked down, their individual effects seem to combine, leading to a more pronounced impact on the worms' survival. *ccf-1* knockdown consistently results in more reduced survival compared to PAL-1 knockdown in these stress conditions. Additionally, when both genes are knocked down, their combined effects result in a more substantial decrease in survival compared to individual knockdowns, indicating that both genes contribute to the worm’s response to stressors.

The presence of stress might change the way these genes interact or function, leading to a more pronounced impact on the animal's longevity. Stress conditions can trigger different molecular pathways and cellular responses, and the simultaneous knockdown of the two genes may disrupt critical processes involved in stress resistance and lifespan regulation, resulting in an additive or synergistic effect on lifespan reduction. Alternatively, impaired mRNA stability as well as insufficient DNA transcription affects both *ccf-1* and *pal-1* in response to stress and might be detrimental to nematode by affecting lifespan. It is also shown that *ccf-1* just had a partial effect on the aging signaling pathways of long-lived mutants meaning the way *ccf-1* interacts with PAL-1 is distinct from other well-known pathways and needs to be further investigated. Several metabolic changes were identified as fingerprints for long-lived mutants including the shift from carbon to amino acid catabolism as an alternative energy source, up-regulation of lipid storage, increased purine metabolism, and increased trehalose stores. Many of these processes were found to be regulated in a DAF-16-dependent manner which encodes the mammalian FoxO transcription factor. Investigating these changes while *ccf-1* is knocked down with and without interacting with other long-lived mutants would be a good future direction.

In conclusion, my research on *ccf-1* has provided valuable insights into its role in various aspects of lifespan and stress response in *C. elegans*. Through our experiments, I have demonstrated that *ccf-1* is essential for stress resistance and survival in wildtype and long-lived worms, indicating its significance in maintaining cellular homeostasis and combating stressors. Interestingly, my findings suggest that *ccf-1*'s influence on lifespan is not entirely dependent on the genetic pathways responsible for lifespan extension in long-lived mutants. This suggests that loss of *ccf-1* may shorten lifespan through distinct mechanisms, independent of the pathways affected by the long-lived mutants studied in this investigation. Furthermore, my research has

unveiled the interaction between the *ccf-1* gene and the PAL-1 protein, demonstrating their additive effects on survival in stress exposure conditions. This interaction sheds light on the intricate regulatory network that governs longevity in *C. elegans*, adding a new dimension to our understanding of the genetic factors influencing lifespan. As we continue to explore its role in the CCR4-NOT complex and its interactions with other longevity pathways, we can gain deeper insights into the fundamental processes regulating aging and stress resistance in this model organism. These findings have the potential to contribute to our understanding of cellular homeostasis and longevity regulation, offering new avenues for future research in aging research.

The main limitation of this study arises from the fact that although *C. elegans* has many homolog genes with humans, further research and a cautious approach are still needed before drawing any definite conclusions about potential impacts of the studied genes on human health and lifespan based on the data presented in this thesis.5.5 Future works and directions

Moving forward, several intriguing aspects and directions emerge from my findings. Firstly, despite *ccf-1*'s involvement in stress resistance and its potential connections to the insulin signaling pathways, further research is needed to fully elucidate the specific mechanisms by which *ccf-1* influences stress response genes and longevity. Investigating the molecular interactions and regulatory networks involving *ccf-1* and the insulin and also mTOR signaling pathway may shed light on the underlying processes that govern stress resistance in wildtype worms. Given the pleiotropic nature of genes involved in stress resistance, exploring other potential factors that contribute to *ccf-1*'s effects on longevity and stress response would be beneficial. Identifying novel genetic components and their interplay with *ccf-1* could deepen our understanding of the intricate mechanisms governing stress resistance and aging.

Finally, considering the impact of *ccf-1* knockdown on the lifespan of *daf-2* mutants and other long-lived mutants, further research could explore the interactions between *ccf-1* and these mutants, possibly leading to a more comprehensive understanding of the intricate genetic and molecular factors influencing lifespan regulation. By delving deeper into these areas, we can advance our knowledge of the fundamental processes governing longevity and stress responses in *C. elegans*.

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