INVESTIGATING THE FUNCTIONAL SIGNIFICANCE OF BRAP-2 IN RESPONSE TO OXIDATIVE STRESS IN *CAENORHABDITIS ELEGANS*

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Abstract

In order to prevent cellular damage caused by reactive oxygen species (ROS), mammals have developed sophisticated defense mechanisms such as the Keap1-Nrf2 oxidative stress response pathway to maintain homeostasis. Deregulation of Nrf2 could result in detrimental neurodegenerative diseases and cancer. The mammalian BRAP2/IMP is a Ras effector protein with a known role in negatively regulating the ERK/MAPK pathway through the KSR scaffold protein. We are interested in studying the homolog of BRAP2 in *C. elegans* known as BRAP-2. Our lab has previously shown that a mutation in *brap-2* causes an enhanced expression of the SKN-1/Nrf2 target gene *gst-4* in the intestine and hypodermis. In this study we utilized genetic and biochemistry approaches to show that BRAP-2 is a negative regulator of SKN-1. Our results demonstrate that *brap-2* mutants have enhanced SKN-1 expression within intestinal nuclei. Western blot analysis indicates BRAP-2 physically interacts with activated LET-60/Ras and KSR-2/KSR. Endogenous expression of pMAPK is also higher in *brap-2(ok1492)*, revealing that BRAP-2 is required in activating Ras and the MAPK cascade for SKN-1 regulation.

To better define the BRAP-2/SKN-1 signaling cascade, an RNAi screen was performed and 20 novel transcription factors or co-activators of SKN-1 were identified to elevate *gst-4* expression in *brap-2* mutants. The screen revealed *elt-3* and *nhr-49* as viable candidates for participation in the SKN-1/Nrf2 signaling pathway to promote this biological effect. Analysis through various experiments show that ELT-3/GATA or NHR-49/PPARα are required for enhanced *gst-4* expression in *brap-2(ok1492)* animals. Our data also indicates that lifespan extension conferred by overexpressing SKN-1 is dependent on functional ELT-3 and NHR-49. Together, this work provides further understanding of how BRAP-2 can regulate SKN-1 in coordination with other transcription factors in response to oxidative stress through the ERK/MAPK pathway. The stress gene regulatory network is a complex model and many of its signaling pathways are evolutionary conserved. My research using the *C. elegans* model may provide a mechanism on how BRAP2 and Nrf2 are regulated in higher organisms, including humans to facilitate research and development of therapies for the treatment of diseases caused by signaling dysregulation such as cancer.

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List of Abbreviations

3' UTR	3' untranslated region
ABC	ATP-binding cassette
ACT	Actin
AGE	Ageing alternation (AGE-1 = ortholog of PI3K)
AKT	Protein Kinase B (PKB)
ARE	Antioxdiant Response Element
BRAP2	Brca-1 Associated Binding Protein 2
BRCA	Breast Cancer
bZIP	Basic Leucine Zipper
cDNA	Complementary DNA
C. elegans	*Caenorhabditis elegans*
ChIP	Chromatin Immunoprecipitation
Cul3	Cullin 3
DAF	Abnormal Dauer Formation
DAPI	4',6-Diamidino-2-Phenylindole
D. melanogaster	*Drosophila melanogaster*
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
ELT	Erythroid/Erythrocyte-like Transcription Factor
ERK	Extracellular signal-regulated kinases
ETC	Electron Transport Chain
FITC	Fluorescein isothiocyanate
FOXO	Forkhead Box
GATA	GATA Family Transcription Factors
GAP	GTPase-activating protein
GCS	γ Glutamyl Cysteine Synthetase
GDP	Guanine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
Grb2	Growth factor receptor-bound protein 2
GSK	Glycogen Synthase Kinase
GST	Glutathione-S-Transferase

GTP	Guanine Triphosphate
H_2O_2	Hydrogen Peroxide
HEK	Human Embryonic Kidney
HMG20A	High Mobility Group 20A
HNF4	Hepatocyte Nuclear Factor
IGF	Insulin growth factor
IIS	Insulin/Insulin-like signaling
IMP	Impedes Mitogenic Signal Propagation
Keap1	Kelch-like ECH-Assocaited Protein 1
KSR	Kinase Suppressor of Activated Ras
LET	Lethal
MAPK/MPK	Mitogen-Acitvated Protein Kinase
MAPKK	Mitogen-Acitvated Protein Kinase Kinase
MAPKKK	Mitogen-Acitvated Protein Kinase Kinase Kinase
MDT	Mediator
MEK	Erk Kinase
modENCODE	Model Organism Encyclopedia of DNA Elements
mRNA	messenger RNA
mTOR	Mechanistic Target of Rapamycin
NAD(P)H	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGM	Nematode Growth Medium
NHR	Nuclear Hormone Receptor
NLS	Nuclear Localization Signal
NSY	Neuronal Symmetry
Nrf2/ NFE2L2	Nuclear Factor (erythroid-derived 2)-like 2
NuMA1	Nuclear Mitotic Apparatus Protein 1
O_2^-	Superoxide
OH^-	Hydroxyl Radicals
OASIS	Online Application for the Survival Analysis
PCR	Polymerase Chain Reaction
p38	Protein 38 MAPK
PD	Parkinson's Disease
PI3K	Phosphatidylinositol 3-kinase
PINK1	PTEN (Phosphatase and tensin homolog)-Induced Kinase 1
p-MAPK	Phosphorylated MAPK
PMK	P38 Map Kinase Family

PPARα	Peroxisome Proliferator-Activated Receptor
qPCR	Quantitative Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
Raf	Rapidly Accelerated Fiborosarcoma
Ras	Rat Sarcoma Oncogene
RICT	Rapamycin-insensitive companion of TOR
RNA	Ribonucleic Acid
RNA Pol	RNA Polymerase
RNAi	RNA Interference
ROS	Reactive Oxygen Species
SEK	SAPK/ERK kinase
SGK	Serum- and glucocorticoid- inducible kinase homolog
SKN	Skinhead
SOD	Superoxide Dismutase
SOS	Son of sevenless
TF	Transcription Factor
Thr-X-Tyr	Theonine-X-Tyrosine
WD40	Beta transducing repeat
WDR	WD repeat-containing protein
WT	Wild Type
Y2H	Yeast 2 Hybrid
YF	Kubiseski Lab strains

List of Mammalian homologs of *C. elegans* genes

Table 1. The *C. elegans* genes and their vertebrate homologs mentioned and discussed in this work. Information of genes were taken from Wormbase and the corresponding references were cited in the study.

***C. elegans* gene name**	**Mammalian homolog**	**Function/ Expression in *C. elegans***
N2 (Wild Type)	---	---
brap-2	Brca1 Associated Protein 2 **(BRAP2)**	- Loss of *brap-2* is sensitive to oxidizing conditions, resulting in developmental arrest or lethality
skn-1	Nuclear factor erythroid-2-related factor 2 **(Nrf2)**	- Initially identified as an initiator required for the development of pharyngeal and intestinal tissue - Also induces the transcription of genes encoding phase II detoxification enzymes for normal lifespan
elt-3	GATA Family	- Initially found to be expressed in the embryonic epidermis during development - Reported to be a potential regulator for aging
gst-4	Glutathion-S-Transferase **(GST)**	- Increased expression resulted in increased resistance to stressors including oxidative stress
gcs-1	γ-glutamine cysteine synthase **(GCS)**	- Expressed in the ASI chemosensory neurons and intestine - Function in a conserved oxidative stress response pathway, as a phase II detoxification enzyme
sod-3	Superoxide Dismutase **(SOD)**	- Encode iron/ manganese superoxide dismutase in the mitochondria by DAF-16/FOXO transcription factor - Defend against oxidative stress and promote normal lifespan
ksr-1/2	Kinase suppressor of Ras **(KSR)**	- Function to positively regulate signaling through the Ras pathway - *ksr-1* functions to control sex myoblast migration - *ksr-2* functions to control the meiotic progression during oogenesis

***C. elegans* gene name**	**Mammalian homolog**	**Function/ Expression in *C. elegans***
let-60	Ras proto-oncogene Family **(RAS)**	- Encodes the GTP-binding Ras proto-oncogene family - Required for viability, vulva development, spicule development, germ line meiotic progression posterior development of the hypodermis, chemotaxis, sex myoblast migration and muscle membrane extension
mpk-1	Mitogen-activated protein kinase **(ERK)**	- Function in vulval cell fate specification, cell migration/ guidance and defense again bacterial infection - Promotes longevity through SKN-1 and insulin-like signaling
mek-1/2	MAP kinase kinase **(MKK7)**	- Involved in the stress response to heavy metals and starvation - Core component of Ras-mediated signal transduction pathways
mdt-15	Mediator subunit **(MED15)**	- Partner with NHR-49 for fat metabolism - Interact with appropriate transcription factor in response to toxins and heavy metals - Interact with SKN-1 in response to oxidative stress
nhr-49	Peroxisome Proliferator-Activated Recetpor **(PPARα)**	- GFP expressional analysis indicated *nhr-49* is highly expressed in somatic tissues and intestinal cells - Activates target genes through conjunction with mediator MDT-15 - A key regulator for fat desaturation and β-oxidation

Co-Authorship

Chapter 2 & 3: The transcription factor RNAi screen was performed by Dr. Lesley MacNeil (now at McMaster University) from Dr. Marion Walhout's lab in the University of Massachusetts. Dayana R. D'Amora assisted in designing experiments and carried out antibody staining, worm gonad dissection and worm lysates western blot.

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Cellular damage caused by reactive oxygen species (ROS) are believed to be a major contributor to the development of detrimental diseases associated with aging [1, 2]. Oxidative stress refers to the cell's inability to maintain the balance between the formation of oxidants and antioxidants, resulting in the accumulation of oxidative damage to macromolecules including DNA, proteins and lipids [3]. The generation of ROS and the corresponding response to oxidative stress are key factors in determining the lifespan of an organism. Both the overproduction and low levels of these oxygen derivatives can lead to extensive tissue damage, and have the ability to cause cell death. To maintain cellular integrity and prevent damage caused by ROS, animals have developed defense mechanisms to protect themselves against various stressors, and maintain homeostasis by removing excess oxygen [4]. Over the years, intensive studies have been done to elucidate the signaling pathways involved in this complex process. Once we understand the essential processes and components needed to fight against oxidative stress at the molecular level, this knowledge can later be applied to further studies that involve pharmaceutical and medical industries.

Complex signaling pathways and their effects in humans are highly conserved and have homologous counterparts in a specific popular set of simpler animals such as mice, *D. melanogaster* and *C. elegans*, making the study of their regulation and execution more feasible in these simple model organisms. In order to understand the cellular signaling

events in stress and aging, the microscopic, free-living nematode *C. elegans*, was utilized in this study. Since Sydney Brenner's pioneering work published in 1974, *C. elegans* has easily manipulated genetic system that lends itself as an ideal model in many biological studies for multiple reasons: it has a completely sequenced genome, mapped cell linage and fate, rapid life cycle, possess transparent cuticles for easy observation; and transgenic strains are readily available within the worm community [5, 6]. Additionally, genetic and proteomic libraries are available for RNAi knockout or yeast-2-hybrid (Y2H) screens, which allows for either high throughput or selective gene and protein studies to be conducted for any pathway of choice. The ability to mutate single or multiple genes in *C. elegans* has become a powerhouse for studies in defining molecular mechanisms in discrete pathways that affect different developmental or physiological aspects. All these advantages have opened avenues to investigate the genetic and molecular mechanisms involved in various processes at the cellular and organismal level. Despite a distant relationship to mammals, *C. elegans* shares 40% of proteins with similar biological functions to humans. Therefore *C. elegans* is often employed as a model organism for the investigation of signaling pathways that can lead to further application in higher organisms [7-9].

The objective of this project was to continue the work of a report published previously by our lab, where it was shown that *brap-2* mutant animals were hypersensitive to hydrogen peroxide and paraquat, leading to developmental arrest or early death in the L1 or first larval stage [10]. The *C. elegans* BRAP-2 has revealed as a key component involved in stress response, at this stage of the investigation we aim to identify and characterize new factors that function with BRAP-2 in phase II

detoxification, as well as to determine the precise signaling mechanism through which BRAP-2 regulates the oxidative stress genes. Here, both molecular and genetic approaches were used to identify a potential new role of BRAP-2 and other novel regulatory components in the Nrf2/SKN-1 detoxification pathway.

1.2 The oxidative stress theory of aging

The oxidative stress theory of aging, originally introduced by Denham Harman, attributes aging and degenerative diseases as a consequence of the damaging effects that free radicals have within cells [11]. Potent sources of ROS in eukaryotic cells include environmental toxin such as UV or free radicals produced during energy metabolism. Later Harman proposed that damage to mitochondria causes an accumulation of ROS that leads to aging and death, therefore this theory was refined as the mitochondrial free radical theory of aging [12]. However this theory was challenged when studies in *C. elegans* showed that their lifespan could be extended when living in moderate ROS conditions [13]. Despite this, two fundamental parts of the theory remained unchanged [14]: the level of oxidative stress is dictated by the imbalance between the formation and sequestration of free radicals during cellular metabolism; and the accumulation of ROS and its byproducts can inflict damage to macromolecules such as DNA, lipids and proteins, and this damage could then lead to the major determinants of the rate of aging, and thereby decrease lifespan [15].

1.2.1 Generation and Detoxification of ROS

ROS refer to highly reactive molecules consisting of unpaired electrons. The production of ROS mainly occurs in the electron transport chain (ETC) in the mitochondria or non-mitochondrial sites such as endoplasmic reticulum. The majority of

ROS is generated in the form of superoxide (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2). During the electron transport process, excessive electrons can directly bind to oxygen and reduce it to the form of superoxide, primarily in complex I of the mitochondria [16]. The production of highly reactive superoxide molecules can oxidize and damage molecules important to cellular function, or react with hydrogen peroxide to form more damaging hydroxyl radicals [17]. To limit the cellular damage caused by ROS, organisms have a number of antioxidant defense systems that employ specific enzymes to fight against these harmful molecules by breaking them down and eliminating them in a sequential manner.

In eukaryotes, the detoxification process often alters ROS in three stages, known as phase I, phase II and phase III. Each phase has evolved and possesses their own variety of antioxidant enzymes to detoxify ROS and protect the cell from oxidative damage. The first line of defense involves the phase I enzyme SOD. SOD is a catalyst that can rapidly react with the O_2^- and convert to a more stable and less harmful intermediate of H_2O_2 [18, 19]. H_2O_2 is relatively stable and membrane-permeable, which can easily travel through compartments via free diffusion or aquaporin channels in the membrane to elicit an intracellular signaling response [20, 21]. H_2O_2 can be further detoxified in phase II. A category of enzymes known as peroxidases or transferases are responsible for reducing, metabolizing and conjugating these reactive radical groups. Finally, the conjugated toxins will be exported from the cells by phase III proteins including the ATP-binding cassette (ABC) transporter. Among these three, phase II is the most extensively studied across species, as transcriptome profiling indicates the master regulator Nrf2 regulates a wide variety of phase II genes, which will be further discussed below.

Biologically, ROS play an important role as they are necessary for survival acting as signaling molecules that activate pathways needed to kill intracellular bacteria by guiding macrophages or regulate inflammatory and healing responses [22]. However, excessive ROS can lead to neurodegenerative diseases such as Parkinson's or Alzheimer's [23]. ROS appears to be both a central signaling compound and potentially toxic. For these reasons, the study of the oxidative stress response in mammals can be challenging, and extensive work is needed to elucidate the mechanisms that maintain homeostasis when exposed to stress.

1.2.2 Oxidative stress response and aging – are they correlated?

As mentioned above, the accumulation of ROS can induce cell damage and tissue health. The study of the oxidative stress response is often conducted in relation to aging. Subsequent experiments using a range of model organisms continue to support and validate the above mentioned hypothesis, which describes that aging in organisms with high ROS production will exhibit a higher mortality rate and increase incidence of age related diseases. The primary evidence to support the correlation of ROS and aging is through exposure of stress inducing compounds or mutations that can change the lifespan in these laboratory organisms [24]. Strong evidence in studies using yeast, *C. elegans* and *D. melanogaster*, have shown that when Insulin/IGF pathway components are mutated and signaling is reduced, the activation of FOXO transcription factor will increase thereby upregulating the transcription of its downstream target detoxification genes (details of which will be explained in **section 1.5.1**). However, recent studies on this topic have suggested that while there is often a correlation between ROS and aging, there are exceptions that may disprove this universal phenomenon. A study by Perez *et al*.

examined the lifespan of mice by mutating or over-expressing the antioxidant genes, *Sod1*, *Sod2*, *Gpx1* and *Gpx4* [25]. Interestingly, results indicated that only *Sod1*$^{-/-}$ decreased lifespan. Additionally, the over-expression of individual antioxidant genes did not show an increase in lifespan despite data indicating an overall increase in oxidative stress resistance. Another study conducted by Schaar *et al*. utilized long-lived *clk-1* mutant worms to define the levels and localization of ROS that are required to promote longevity [26]. Using a genetic approach, Schaar and colleagues compared the resulting lifespans of worms with mitochondrial or cytoplasmic *sod* gene knockouts. It was found that the deletion of mitochondrial *sod* genes increased lifespan, while the animals have shortened lifespan when cytoplasmic *sod* gene function is lost. Their work suggests that elevated ROS production in different cellular compartments is imperative for the regulation of lifespan. The above studies are only a few examples that demonstrate that resistance to oxidative stress does not lend itself to an extension in lifespan. Other seminal papers have also suggested that ROS and long-lived animals are sometimes uncoupled or poorly correlated [27]. Thus has led to the idea that perhaps molecular damage caused by oxidative stress is not the cause of aging [28-30]. Although there is a direct contradiction in the ROS theory of aging, the ultimate goal of stress and aging studies is to discover processes that promote longevity and healthspan, as well as to ensure a greater quality of life overtime.

1.3 Mammalian Nrf2 and its role in the response to oxidative stress

Cellular processes function like a highly specialized machine. Each individual process is tightly regulated, maintained and necessary for survival in order to maintain cell integrity. Cells are constantly encountering various kinds of exogenous stresses such

as toxins, or endogenous stresses including reactive oxygen species. In order to maintain cellular homeostasis there must be a balance between the production of oxidants and anti-oxidants. The eukaryotic genome possesses regions known as antioxidant response elements (ARE) that are responsible for transcribing and encoding phase II detoxification enzymes and antioxidant proteins. Activation of ARE related genes is controlled by the basic leucine zipper (bZIP) transcription factors (TF). Moi and colleagues were the first group to clone and characterize the mammalian bZIP NF-E2-related factor 2 (Nrf2) [31]. Nrf2 is a master transcriptional regulator of stress genes and plays a vital role in inducing enzymes that are involved in the repair or removal of damaged proteins caused by oxidative stress, as well as to promote longevity [32]. When Nrf2 is translocated into the nucleus, it can associate with other enhancers or TFs to promote transcriptional activation of antioxidant or detoxifying enzymes by binding to the ARE in the promoter regions of target genes. Genetic mutations of Nrf2 have been associated with respiratory, cardiovascular, and neurodegenerative diseases, as well as cancer [33]. Nrf2 is required to protect cells from oxidative stress, and due to its involvement in a range of deleterious diseases, extensive work has been done to understand possible Nrf2 regulated pathways, centering itself as a potential therapeutic target in drug development.

Mechanism of Nrf2-Keap1 Signaling Pathway

TF activity regulation is important in controlling the resulting abundance of its target genes. However, accumulation or insufficient expression of certain target genes could prevent cell survival, thus, a tightly regulated mechanism is required to maintain a steady state of these TF. Dysfunctions in protein degradation and removal may lead to the deterioration of many fundamental cellular processes. The stress regulated Nrf2 is no

exception; mis-regulation of Nrf2 can result in catastrophic diseases such as arteriosclerosis or cancer [33]. Though Moi *et al*. have shown that Nrf2 is expressed ubiquitously, they were not able to explain how continuous expression of Nrf2 did not hinder cellular health. Detailed analysis of Nrf2 regulation and activity identified that Nrf2 is post-transcriptionally regulated by binding to its inhibitor Keap1 (Kelch-like ECH-associated protein 1) which targets it for proteosomal degradation, thereby balancing Nrf2 presence and activity [32]. Keap1, first introduced by Itoh *et al*., is a thiol-rich cytoplasmic protein essential for the regulation of Nrf2 activity [34]. Kobayashi and colleagues provided evidence that Keap1 acts directly as an adaptor protein for the Cul3-based E3 ligase, for rapid and effective degradation of Nrf2 when cells are not exposed to oxidative or electrophilic stresses [35-37]. Under non-stressed conditions, Nrf2 is sequestered in the cytoplasm via anchoring to Keap1 and will be degraded through the ubiquitin-proteasome pathway. In contrast, the presence of electrophiles or ROS discontinues Nrf2 degradation. Upon exposure to ROS, the cysteine residues of Keap1 bind stress molecules to induce conformational changes of Nrf2-Keap1 complex, and thus impair the structural integrity of the Keap1-Cul3 interaction. This results in a decline of ubiquitination activities and facilitates the accumulation of Nrf2. Thus, Keap1 not only plays a role as a stress sensor, but also as a molecular switch for Nrf2 degradation [38].

1.4 Transcriptional regulation and stress/ aging signaling pathways in *C. elegans*

An organism is able to respond and defend against oxidative stress because it can detect stress-induced compounds and regulates the expression of appropriate genes needed for detoxification. Like mammals, the nematode *C. elegans* have well-developed

lines of defense to protect themselves from toxic compounds such as arsenite or paraquat [39-41]. If unable to avoid contact with these compounds, worms will elicit an avoidance response by initiating the transcriptional regulation activities to detoxify these stress compounds. The *C. elegans* Nrf2 ortholog, SKN-1, is a member of the bZIP TF family and it consists of three isoforms (Figure 1.1A): *skn-1a*, *skn-1b* and *skn-1c*, each isoform has their own distinct tissue expression and function (Figure 1.1B). SKN-1 was initially identified as an initiator required for the development of pharyngeal and intestinal tissue [42]. During *C. elegans* embryogenesis, SKN-1 needs to be maternally provided to subsequent progeny for specification of the EMS blastomere in order to give rise to somatic tissue (Figure 1.1C) [43]. The post-embryonic function of SKN-1 was later discovered, and like Nrf2 is a master regulator that induces phase II detoxification genes such as *gcs-1* or *gst-4* to defend against stress and relate aging [44]. An and Blackwell reported that SKN-1 is required for normal lifespan and regulates phase II detoxification genes in response to endogenous stresses [44]. SKN-1 is also expressed in amphid chemosensory neurons and the intestine under basal conditions, but accumulates in intestinal nuclei upon exposure to stress. With a mutation in *skn-1*, expressional analysis of *gcs-1* using a GCS-1::GFP transgenic strain, displayed undetectable expression of *gcs-1* in the pharynx and intestine under normal and oxidative stress conditions. It has also been shown that SKN-1::GFP expression levels are elevated in intestinal nuclei under oxidative stress or heat shock, suggesting SKN-1 travels from the cytoplasm to nucleus in response to stress. Like Nrf2, SKN-1 plays a central role in extending lifespan. Lifespan analyses have demonstrated that the mean lifespan was reduced by 25-30% in worms with a *skn-1* mutation in comparison to the wild type, indicating that *skn-1* is required for

a normal longevity [44]. While SKN-1 has defined an essential role in development and stress response, more recent studies on SKN-1 have focused on its potential role in dietary restriction and fat metabolism. In light of this, new avenues of SKN-1 research should not only focus on its function in development, but should continue to be explored in the context of stress and aging.

However, unlike humans, *C. elegans* lacks a Keap1 homolog to regulate the proteasomal degradation of SKN-1. Yet, both the cytoplasmic and nucleic homeostasis of SKN-1 *in vivo* has been found to be regulated through ubiquitin-mediated proteolysis mechanisms. A study done by An *et al*. showed that the *C. elegans* glycogen synthase kinase-3 (GSK-3) is able to phosphorylate SKN-1 and prevent it from accumulating within intestinal nuclei in the absence of stress [45]. The inhibition of SKN-1 by GSK-3 can be counteracted when p38 MAPK signaling is activated, allowing for the dramatic induction of SKN-1 phosphorylation under stress conditions. This study provided evidence that GSK-3 can influence SKN-1 intestinal accumulation and act as an important regulator of phase II detoxification genes. Additionally, a genome wide RNAi screen revealed a WD40 repeat protein known as WDR-23 that has a functional complement to Keap1 [46]. Choe and colleagues found that the constitutive accumulation of SKN-1 within intestinal nuclei can be repressed through binding of WDR-23, an E3 ubiquitin ligase adaptor, to SKN-1 through the CUL-4/DDB-1 ubiquitin ligase complex under non-stress conditions. Loss of *wdr-23* causes an increase in SKN-1 transcript and protein levels, and constitutive expression of phase II detoxification genes in intestinal nuclei, leading to increased longevity and stress resistance in the worm [46]. These findings provided a potential mechanistic framework for SKN-1 in regulating antioxidant

genes for stress response, but the gene regulatory network is a complex circuit, and the components upstream of SKN-1 that initiate its transcriptional response have not been clearly identified. Therefore a more comprehensive analysis is necessary to uncover these regulatory factors and their associated pathways.

Given the newly published role of SKN-1 in stress resistance and longevity, extensive research has been devoted to understanding SKN-1 pathways that protect against endogenous and exogenous forms of stress. Intensive studies have been done on this topic and they have provided evidence of the complex nature of SKN-1 function, ranging from detoxification and immunity, to proteostasis and metabolism. In addition, the mechanisms that control nuclear accumulation of SKN-1 and its regulation continues to be a promising and active area of study and will be discussed in detail in the following section.

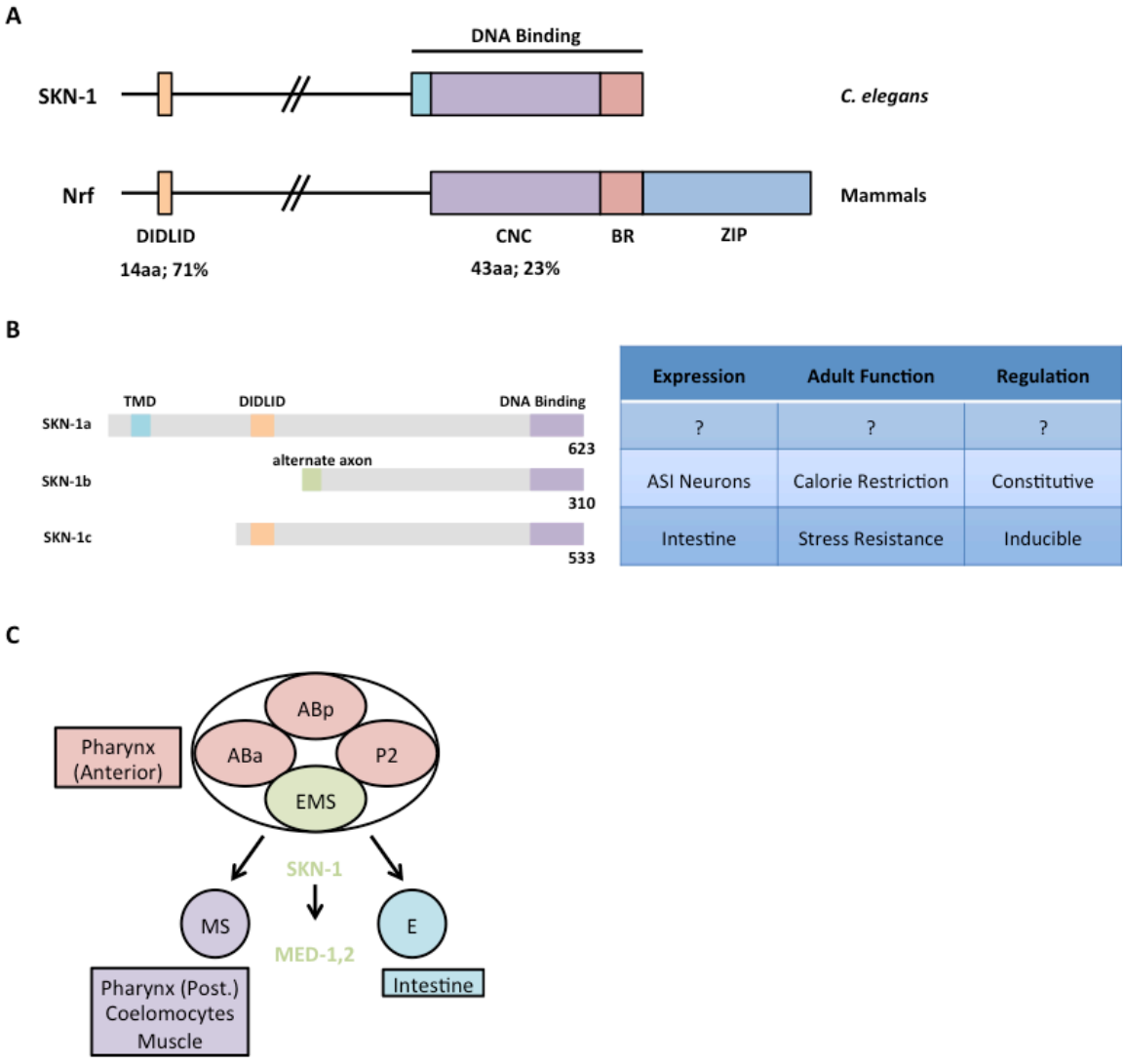

Figure 1.1 *C. elegans* SKN-1 is highly conserved to mammalian Nrf2 and has several essential functions

(A) Schematic figures representing the general structure of *C. elegans* SKN-1 in comparison to mammalian Nrf2. The conserved regions and percent identity are also indicated (CNC: Cap'n'Collar; BR:Binding Region). **(B)** *C. elegans skn-1* encodes three splice variants, each with their own distinct expression pattern and function. **(C)** The embryonic functions of SKN-1 are linked to cell fate specification. At the 4-cell stage of worm embryogenesis, *skn-1* is required to initiate mesododermal development in the EMS blastomere, which will give rise to pharyngeal, muscle and intestinal cells. Adapted and modified from An and Blackwell and Blackwell *et al.* [44, 56].

1.4.1 The Insulin/Insulin-like signaling (IIS) pathway in *C. elegans*

One of the most important and highly conserved pathways across species that regulates stress and aging is the IIS pathway [47]. In the last 20 years, increased attention has been paid to attempt to understand the role and function of the IIS in this context. The pathway was originally identified in *C. elegans* using reverse genetics approaches and it is closely associated with metabolism, development, aging and lifespan. The two main components of the pathway, *daf-2* and *daf-16* [48], encode the mammalian homologues insulin/insulin growth factor (IGF) and forkhead transcription factor FOXO3a, respectively [49]. In light of the evolutionary conservation of IIS, its biological functions and regulation have been expansively studied and experimentation has been conducted in higher organisms such as humans [50]. However, a broad range of evidence has shown that mammals have multiple insulin-like receptors, and this apparent functional redundancy only further complicates the pathway's association with enhanced lifespan, making it a controversial area of research. Therefore simple organisms such as *C. elegans,* although they have multiple insulin like ligands only have one common insulin-like receptor. This makes them ideal for revealing the "big picture" or main role of the IIS pathway in longevity and other pertinent biological processes. This is beneficial to further propagate more focused studies in higher organisms to dissect the components and their links to stress response signaling and survival.

The *C. elegans* Insulin/IGF-1 signaling pathway is required for extended lifespan; under extreme conditions worms will enter into a larval diapause or developmental arrest known as Dauer. Due to its many functional homologous counterparts to humans, worms have been used and manipulated for the study of age-related diseases such as Alzheimer's, Parkinson's, cancer and diabetes. Despite their diversity, these animals

share conservation of the core IIS pathway components (Figure 1.2). Upon cell stimulation or in response to stress, the DAF-2 insulin-like receptor signals to AGE-1/PI3K to activate AKT-1/AKT-2 serine/threonine kinases through binding to a pleckstrin homology (PH) domain. Phosphorylated AKT-1/AKT-2 will then be able to act on its main target, the transcription factor DAF-16/FoxO and inhibits its accumulation into nuclei. The role of DAF-16/FoxO in the DAF-2 pathway and its subsequent involvement in dauer formation and longevity has been well documented in literature *daf-2* or *age-1* mutations display a 2-fold increase in lifespan and induce metabolic changes such as glucose intake or ubiquinone synthesis [48, 51]. Studies have shown that *daf-2* or *age-1* mutations cause worms to undergo an alternate pathway known as Dauer, instead of entering the normal L3 larval stage [52]. Wild type worms exposed to unfavourable conditions such as stress, or food shortages could also induce dauer formation. Dauer worms tend to be more resistant to prolonged stress conditions, due to slowed metabolic rates and high fat storage, resulting in longer lifespans as less energy is being consumed. Promoting IIS-regulation can restore this phenomenon and this enables the worm to exit dauer and resume growth and reach adulthood [52].

A great deal has been done to demonstrate how DAF-2 regulates the nuclear accumulation of DAF-16/FoxO, as well as to identify genes acting downstream of DAF-16 to influence lifespan [53, 54]. Interestingly, recent work carried out by Tullet and colleagues showed DAF-2 is also capable of directly inhibiting SKN-1 in parallel [55]. *daf-2* knockdown also shows an increase in SKN-1 nuclear localization. Furthermore, *in vivo* and *in vitro* analysis also revealed that the DAF-2 downstream kinases AKT-1/AKT-2 phosphorylate SKN-1, suggesting that direct phosphorylation of SKN-1 can alter its

localization. Additionally, a *skn-1* mutation represses the long-lived phenotype of *daf-2(e1370)*, indicating that SKN-1 is essential for lifespan extension. In contrast to DAF-16 Dauer regulation, *skn-1* rescues longevity but not Dauer induction in *daf-16;daf-2* double mutants. This is the first time SKN-1 has been shown to promote longevity through direct regulation by DAF-2/IIS and independently of DAF-16. This novel finding of a DAF-2/SKN-1 pathway in worms provides us with more evidence of the cross-talk between pathways with the same physiology outputs and requires further exploration.

Studies that dampen the insulin-like signaling pathway have already shown that it is a prominent model to slow down aging by allowing DAF-16 to activate dauer-associated mechanisms. Surprisingly, a new study done by Ewald and colleagues discovered reduced IIS can also extend lifespan independently of the dauer pathway, which is through increasing expression of collagen and extracellular matrix (ECM) genes that are mediated by SKN-1 [56]. Ewald *et al*. performed a high-throughput microarray to identify genes that are upregulated by SKN-1 in *daf-2* mutants. The analysis produced a set of collagen genes essential for the formation of the *C. elegans* cuticle, indicating ECM remodeling can be regulated directly or indirectly by SKN-1. However, the possible significance for the reduced IIS mediated mechanism for longevity and for the promotion of collagen maintenance has not yet been determined. In humans, the production of collagen and ECM components plays a vital role in longevity, as they are the main structural proteins in connective tissue. Aging leads to ECM deterioration with devastating consequences, such as increased risk of complications in diabetes sufferers or the onset of kidney diseases. Therefore the investigation of collagen expression in later years is essential for a longer lifespan, as it enhances cuticle structure integrity. Using *C.*

elegans for studies in anti-aging and collagen maintenance has shed light in identifying new processes that influence aging, as well provide significant clues of how changes in collagen expression may affect aging.

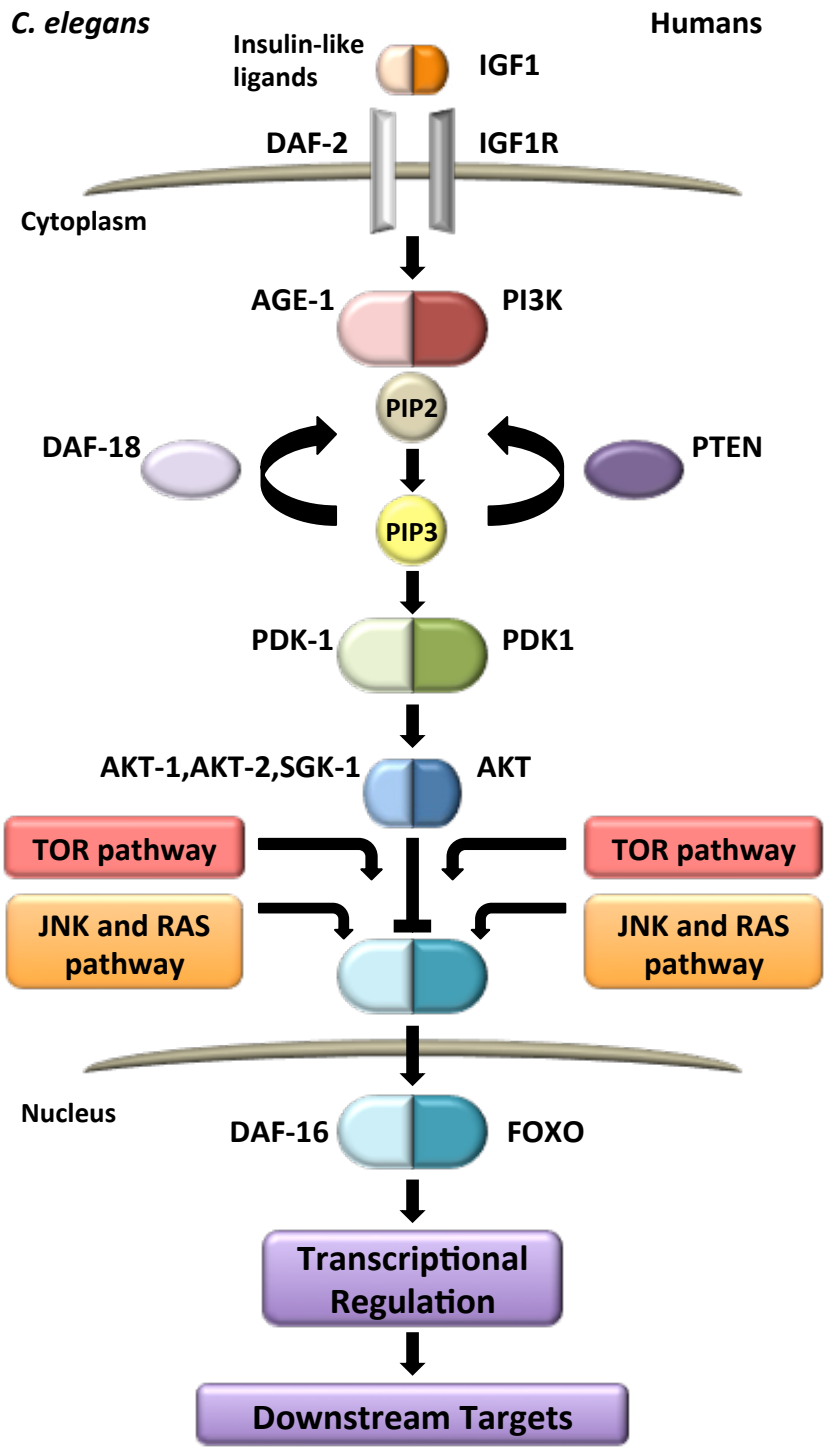

Figure 1.2 Diagram depicting the *C. elegans* DAF-2 pathway and its high conservation to mammalian insulin-like signaling pathway.

Respective components of the organism's pathway are shown down the vertical axis. Adapted from Christensen *et al.* [50].

1.4.2 The mTOR pathway in *C. elegans*

The mechanistic target of rapamycin (mTOR) is a highly conserved, serine/threonine kinase in PI3K/AKT/PKB family pathway that serves as a central regulator in cellular growth and metabolism, proliferation and survival [57]. Deregulation of mTOR in humans has been linked to diseases such as cancer or type II diabetes. mTOR signals through two functionally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Each complex has distinct constituent proteins and regulate different processes downstream of their initial activation, resulting in distinct functions (Figure 1.3). mTORC1 has a well-established role in cell growth and proliferation. Aberrant mTOR signaling is often associated with diseases such as cancer. Loss of this tumour suppressor will reduce PI3K-dependent mTORC1 signaling, and overactive mTORC1 could promote tumourigenesis [57]. The antibiotic rapamycin can bind to and form a complex with the adaptor protein FKBP12 (FK506-binding protein), acting as an allosteric inhibitor of mTORC1. The formation of rapamycin /FKBP12 is believed to disrupt the activation of mTOR pathway by dissociating mTORC1 from its downstream substrates [58]. The inhibition of mTORC1 by rapamycin increases lifespan in species from yeast to mice by reducing the overall rates of protein synthesis. In contrast to mTORC1, relatively little is known about mTORC2. What is known is that mTORC2 does not bind to rapamycin/FKBP12 and is resistant to acute rapamycin treatment. However, prolonged exposure to rapamycin can sequester mTOR and inhibit the assembly of mTORC2 [58]. The signaling pathways that lead to the activation to mTORC2 is not well understood, but current literature has presented that increasing related kinase activity and AKT phosphorylation could promote cell survival, metabolism and proliferation [57-59].

There is growing interest for mTOR and rapamycin in the aging field, since the inhibition of mTORC1 has been shown to extend the lifespans of many model organisms [60]. In *C. elegans,* the connection between mTOR and aging was not clear until Robida-Stubbs and colleagues were the first to discover that the pathway can influence longevity by regulating SKN-1 and DAF-16 [61]. Through genetic and drug analyses, Robida-Stubbs *et al*. showed that inhibiting TORC1 increased the expression of SKN-1/Nrf and DAF-16/FOXO target genes. Furthermore, inhibiting TORC1 activities using rapamycin confers *skn-1*-dependent and *daf-16*-independent longevity, implying that the mTOR pathway is linked to the transcriptional regulation of SKN-1. Using *C. elegans*, Mizunuma and colleagues later illuminated work on the effect of RICT-1/TORC2 on longevity [62]. They provided evidence that loss of *rict-1* indeed influenced aging by inhibiting SGK-1 phosphorylation of SKN-1. Their results suggest that the regulation of SGK-1 by TORC2 regulates stress response and lifespan in one of two ways: TORC2 directly binds to SGK-1 to inhibit SKN-1 localization into intestinal nuclei; or TORC2 activity in neurons is required for SGK-1 to promote longevity at lower temperatures through the TRPA-1/SGK-1 pathway [62, 63]. In data consistent with mammalian studies, the drug rapamycin could reduce TORC1 and TORC2 activities through distinct mechanisms in *C. elegans* to defend against environmental stresses. Since *C. elegans* and mammalian Nrf signaling are highly conserved, the fundamental TOR mechanisms uncovered in *C. elegans* would strongly provide some hint that Nrf2 may also mediate longevity in humans by reducing mTOR activity, and this knowledge will in-turn be beneficial for future pharmaceutical studies and drug design.

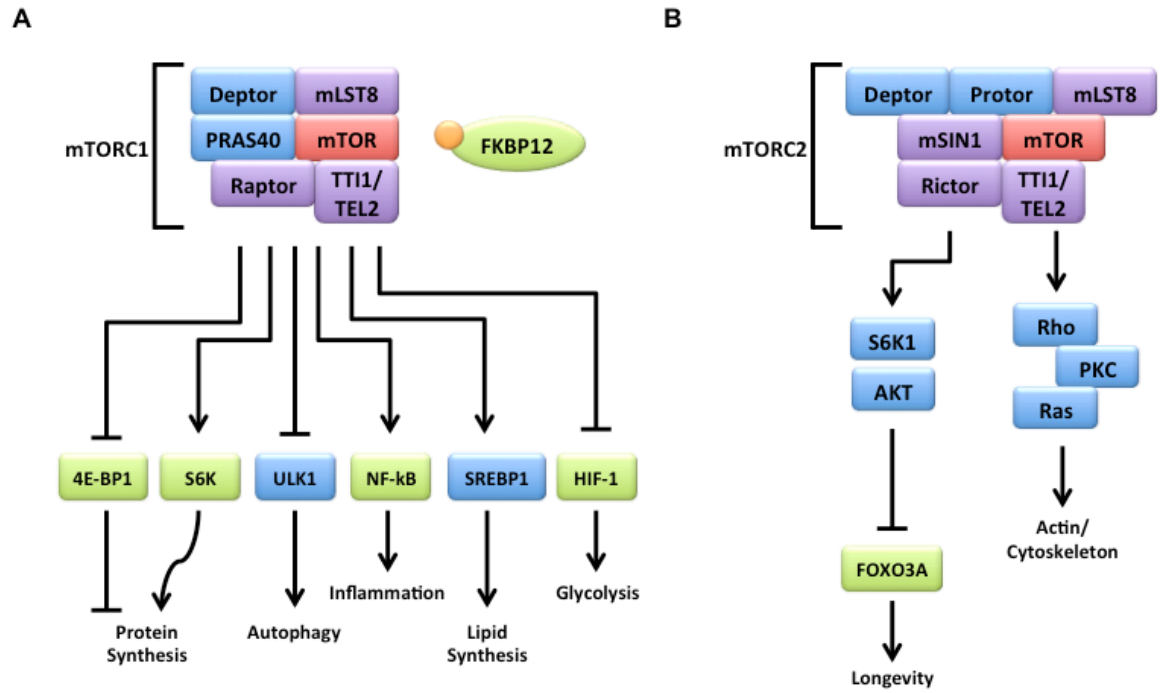

Figure 1.3 Pathways of the mammalian mTOR1 and mTOR2 complex (also known as mTORC1 and mTORC2) components and their distinct downstream targets and cellular processes.

mTORC1 and mTORC2 have their own distinct constituent proteins to activate a specific subset of downstream effectors. **(A)** mTORC1 has a known roles in several diverse cellular activities from protein synthesis to glycolysis. Inhibtion of mTORC1 occurs when the antibiotic rapamycin binds to the protein FKBP12, a complex which will interrupt the interaction between mTOR and raptor. **(B)** Contributions of mTORC2 in cellular physiology has not been fully elucidated. There is no evidence showing its activities can be affected by direct binding of rapamycin/FKBP12. However, chronic exposure to rapamycin can sequester and prevent mTORC2 activities.

1.4.3 The MAPK pathways in *C. elegans*

The Mitogenic-activated protein kinase (MAPK) superfamily is a highly conserved signal cascade essential to the functioning of vital biological processes such as cell differentiation and proliferation, stress response, apoptosis and survival. The MAPK pathways can be initiated transiently in response to extracellular stimuli. The basic assembly of the signaling cascade consists of three components that are necessary for sequential phosphorylation events: MAPKKK, MAPKK and MAPK [64]. MAPKKK is typically activated by a small GTPase, which then directly phosphorylates and activates its downstream kinase MAPKK, and thereby activates MAPK through dual phosphorylation of both threonine and tyrosine residues within a conserved Thr-X-Tyr motif. The activation of MAPK can lead to phosphorylation of downstream substrates such as transcription factors for the execution of an appropriate biological response. Regardless of differences across species, eukaryotes all follow the core module of the MAPK cascade. In *C. elegans*, two MAPK pathways have been characterized, and each pathway is named for a particular MAPK that activates transcription directly: the p38/MAPK pathway - important for regulating stress and innate immunity; and the ERK/MAPK pathway – involved in vulval development and stress response (Figure 1.4) [65].

p38 MAP kinase pathway

Similar to mammals, the *C. elegans* p38 MAPK pathway has significant roles in neuronal development, immune response and the oxidative stress response [66-69]. The p38 homolog *pmk-1*, has been extensively studied to determine its role in pathogen resistance [68]. Regulation of *C. elegans* p38 MAPK pathway is carried out by SEK-

1/MAPKK and NSY-1/MAPKKK [66, 67]. More recently, there has been increased interest to understanding the underlying mechanism of *pmk-1* in stress response through SKN-1. Inoue and colleagues were the first group to introduce the p38 model system for stress-induced signal transduction [68]. They found that the deletion of *sek-1* reduced endogenous PMK-1 protein levels and GCS-1::GFP expression. In addition, PMK-1 is no longer able to directly phosphorylate SKN-1 when the SKN-1 phosphorylation sites Ser164 and Ser430 were mutated. Taken together, it was proposed that after exposure to ROS, the immune-related NSY-1/SEK-1/PMK-1 pathway will be activated to phosphorylate SKN-1, and subsequently influence SKN-1 nuclear localization to induce the activation of stress genes. However, the p38 MAPK pathway is a receptor independent pathway, and how ROS directly activates the p38 MAPK cascade in *C. elegans* is not well defined. Research in mammals looking at the mechanism of how ROS induces the activation of MAPK pathway suggests that direct exposure of ROS causes oxidative modification of intracellular kinases, and this may be applicable to *C. elegans*.

ERK1/2 MAP kinase pathway

Among the known MAPK signaling networks, the ERK pathway is the best defined. MAP kinases play an important role in the Ras signaling pathway, as they are essential in activating downstream substrates through phosphorylation. Mutations in mammalian MAP kinases can cause severe syndromes such as Noonan syndrome or Costello syndrome and various types of cancers [70-72]. In *C. elegans*, ERK/MAPK is also linked to the Ras pathway, and it was first shown to have a role in vulva induction and germline development [73]. Studies showed that constitutively active LET-60/Ras in the worm could cause the development of multiple vulvas [74, 75]. This abnormal vulval

development can be rescued by suppressing MAPK activity through *mpk-1* deletion worms. Genetic analysis also indicated that *mpk-1* null mutants exhibit a vulvaless phenotype [76]. This suggests that the regulation of MAPK activity needs to be tightly regulated for normal development.

Although several articles have been published showing the importance of Ras/MAPK pathway in mammalian aging, there has been a lesser focus within the *C. elegans* research community on this topic [77]. In a recent study, Okuyama *et al*. identified a novel pathway presenting that ERK/MAPK regulates longevity and oxidative stress through SKN-1 [78]. Phosphorylation of SKN-1 on key sites by MPK-1 is required for SKN-1 nuclear accumulation. RNAi and drug treatment analysis using N2 worms was also performed to knockdown *mpk-1* and *mek-1* function and measure the average lifespan of these animals. The loss of either gene shortened the lifespan compared to the control, indicating that the ERK/MAPK cascade is necessary to promote longevity. This same group also defined the upstream pathway for ERK/MAPK longevity regulation. Reducing the activity of DAF-2/insulin-like signaling pathway has proven to extend lifespan in *C. elegans*. Therefore lifespan analysis was conducted in *daf-2(e1370)* mutants treated with *mpk-1* and *skn-1* knockdown using RNAi, and in contrast to Tullet *et al.* [55], no lifespan reduction was observed. In contrast, they showed that SKN-1 could promote longevity through the transcriptional repression of insulin-like genes such as *ins-39* and *daf-28*. A positive feedback loop consisting of increased SKN-1 by MAP Kinase activity has proven that lifespan extension in *C. elegans* is partially mediated by the DAF-2/DAF-16 pathway.

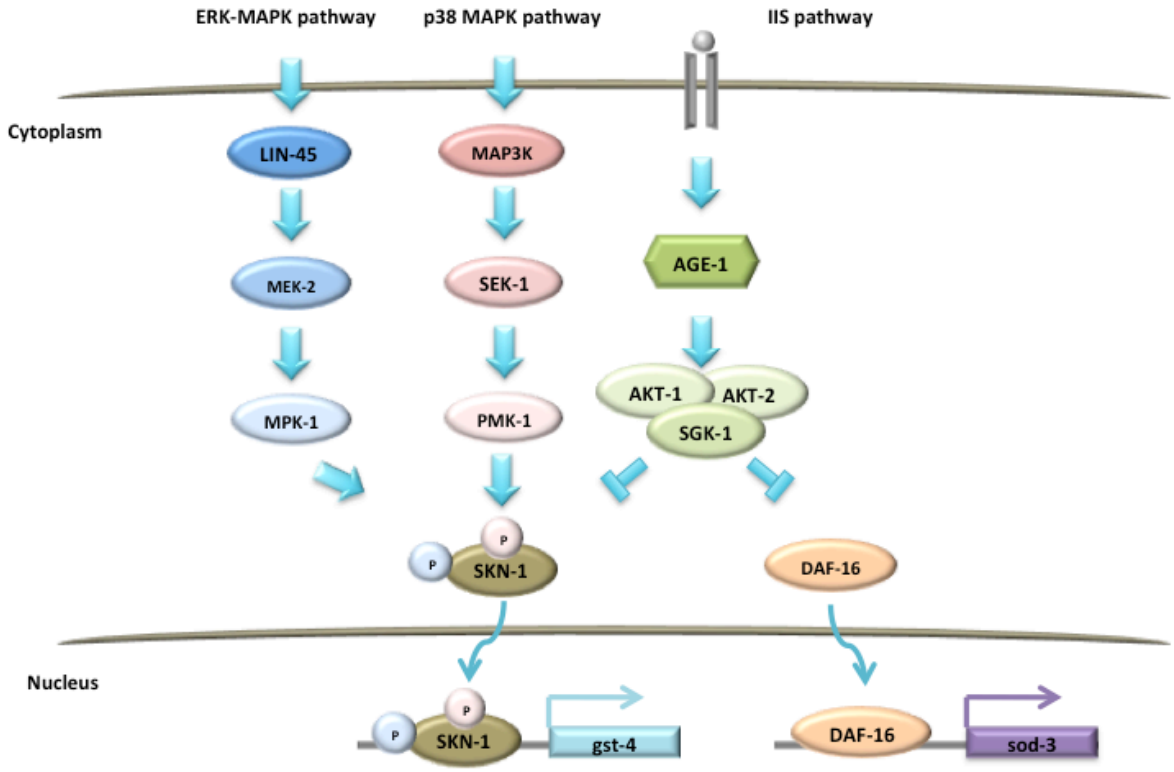

Figure 1.4 Schematic representing the *C. elegans* ERK/MAPK, p38 MAPK and DAF-2 insulin-like pathways in regulating SKN-1

Respective components of each stress pathway in *C. elegans* are shown down the vertical axis. Adapted and modified from Inoue *et al.*, Tullet *et al.* and Okuyama *et al.* [55, 68, 78].

1.5 GATA: The pioneer transcription factor

Eukaryotic chromosomal DNA is organized and condensed in the form of chromatin and safely packaged into nuclei. The condensed DNA is comprised of eight histones that form a nucleosome. In order to initiate the transcriptional process, DNA must be "untangled" for TFs to traverse the highly packaged DNA and locate their target sequences. This process of "untanglement" is known as histone modification and chromatin remodeling. A subset of eukaryotic TFs that have the unique ability to remodel chromatin structure just enough to initiate transcriptional events in closed chromatin were identified and named "pioneer transcription factors". Pioneer factors are a class of TFs that play a primary role in cell programming during eukaryotic embryonic development [79]. Genetics and biochemical studies have suggested that these factors have the ability to target silent chromatin before histone H3K4 methlyation. Pioneer factors can be characterized into two categories or roles: active or passive [80, 81]. The active role of pioneer factors are first to function to help carefully open up the local chromatin, and allow for the recruitment of other TFs, chromatin or nucleosome remodelers. In contrast, the passive role of pioneer factors has a major role in tissue-specific expression or embryogenesis. They are able to bind to condensed chromatin along with an enhancer to form a pre-initiation complex for a rapid transcriptional response [80]. The GATA4 protein was the earliest identified pioneer factors in mammals [82, 83]. The discovery of this unique property has provided successive steps towards the study of cell differentiation and organogenesis. It is anticipated that understanding the fundamental role and mechanism of pioneer factors will lead to prospective therapeutic targets and applications for treatment of diseases associated with cancers.

ELT-3: The *C. elegans* GATA transcription factor that is essential for embryonic development and aging regulation

The *C. elegans* ELT (Erythroid-like transcription factor family) was first identified as a homolog of the GATA family. These factors contain a single GATA-type zinc finger that specifically binds to the DNA sequence (A/T)GATA(A/G) in the regulatory or promoter regions of target genes [84]. Comparative genetic analysis of DNA binding domains revealed that the *C. elegans elt-1, -2* and *-3* are highly conserved to vertebrate GATA-1 and -4 [85]. Additionally, subsequent work has suggested both the vertebrate and *C. elegans* GATA factors are involved in cell differentiation and lineage-specific gene expression. The vertebrate GATA-1, - 2 and -3 have known roles in differentiation of specific haematopoietic cell lineages [86, 87], whereas GATA-4, -5 and -6 are involved in gut, liver and cardiac development [88-90]. In *C. elegans*, the GATA factors were initially shown to be essential in hypodermal differentiation and endodermal development, demonstrating their functional conservation across species.

Among the identified GATA factors in *C. elegans*, *elt-1* and *elt-3* have been extensively studied and have been found to be required for the activation of hypodermal differentiation during embryonic development, and are highly expressed in the embryonic hypodermis (epidermal precursor) [91]. A DNA microarray analysis conducted by Budovskaya and colleagues identified that the GATA factors ELT-3, ELT-5 and ELT-6 are responsible the regulation of aging [92]. They also reported a new role for ELT-3, as a potential regulator for adult lifespan, where it is functionally important in changing the expression of target somatic aging genes such as *sod-3*. However, this hypothesis was criticized and re-evaluated by Tonsaker *et al*. who showed that *elt-3* was not expressed to a significant degree in the worm intestine, a major organ for digestion and metabolism

[93-96]. Interestingly, studies have suggested that GATA TFs could work with other DNA binding proteins or co-activators in a combinatorial fashion to establish specific patterns of gene expression [79, 97]. Gilleard and McGhee showed that ELT-3 is expressed in the *C. elegans* digestive tract in adulthood [85]. Thus, it is not surprising ELT-3 might also act as a pioneer factor in the worm intestine and potentially able to interact with other TFs or enhancers for promoting longevity. Further investigation is thus needed to provide further insight into the role ELT-3 may play in aging and stress response.

1.6 Nuclear hormone receptor and gene expression

The eukaryotic transcriptional machinery is crucial for development and survival, as it is a process required for execution and implementation of the genetic information stored in DNA. The mammalian nuclear hormone receptor (NHR) family is a group of ligand-activated TFs that require steroid hormones or lipid-soluble signals for its activation and subsequent modulation of cellular functions [98]. These TFs are shown to impact particularly the regulation of lipid metabolism, innate immunity and stress response. To transcribe and enhance the transcriptional activity of target genes, the NHR family TFs often employ a co-regulator that acts as a bridge to form a regulatory complex between them and RNA polymerase at the promoter element [99]. The *C. elegans* genome encodes 284 putative NHR-related genes, with only 50 NHRs having detectable phenotypes and even fewer still have been well studied [100, 101]. In spite of this, their identified functions involve in a wide range of biological processes from development, cell fate specification, to nutrient sensing and fat metabolism. Although there is a distant relationship to mammals, the unveiling of these highly conserved TFs in nematodes has

offered us a more refined focus and a powerful window into the NHR-regulated transcriptional network.

The *C. elegans* nuclear hormone receptor NHR-49 and its mediator MDT-15 – Regulation from fat metabolism to oxidative stress response

A growing number of studies show lipid biology and dietary restrictions including fasting are factors that influence aging. Pathways that govern longevity control would affect storage lipid levels, thus changing the balance of energy to prolong lifespan. *C. elegans* NHR-49 encodes the mammalian Hepatocyte Nuclear Factor 4α (HNF4α), which has a functional homolog of vertebrate Peroxisome Proliferator-Activated Receptor (PPARα) that plays essential roles in energy metabolism and regulating fatty acid composition. Previous work has proven that *nhr-49* also has functions in body fat metabolism, where depletion of *nhr-49* will result in an impaired response to fasting and shorter lifespan due to an elevated ratio of saturated to mono-unsaturated fatty acids, leading to lipo-toxicity in these animals [102, 103]. PPARα function is engaged as a transcriptional co-activator to mediate metabolic and biogenic responses. However, conserved mediators remained obscure in *C. elegans* [104]. For this reason, Taubert *et al*. conducted an Y2H screen in order to search for potential co-regulators of NHR-49 involved in energy homeostasis, and the screen resulted in 6 isolated clones representing full-length MDT-15/MED15 (mediator subunit). Knockdown of *mdt-15* altered downstream gene expression and membrane lipid composition. There is evidence that MED15 regulates sterol responsive gene expression, and this regulation is implied to have conserved biological function to the NHR-49/MDT-15 interaction, suggesting MDT-15 can act as a co-activator of NHR-49 for lipid synthesis [105]. In addition, studies prove *mdt-15* and *nhr-49* have a role in fatty-acid desaturation, but are also

required for expressing β-oxidation genes in response to fasting (Figure 1.5). A recent publication showed that the activation of select fasting responsive genes are *nhr-49*-in dependent, and can only be stimulated through an MDT-15 to SKN-1 interaction [106, 107]. These studies not only indicate that *mdt-15* is needed to express genes required for lipid breakdown and synthesis, but also open a new window for SKN-1 study, with a physiological role in fat metabolism.

Besides its role in lipid breakdown and synthesis, MDT-15 is also involved in stress response. There are a number of identified putative xenobiotic detoxification genes whose activation is dependent on *mdt-15* expression [108]. In a recent study done by Goh and colleagues, the role of *mdt-15* in adult worm stress response to arsenite was also explored [109], and they found that the *mdt-15(tm2181)* mutant is sensitive to exogenous oxidative stress. Thus *mdt-15* is required to induce anti-oxidative gene expression for survival. In addition, they showed MDT-15 physically interacts with SKN-1 via an uncharacterized region of MDT-15 using an Y2H assay. Co-incidentally, this SKN-1/MDT-15 interacting region contributes to NHR-49 binding. RNAi analysis also indicated MDT-15 is required for the induction of SKN-1 target genes. These experiments carried out by Goh *et al*. suggest that MDT-15 has a new role to co-regulate SKN-1 in the regulation of stress and aging. However, Taubert *et al.* showed that *nhr-49* and *mdt-15* are required for inducing the oxidative response to tert-butyl hydroperoxide (tBOOH) in a *skn-1*-independent manner [109, 110]. Whether the role of MDT-15/SKN-1 in promoting lifespan is parallel to MDT-15/NHR-49 dependent lipid signaling is a question that needs to be addressed. SKN-1 may not be the only TF co-regulated by MDT-15 for stress response, and this study also proposed MDT-15 might contribute to

other transcriptional responses to oxidative stress with multiple TFs. Since NHR-49 has shown to regulate lifespan through lipid metabolism and it has also been identified as an interacting partner of MDT-15, NHR-49 could be a potential candidate for SKN-1/MDT-15 regulation, and would be interesting to pursue its role in regulating stress genes.

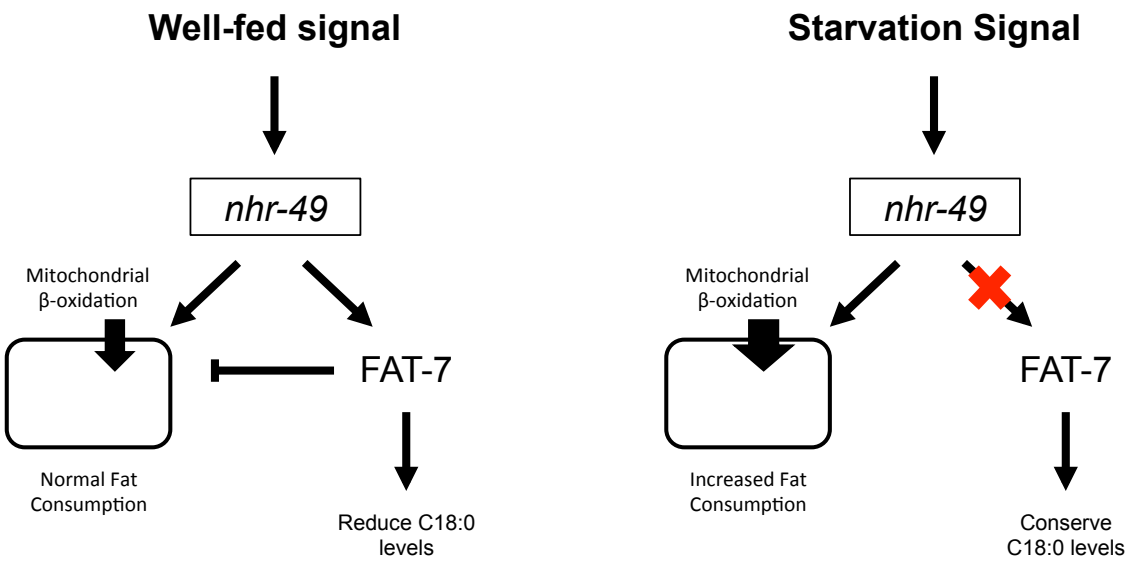

Figure 1.5 Model illustrating the role of *nhr-49* in regulating fat metabolism dependent on food availability.

nhr-49 is the key component in regulating multiple aspects of nutritional response. **Left Panel:** When worms are at fed stage, *nhr-49* stimulates the activation of mitochondrial fatty acid β-oxidative genes such as *acs-2* (fatty acid Co-A synthetase family) or *gei-7* (also known as *icl-1* isocitrate lyase homolog) to promote fat breakdown. *nhr-49* also regulates fatty acid desaturation through the activation of *fat-7* (fatty acid desaturase) to enhance the conversion of stearic acid (C18:0 saturated fatty acid) into oleic acid (unsaturated fatty acid) in preventing the excess accumulation of saturated fats. **Right Panel:** In contrast, during fasting condition, *nhr-49* will strongly induce the mitochondrial β-oxidative genes and increase the mobilization of fat for energy production. Moreover, *nhr-49* will also inhibit the activation of *fat-7* for conservation of stearic acid. Adapted and modified from Van Gilst *et al.* 2005 [102].

1.7 Current understanding of Brap2/IMP and its diverse role

The nucleocytoplasmic trafficking of protein molecules in eukaryotes is tightly regulated, as it is essential for all aspects of cellular physiology including maintaining cell integrity, regulating cell differentiation and development. As such, substantial studies have been conducted in order to understand and dissect the different pathways and the protein factors that regulate them. Most macromolecules greater than 40-60 kDa require a nuclear localization signal (NLS) motif for transportation between cytoplasm and the nucleus [111]. Misregulation of these proteins between these compartments may lead to a wide range of disorders in humans. One recently characterized cytoplasmic retention factor is BRAP2 (also known as IMP for Impedes Mitogenic Signal Propagation). BRAP2 (Brca1 associated binding protein 2) was first identified by Li *et al*. through a Y2H screen [112]. They showed that BRAP2 serves as a cytoplasmic retention protein that recognizes the NLS motif of the tumour suppressor gene BRCA1 (Breast Cancer), preventing its movement into the nucleus [113]. In general, the aberrant subcellular localization of tumour suppressors in general can increase the incidence of cancer.

Since the initial discovery of BRAP2 as a cytoplasmic retention protein, research efforts were dedicated to understanding the biological role of BRAP2, leading to a substantial amount of literature being published on the subject. The C-terminal region of BRAP2 has also been shown to associate with the NLS of other cargo proteins such as the cell cycle inhibitor p21 during monocyte differentiation [114]; and interaction with viral proteins including SV40 large T antigen or human papilloma virus proteins [37]. BRAP2 also seems to play a role in the regulation of the centrosome cycle, mitosis and cytokinesis through binding to the protein phosphatase Cdc14 [115]. The interaction of

BRAP2 with all these proteins leads to their retention in the cytoplasm. Thus, the mis-regulation of BRAP2 may lead to human diseases associated with cancer or inflammatory dysfunction of the cardiovascular system. BRAP2 is also known to be a novel regulator that mediates the NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells) to enhance its nuclear translation through the inflammatory response [116].

Recently, BRAP2 was shown to express in the testis more abundantly than in other tissues in humans, and it is therefore believed that BRAP2 has a potential role in testicular development [117]. This leads to research groups to establish a human testis cDNA library in order to gain a better understanding of the BRAP2 interactome. Accordingly, a Y2H screen identified BRAP2 interacting protein partners which has recently been published, includes a wide variety of proteins involved in formation of the actin cytoskeleton, and processes of ubiquitinylation, cell cycle/apoptosis, and transcription. Davies and colleagues validated that BRAP2 interacts with nuclear proteins HMG20A and NuMA1 in mice, resulting in cytoplasmic retention of the two proteins and prevention of nuclear localization during spermatogenesis. Further study done by the same group provided evidence with both *in vitro* and *in vivo* analyses, that BRAP2 contributes to localization of certain proteins to specific areas in the later stage of sperm development. It is the first time suggesting BRAP2's role is not only as a cytoplasmic retention protein, but it is also potentially a scaffold protein to anchor proteins and support their interacting partners [118]. BRAP2 has shown to have a vital role in modeling subcellular localization of a range of testicular proteins during spermatogenesis. These findings propose a wider scope for BRAP2 function, and the characterization of new interacting partners now provides a more solid framework for

how two proteins previously believed to be can function together for cell signaling regulation.

BRAP2/IMP in the Ras signaling pathway

The Ras signaling pathway has been characterized as an essential signaling network in controlling cellular proliferation, development and survival. The pathway and its associated proteins have been extensively studied, as experimental observations have shown that the Ras pathway is mutually hyper-activated in tumours and could lead to the development of cancer. Therefore, a highly regulated system is needed to coordinate these events. Ras is a membrane-bound guanine nucleotide-binding protein and acts as a molecular "switch" upon cell stimulation. The chain of events for Ras activation begins with a mitogen signal binding to a tyrosine kinase receptor, followed by the phosphorylation of SOS (Son of sevenless) through the adaptor protein Grb2 (Growth factor receptor-bound protein 2). Once SOS-Grb2 is recruited to the receptor, the complex will be brought into close proximity with Ras proteins. In quiescent cells, Ras is found to bind to GDP (Guanine diphosphate) and the upstream stimulatory signal can trigger Ras activation by GEF (Guanine nucleotide exchange factor) for signaling configuration. When the signal has been transmitted, GTP (Guanine triphosphate) is hydrolyzed back to GDP by the GTPase-activating protein (GAP) for inactivation (Figure 1.6).

Recently, BRAP2/IMP was found to be a Ras effector protein and E3 ligase. Structural analysis indicates BRAP2 contains Zinc Finger and RING Finger domains, two components that are necessary to facilitate ubiquitination [112, 119]. As described by Matheny *et al*., BRAP2/IMP has an important role as an "ON/OFF" switch in the Ras

signaling cascade [120]. It can bind to the scaffold protein KSR1 and postulated to negatively regulate the ERK/MAPK pathway by limiting the formation of RAF-MEK in quiescence cells; or BRAP2 may serve as an E3 ubiquitin ligase that can trigger self-degradation when Ras is activated upon cell stimulation, thereby modulates the RAF-MEK-ERK kinase cascade and regulates downstream nuclear TFs or signaling proteins. This mechanism of controlling the activation intensity of the Raf kinase family could affect cell fate [121, 122]. The observation suggests that BRAP2/IMP functions as a modulator of the Ras signaling pathway. While some properties of BRAP2 have been described, its biological significance is less well understood. Therefore, it is likely that further studies are required to grasp the full scope and understanding of BRAP2.

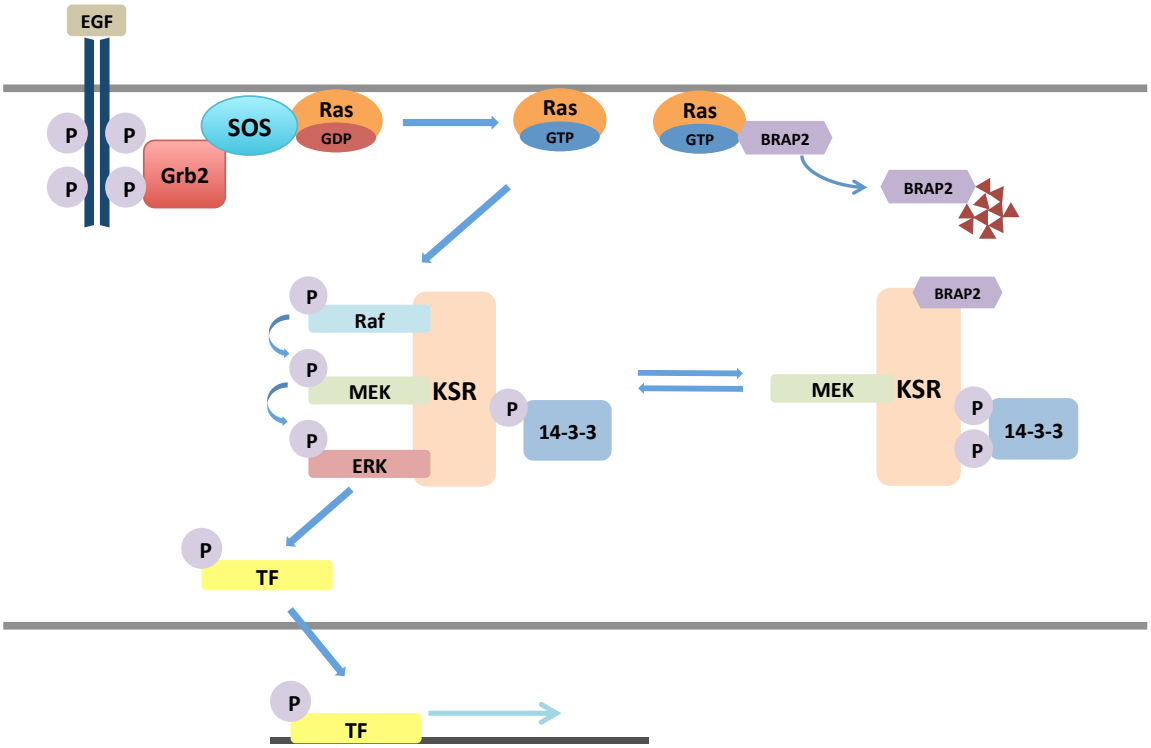

Figure 1.6 Illustration of the known role of mammalian BRAP2/IMP in the Ras signaling pathway

The induction of Ras signaling causes Ras-GDP to become active in the form of Ras-GTP, which allows it to bind to the BRAP2/KSR complex. The binding of Ras-GTP to BRAP2 triggers BRAP2 auto-ubiquitylation and degradation, thereby releasing KSR from the BRAP2/KSR complex. Free KSR will activate downstream kinases. Activated KSR initiates the activation of the MAP kinase cascade, which can phosphorylate substrates such as transcription factors and increases their nuclear localization. Adapted and modified from Kolch [119].

1.8 Objective and Hypothesis

BRAP2 has not been well established and knowledge of its function in *C. elegans* is limited. In *C. elegans*, the *brap-2* gene sequence was analyzed and deletion sites were identified [10]. The recognized domains present in BRAP-2 consists of conserved RING and Zinc Fingers, followed by a leucine heptad repeats, a coiled coil structural motif which is necessary for homodimerization and involved in major biological functions such as gene expression. The C-terminal region consists of a low complexity region, which has recently been shown to have a role in stress responses (Figure 1.7). Koon and Kubiseski also characterized a mutated allele *brap-2(ok1492)*, which has a deletion of 4 introns and exons in the C-terminal region (Figure 1.8). When exposed to oxidative stress these mutant worms showed developmental arrest at L1 larval stage or caused lethality. The *C. elegans brap-2* gene is highly conserved to its mammalian counterpart, and it also shares the same operon as the human homolog to PINK1 (PTEN-Induced Kinase), known as PINK-1 (Figure 1.9). In mammals, PINK1 expression is associated with an early onset of Parkinson's disease (PD). Studies have shown that accumulation of ROS in dopaminergic neurons can lead to chronic neuroinflammation, resulting in the progression of PD. Since genes in the same operon tend to share the same biological functions and pathways, it is fair to hypothesize that BRAP-2 may have a crucial role for oxidative stress regulation in *C. elegans*.

Although we have shown that *brap-2(ok1492)* is important in responding to stress, its role and biological significance is not yet well understood. Furthermore, we have detected an increase in expression of the SKN-1/Nrf2 regulated gene *gst-4*, in the absence of *brap-2*. At this stage of the investigation, we aim to identify a precise mechanism for

this potential BRAP-2/SKN-1 pathway, using genetics and biochemical techniques to determine the regulatory function for BRAP-2 and the induction of SKN-1 target genes in *brap-2(ok1492)* mutant. According to previous studies, BRAP2 in mammalian systems inhibits the Ras signaling cascade through KSR, a scaffold protein that is needed to phosphorylate the MAPK downstream kinases [120]. While another study revealed a novel pathway in *C. elegans*, where the ERK-MAPK regulates longevity where MPK-1 (MAPK) kinase is necessary to phosphorylate SKN-1 for nuclear accumulation [68]. Based on these findings, we hypothesized that BRAP-2 can regulate SKN-1 and its target genes through the Ras signaling pathway and the MAPK cascade.

In deciphering signaling events, research investigations often employ high throughput studies to dissect the genes important to stress response and the ways in which they are regulated. One way to attempt this is to identify TFs that work in combination with target genes of interest. Thus, our study took advantage of such high throughput technology and utilized a transcription factor RNAi screen to characterize novel regulators or co-regulators of SKN-1 that may participate in BRAP-2 regulation. The *C. elegans* TF RNAi library consists of 940 clones; using a *brap-2(ok1492);gst-4p::gfp* transgenic worm we were able to identify 20 new candidates. In this study ELT-3 and NHR-49 were chosen for further investigation into their roles and importance in the regulation of stress response and prolonging lifespan. The significance of this project is to discover novel pathways and how the newly identified roles of TFs could function together with SKN-1 in stress genes regulation, through which future work on this subject may then in turn provide potential new targets for therapeutic drugs for treatment of

debilitating diseases caused by the dysregulation and improper function of these signaling pathways.

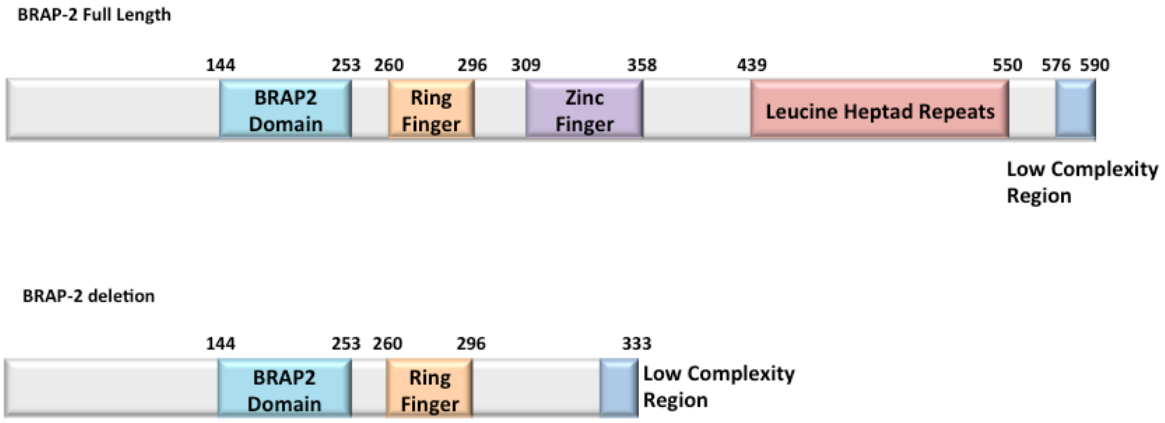

Figure 1.7 Structures of *C. elegans* BRAP-2 and the BRAP-2 deletion mutant

The major domains of BRAP-2 are shown. *C. elegans* BRAP-2 contains a BRAP-2 domain (residue 144 to 253) in the N-terminal region, which has been shown to permit binding to nuclear localization signals motifs of proteins such as BRCA1. The Ring Finger (residues 260 to 296) and Zinc Finger (residue 309 to 358) domains confer E3 ubiquitin protein ligase activity. Leucine heptad repeats located in the C-terminal (residue 439 to 550), is a coiled coil structural motif that is necessary for homodimerization, and is involved in major biological functions such as gene expression. A low complexity region (residue 576 to 590) is also present and is predicted to be involved in the translation of stress response-related genes. The BRAP-2 mutant, at 333 amino acids has a partial deletion in the C-terminal portion (residue 319 to 578), giving rise to an in-frame deletion that retained residues 1-318 in the N-terminal region and residues 579-590 in the C-terminal region.

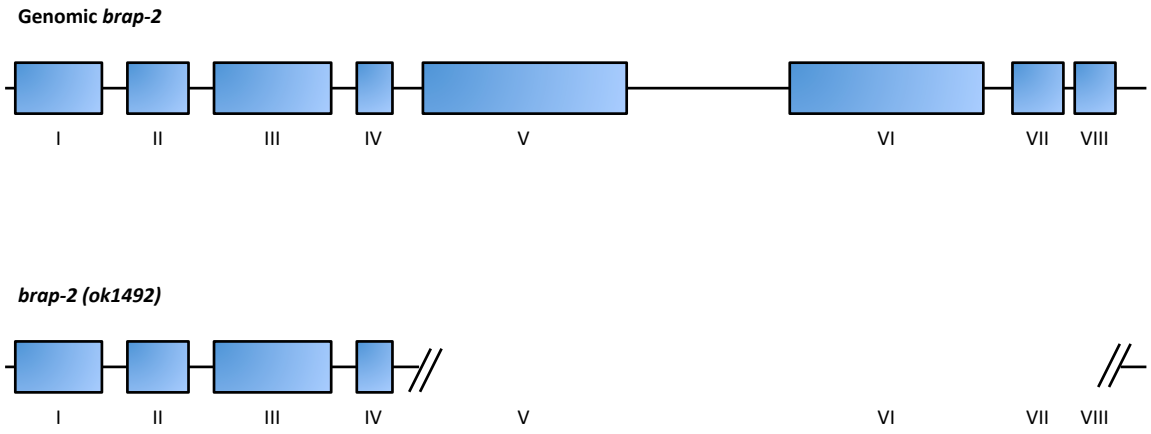

Figure 1.8 Schematic genomic illustration of the *brap-2* full length gene, and the deletion *brap-2(ok1492)*

Top Panel: *brap-2* in *C. elegans* is located on chromosome 2 and consists of 8 exons, with a transcript length of 2963 bp. The coding sequence length is 1773 bp which translates into a 590 aa protein. **Bottom Panel:** The *brap-2* mutant used in this study is called *brap-2(ok1492)*. The allele *ok1492* has 1540 bp deletion that spans from exon 5 to 8, leaving 15 bp intact in the C-terminal region.

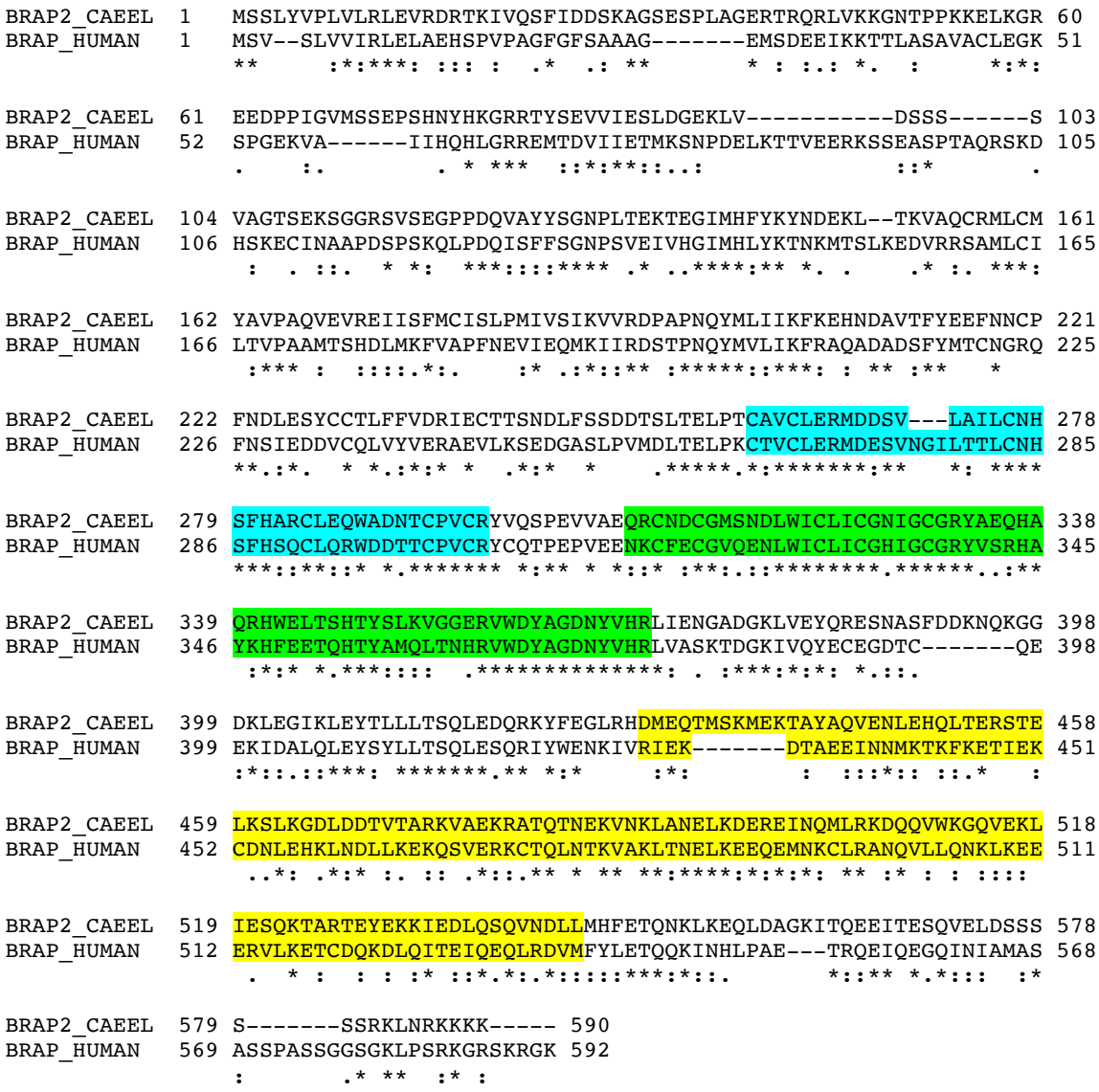

Figure 1.9 Comparative analysis of the BRAP-2/BRAP2 protein sequences in *C. elegans* and *H. sapiens*.

Multiple Sequence Alignment of *C. elegans* BRAP-2 and Human BRAP2 protein sequences was performed using ClustalW. Alignment of conserved residues were compared and highlighted in various colours. The percent homology of each protein domain was derived by using BlastP. Colour indication: Blue – Ring Finger (80%), Green – Zinc Finger (81%), Yellow – Coiled-coil Region (58%).

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Chapter 2

Caenorhabditis elegans Brap2 regulates SKN-1/ELT-3 in response to oxidative stress through the ERK/MAPK pathway

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Manuscript in preparation

All figures and the data presented in the manuscript are mostly my own efforts with the following exception. Lesley T. MacNeil and Marian Walhout performed the RNAi screen as shown in Table 2. Dayana R. D'Amora assisted in designing and carried out worm lysates western blot, antibody staining and worm gonad dissection experiments presented in Figures 2.5C, D and Figures S2.1A, B.

Terrance J. Kubiseski, Dayana R. D'Amora and I are responsible for drafting the manuscript.

2.1 Summary

Cellular damage caused by reactive oxygen species (ROS) is believed to be a major contributor to detrimental diseases associated with aging, therefore eukaryotes have developed defense mechanisms to protect themselves from these stressors. The Nrf2 transcription factor is a conserved master regulator of antioxidant responses among species. The *C. elegans* Nrf2 homolog SKN-1, is essential and involved in various aspects of organismal development, longevity and stress resistance. However, the molecular mechanisms of SKN-1 and its upstream regulation remain poorly understood. We have used both genetic and biochemistry approaches in order to identify genes and discover novel regulatory components that function in the SKN-1/Nrf2 detoxification pathway. Here we show that the *brap-2(ok1492)* mutant displays an enhanced expression of the SKN-1 dependent gene *gst-4* in the intestine and hypodermis, indicating that functional BRAP-2 may act as a potential inhibitor of SKN-1. It was observed that the loss of BRAP-2 increases p-ERK1/2 protein activity *in vivo*. In addition, BRAP-2 physically interacts with LET-60/Ras and KSR-2 *in vitro*, suggesting that the MAPK pathway is involved in BRAP-2/SKN-1 regulation. A subsequent RNAi screen was also conducted using a transcription factor library to identify proteins required for the enhanced *gst-4p::gfp* expression in the *brap-2(ok1492)* strain. From this screen, *elt-3* was identified as a potential candidate to promote this biological effect in the SKN-1/Nrf2 signaling pathway. Our results show that ELT-3/GATA is required for enhanced *gst-4* expression in the *brap-2(ok1492)* mutant, through binding to SKN-1. Further analysis also revealed the activation of *gst-4* by SKN-1 requires ELT-3, indicating the two transcription factors may dimerize to increase *gst-4* expression and transcriptional

activity. Lastly, our results also indicate that lifespan extension observed in overexpressing SKN-1 is dependent on functional ELT-3. Taken together, these results suggest a model where BRAP-2 acts as negative regulator of SKN-1 through inhibition of KSR and MAPK activity, and that ELT-3 along with SKN-1, is required for *gst-4* expression.

2.2 Introduction

Reactive oxygen species (ROS) are harmful molecules that can cause damage to a variety of cellular components and are believed to contribute to the development of detrimental diseases associated with aging such as Alzheimer's or Parkinson's. The overproduction or low levels of these oxygen derivatives can lead to extensive tissue damage, and have the ability to cause cell impairment and even death. In order to cope with changes in ROS levels, maintain homeostasis, and cell survival organisms possess a sophisticated defense mechanism that induces the transcriptional expression of detoxification enzymes that remove excessive oxygen radicals. The most prominent and well-studied transcription factors involved in the regulation of the mammalian stress response are FOXO and the NF-E2-related factor (Nrf2), which are responsible for detoxifying stress compounds and promoting longevity [1-3].

Nrf2 is evolutionarily conserved and is present in a number of invertebrates, including *C. elegans*, where it is known as SKN-1 [4]. SKN-1 was initially identified as the transcription factor that initiates endoderm specification during embryogenesis [5, 6]. However, like its ortholog in humans, SKN-1 also functions in regulating the expression of the phase II detoxification response in *C. elegans* [7]. In the intestine, which is the main detoxification organ in *C. elegans,* SKN-1 accumulates in nuclei and activates target genes in response to environmental stresses. Genes up regulated by activated SKN-1 include glutathione-S-transferase 4 (GST-4) and NAD(P)H: quinone oxidoreductase, and a wide range of genes that are involved in membrane, lysosomal, proteasomal, and metabolic processes [8]. However the induction of these detoxification genes are often regulated through complex transcriptional networks, as some SKN-1 target genes are also

under the regulatory control of SKN-1 independent mechanisms, therefore it is important to determine how regulatory inputs modulate expression of these target genes.

The known mechanisms of regulation of Nrf2 and SKN-1 activity involve the control of their localization within the cell by phosphorylation and proteasomal degradation. Direct phosphorylation of Nrf2/SKN-1 by specific kinases can either promote or prevent translocation from the cytosol to the nucleus, where gene expression can then be initiated. Kinase signaling pathways that are thought to positively regulate (i.e. phosphorylation induces translocation to the nucleus) Nrf2/SKN-1 include the p38/ERK MAP kinase pathways and the JNK stress activated pathway [9, 10]. While negative regulators (i.e. phosphorylation that promotes cytosolic localization) of Nrf2/SKN-1 include GSK-3β and SGK-1 pathways [11, 12]. Regulation of Nrf2 also involves the oxidative stress sensor Keap1 that promotes cytoplasmic localization and degradation of Nrf2 [13]. Proteasomal degradation of Nrf2 also occurs in a Keap1 independent manner, demonstrated by mutations in Nrf2 that abolish the Nrf2/Keap1 interaction, yet still exhibits targeted degradation [14]. However unlike humans, *C. elegans* does not have a Keap1 homolog to regulate SKN-1. Yet the WD40 protein WDR-23 was identified and has a functional complement to Keap1, which prevents the accumulation of SKN-1 and targets the protein for degradation [15].

Our interest lies in further understanding the mechanisms of an organism's response to oxidative conditions, in that pursuit we have been studying the role of the Brap2 (Brca1 associated binding protein 2) gene product in this response. Mammalian Brap2 is a proposed cytoplasmic protein capable of binding the nuclear localization signal (NLS) motifs of BRCA1 [16]. Brap2 also has been shown to bind the SV40 large

T antigen, cdc-14 and $p21^{WAF1/CIP1}$ during monocyte differentiation and proposed to regulate the assembly of the mitogenic signaling complex in response to Ras activation [17-21]. In addition, Brap2 has also been identified as a scaffold or adaptor protein for the maturation of spermatozoa [22, 23]. Previous studies conducted by our lab has identified the *C. elegans* Brap2 ortholog known as BRAP-2 [24]. Koon and Kubiseski showed that BRAP-2 is necessary for preventing an inappropriate response to elevated levels of ROS by countering premature activation of BRC-1 and CKI-1. However, the exact mechanism has not yet been defined. Therefore we are interested in furthering the study of the role of BRAP-2 in response to stress as well as identify the pathway it associates with for ROS detoxification.

Since there is increased sensitivity of *brap-2* mutant worms to oxidative stress, we were interested in investigating the expression levels of various ROS detoxification enzymes in this strain. The work presented here provides a new role for BRAP-2 in response to oxidative stress through the transcription factor SKN-1. Here we report that the *brap-2(ok1492)* mutation triggers a SKN-1 dependent induction of the antioxidant glutathione-S-transferase gene *gst-4*. We also confirmed that like its human ortholog, *C. elegans* BRAP-2 is a Ras/LET-60 effector protein, interacts with KSR-2 and is part of the worm MAP kinase pathway, and thus responsible for enhanced SKN-1 activity [10, 21]. An RNAi screen showed that this enhancement is dependent on the transcription factor ELT-3, a member of the GATA transcription factor family. We also found that ELT-3 formed a heterodimer with SKN-1 that activates *gst-4* transcription *in vitro* and that worms overexpressing SKN-1 required ELT-3 for lifespan extension. Together, these observations suggest a model where BRAP-2 acts as a negative regulator of the

Ras/MAPK signaling pathway, and that the disassociation of KSR-2 from BRAP-2 leads to the activation of SKN-1 and ELT-3 to promote expression of the nematode phase II detoxification response.

2.3 Experimental Procedure

2.3.1 *C. elegans* Strains

All *C. elegans* strains were maintained as described by Brenner [25]. Experiments were performed at 20°C unless stated otherwise. The following strains were used in this study: Bristol strain N2, *muIS84* (CF1553), *dvIs19* (CL2166), *skn-1(zu67)* (EU1), *elt-3(vp1)* (JG1) (gift from James McGhee lab), *vpIs1* (JG5), *ldIs007 (LD001), ldIs008 (LD1250*) (gift from Keith Blackwell lab), *wgIs178* (OP178), *brap-2(ok1492)* (YF15), *brap-2(ok1492);dvIs19* (YF67), *brap-2(ok1492);Is007* (YF68), *brap-2(ok1492);vpIs1* (YF112), *brap-2(ok1492);elt-3(vp1)* (YF114*), brap-2(ok1492);elt-3(vp1);dvIs19* (YF121*), brap-2(ok1492);skn-1(zu67)* (YF126), *brap-2(ok1492);muIs84* (YF127), *brap-2(ok1492);wgIs178* (YF165). Unless noted, all *C. elegans* strains were obtained from *Caenorhabditis* Genetics Center.

2.3.2 Transcription Factor RNAi Screening and RNAi assay

The RNAi screen was performed using a transcription factor RNAi library generated by the Walhout lab. The screen was carried out using the liquid culture method as described by Conte *et al*. [26]. The RNAi screen was performed in a 96-well plate, with each transgenic strain screened in three independent trials. Transcription factors that scored positive in at least two experiments were considered as true positives. *dvIs19* and *brap-2(ok1492);dvIs19* strains were used in this screen. In order to identify new regulators or co-activators of SKN-1 to promote *gst-4* activity in *brap-2(ok1492)* background, animals were fed in RNAi encoding a specific transcription factor. The change in *gst-4p::gfp* expression in the intestine was visually monitored in the RNAi-treated worms. Animals that showed a diminished *gst-4p::gfp* expression were scored as positives. RNAi assay was carried out by feeding worms dsRNA on bacterial NGM plates containing ampicillin

(100 µg/mL), IPTG (0.4 mM) and tetracycline (12.5 µg/mL) to further validate the potential positive candidates.

2.3.3 Phenotypic Analysis

gfp expression was visualized using various cell-type specific *gfp* reporters. In order to investigate *gfp* expression in a *brap-2(ok1492)* background, transgenic worms were generated using standard *C. elegans* techniques and confirmed by single worm PCR. L4 stage animals were picked and anesthetized using 25 mM Levamisole (Sigma L9756) and mounted on 2% agarose pad. Images of fluorescent animals were taken using a Zeiss LSM 700 confocal laser-scanning microscope with Zen 2010 Software®.

2.3.4 Cell Culture and Transfection

Human Embryonic Kidney (HEK) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. For transfection, 5 µg (Pull-down Assay) or 1 µg (Luciferase Assay) of mammalian expressing constructs were co-transfected in HEK-293T cells using transfection reagent polyethylenimine (PEI).

2.3.5 Pull-Down Assay

A GST "pull-down" assay was performed to confirm the mGST::BRAP-2/3xFlag::KSR-1, mGST::BRAP-2/3xFlag::KSR-2, mGST::BRAP-2/myc::LET-60 and EGFP::SKN-1/mGST::ELT-3 interactions with co-transfection in HEK 293T cells. Cells were lysed in TBS containing 1% NP-40 and complete protease inhibitor cocktail (Calbiochem #D06159166) after 72 hours. The mGST tagged protein was then pulled down from cell lysates using glutathione sepharose beads (Roche #17-0756-01), according to the manufacturer's protocol. Samples were analyzed by standard SDS polyacrylamide gel, followed by Western Blot analysis. The antibodies used were: anti-FLAG M2 (1:5000)

(Sigma #F1804), anti-BRAP-2 (1:5000), anti-myc 9E10 (1:5000), anti-GFP (1:5000) (Santa Cruz #SC-9996).

2.3.6 Promoter Cloning

The *gst-4* promoter region (724bp) was amplified from wild type *C. elegans* genomic DNA by PCR, and the DNA product was cloned in frame to a KpnI digested pGL4.10 Luciferase vector using the In-Fusion HD Cloning Plus, following the manufacturer's instructions (Clontech #638909). The constructed plasmid was sent to ATCG DNA/Sequencing Facility at SickKids (Toronto) for sequencing. Primer sequences will be provided upon request.

2.3.7 Luciferase Assay

HEK293T cells were transfected with the promoter plasmid, designated transcription factor plasmids and the pRL-TK Renilla internal control vector in a 6-well plate. Cell lysates were collected after 48 hours transfection and Luciferase assay was performed using the Promega Dual-Glo Luciferase Assay System (E2920) according to the manufacturer's protocol. Each transfection was done in triplicate and samples were then averaged. The Firefly and Renilla luminescence signals from the cells were detected using the Wallac 1420 plate reader.

2.3.8 Immunostaining

Whole worm staining: Antibody staining was performed according to the Koelle Lab protocol [27]. Synchronized L4 worms were collected, stained with the designated antibodies and were mounted on 3 μL Prolong Gold Anti-fade reagent (Life P36930). Worms were then visualized with Zeiss LSM 700 confocal microscope at 20X. Fluorescence intensity was quantified from 20 worms for each genotype using ImageJ

software. Antibodies used were: rabbit anti-BRAP-2 (1:500), anti-pMAPK (1:500) (Sigma #M8159) and DAPI (1 µg/mL) (Sigma #D9542). Alexa Fluor 488 (1:1000) (Invitrogen #A11001) secondary antibody was used for visualization.

Whole worm lysates Western blot: Synchronized adult worms were grown for each genotype. Packed worms (100 µL) were washed with M9 buffer and resuspended in worm lysis buffer (50 mM HEPES pH7.5, 100 mM NaCl, 10% Glycerol, 1mM EDTA, protease inhibitor and phosphatase inhibitor cocktail (Sigma P5726 & P0044)) followed by sonication (10s ON/ 10s OFF, 10% amplitude for 2 minutes). The lysates were cleared by centrifugation and the total protein lysate concentrations were quantified using BCA assay according to the manufacturer's protocol (Thermo Scientific #23227). Western blot was performed by loading 50 µg of total protein lysates and probed with anti-ERK1/2 (Santa Cruz #SC-94) (1:200), anti-pERK1/2 antibody (1:1000) (Sigma M8159) and anti-tubulin (1:1000) (Cell Signal #3873). The MAPK band density was measured and normalized to their respective anti-tubulin protein levels using Image J software.

2.3.9 Drug Treatment

U0126: The *gst-4p::gfp* expression pattern was observed by suppressing MAPK signaling, using the MEK1/2 inhibitor U0126 (Cell Signal #9903). U0126 was dissolved in dimethyl sulfoxide (DMSO) and plated at 20 µM onto agar plates and DMSO alone was used as a control. Young adult hermaphrodites were placed onto pre-treated agar plates and allowed to lay eggs. Progeny that reached the late L4 were visualized by confocal microscopy and RNA was quantified by qRT-PCR.

Paraquat or Arsenite: N2 and *elt-3* strains were collected with M9 buffer and treated with 100 mM paraquat (Sigma #856177) or 5 mM sodium arsenite (Sigma #35000-1L-R) for 2 hours to induce stress shock, followed by RNA isolation and qRT-PCR for *gst-4* mRNA quantification. M9 buffer treatment was performed simultaneously as control. Experiment was completed in three independent trials.

2.3. 10 Chromatin Immunoprecipitation (ChIP)

Protein lysates were prepared and ChIP was performed as previously described in Mukhopadhyay *et al.* and Claycomb *et al.* with some modification [28, 29]. Briefly, synchronized L4 worms were collected and worm "popcorn" was prepared using liquid nitrogen prior to sonicating and cross-linking. Lysates were collected by centrifugation and the total protein concentration was determined using Thermo Fisher BCA assay kit (#23227). An amount 0.5 mg of total protein lysates along with IgG (Abcam ab37415) or anti-GFP (Abcam ab290) antibody was used for overnight incubation. DNA collected from reverse-crosslinking was resuspended in 25 µL ddH_2O followed by qRT-PCR.

2.3. 11 RNA Isolation and Quantitative Real Time PCR (qRT-PCR)

For each strain, 50 µl of mixed-stage worms were collected and washed three times with M9 buffer. Total RNA was isolated using TRI Reagent (Sigma #93289) according to the manufacturer's protocol. Total RNA concentration was determined using Fisher Thermo NanoDrop2000 and 0.5 µg of RNA was used to carry out reverse transcription PCR with Applied Biosystem RNA to cDNA kit (#4387406) in a 20 µl reaction following the manufacturer's protocol. SYBR Green (Clontech #639676) qRT-PCR was conducted using Qiagen Rotor-gene Q detection system and analyzed using the comparative method (ΔΔCt). Oligos will be provided upon request. qRT-PCR data were derived from three

independent replicates. Results were graphed and the mRNA expression level of each strain was compared relative to N2. The endogenous control used for normalization was *act-1* unless otherwise specified. Statistical analyses were carried out using one-way ANOVA with Tukey's test or Student's T-test.

2.3. 12 Lifespan Assay

Lifespan assay was performed as previously described in Wilkinsin *et al.* [30]. The long-lived strain *ldIs008*, and N2 were raised at 20°C on worm plates with HT115 *E. coli*. At L1, 40 worms were placed onto *elt-3* RNAi seeded plates. Worms were transferred to fresh plates (away from progeny) and survival was scored every day as response to prodding. Statistical analysis was completed using the online survival analysis program OASIS® [31].

2.4 Results

2.4.1 *brap-2* mutants display increased expression of phase II detoxification gene

Our current interest in studying the mechanisms of an organism's response to oxidizing conditions came about from studying the physiological role of *C. elegans* BRAP-2. To identify the localization of BRAP-2, whole worm antibody staining was performed and showed BRAP-2 expression in the gonad and intestine (Figure S2.1). Since the *C. elegans* intestine has been well characterized as a major site for energy metabolism and stress response, the expression of BRAP-2 in the intestine may imply that it is important in regulating detoxification pathways.

We have previously identified that *brap-2(ok1492)* worms arrest at the L1 larval stage when exposed to oxidative stress. Due to an aberrant response to elevated levels of ROS, we hypothesized that the *brap-2(ok1492)* mutant may have modified expressions of ROS detoxification enzymes. To test this, we used transgenic animals carrying the transcriptional reporter constructs *gst-4p::gfp* or *sod-3p::gfp*, and analyzed GFP expression levels in N2 and *brap-2(ok1492)* nematodes. We found that *brap-2(ok1492)* worms expressed elevated levels of *gst-4p::gfp* in intestinal and hypodermal cells compared to N2, while no difference was seen with *sod-3p::gfp* (Figure 2.1A). To confirm that this was not an artifact of the transgenes used, mRNA levels were quantified using qRT-PCR and were consistent with the results obtained from fluorescent microscopy, as *brap-2(ok1492)* mutant showed at least 15-fold increase in *gst-4* compared to N2, while *sod-3* levels were identical in the *brap-2* mutant compared to N2 (Figure 2.1B). Five additional phase II genes were also studied (*gst-7*, *gst-10*, *gst-19*, *rhy-1* and *gcs-1*), and amongst them we identified *gst-4* as having the most significant change, with a 22-fold increase compared to the wild type (Figure 2.1C). Since higher

mRNA levels may not be due to an increased in transcription, we decided to measure pre-mRNA levels of the SKN-1 targets with qPCR to test the mRNA stability. Results showed an increase in the pre-mRNA levels, thereby indicating the relative transcript levels reflected the transcriptional activity (Figure 2.1D).

The phase II detoxification genes in *C. elegans* are mainly regulated by the transcription factor SKN-1. To show that the activation of the stress-dependent genes in the intestine is mediated by SKN-1 in *brap-2(ok1492)*, an RNAi assay was performed to knockdown *skn-1* in *brap-2(ok1492)* and examine the change in *gst-4p::gfp* expression level in the intestine (Figure 2.2A). As expected, a reduction of *gst-4* was seen with *skn-1* depletion. In addition, *skn-1(zu67*) and *brap-2(ok1492);skn-1(zu67)* mutants demonstrated a decrease in *gst-4* levels (Figure 2.2B). As shown in Figure 2.1, the *brap-2(ok1492)* worms expressed elevated levels of *gst-4p::gfp* in the intestine. However, *gst-4* expression remained in the body wall and not in the intestine when *skn-1* was disrupted. Taken together, these results indicate that the specific expression of *gst-4* in the intestine of *brap-2(ok1492)* mutants is dependent on the transcription factor SKN-1.

Previous research has reported various kinases control SKN-1 activity by regulating its transport into the nucleus. Therefore, we were interested in investigating the localization of SKN-1 in N2 and *brap-2(ok1492)* worms. We examined the intestinal expression pattern in L4 nematodes containing the translational fusion construct that produced a *skn-1::gfp* fusion protein and examined the GFP expression pattern in *brap2(ok1492)* worms (Figure 2.3A). An increase in *skn-1::gfp* expression was observed, where expression is shown in both the body muscles and the intestinal nuclei in *brap-2(ok1492)* mutants but not in N2 (Figure 2.3B). Furthermore, in the absence of *brap-2*,

the incidence of SKN-1 localization within intestinal nuclei is 67% (Figure 2.3C). Taken together, these results suggest that BRAP-2 functions to inhibit the nuclear localization of SKN-1.

Literature indicates that *C. elegans skn-1* encodes three splice variants (*skn-1a*, *skn-1b* and *skn-1c*), each with their own distinct expression patterns and function [7, 32, 33]. While the expression and function of *skn-1a* is not clearly known, *skn-1b* and *skn-1c* have roles in calorie restriction and stress resistance, respectively. qRT-PCR was performed and a 1.5-fold increase in *skn-1b* and 2.75-fold increase in *skn-1c* mRNA levels were observed in *brap-2(ok1492)*, this suggests that an accumulation of SKN-1 occurs in the intestine to activate detoxification genes in *brap-2* mutant worms (Figure 2.3D). SKN-1 is a master gene regulator that controls defences against stress and can directly bind to the promoter regions of its target genes in order to increase their transcriptional activities. Therefore identifying the exact transcription factor binding sites is necessary in order to gain a better understanding of this gene regulatory network. The modENCODE project has recently mapped the SKN-1 transcription factor binding sites at every stage of *C. elegans* development [34]. They were able to show an enrichment of SKN-1 binding to various sites of *gst-4* promoter at L1-L4 stages of the worm. Using chromatin immunoprecipitation, we examined two *gst-4* promoter regions containing the canonical SKN-1 binding sites WWTRTCAT or TCTTATCA [7]. ChIP-qPCR was performed and there was an at least 2-fold or greater enrichment of SKN-1 binding at two *gst-4* promoter sites in *brap-2(ok1492);wgIs178* compared to *wgIs178* at the L4 stage (Figure 2.3E). These findings indicate the activation of *gst-4* genes in *brap-2(ok1492)* is dependent on SKN-1. Furthermore, a pull-down assay did not detect a physical

interaction between BRAP-2 and SKN-1 (data not shown), indicating that BRAP-2 does not directly inhibit SKN-1 to regulate its function. However, the possibility of co-activators present that function to co-ordinate SKN-1 activities cannot be ruled out. Thus an RNAi screen was conducted to determine the mechanism through which BRAP-2 regulates SKN-1.

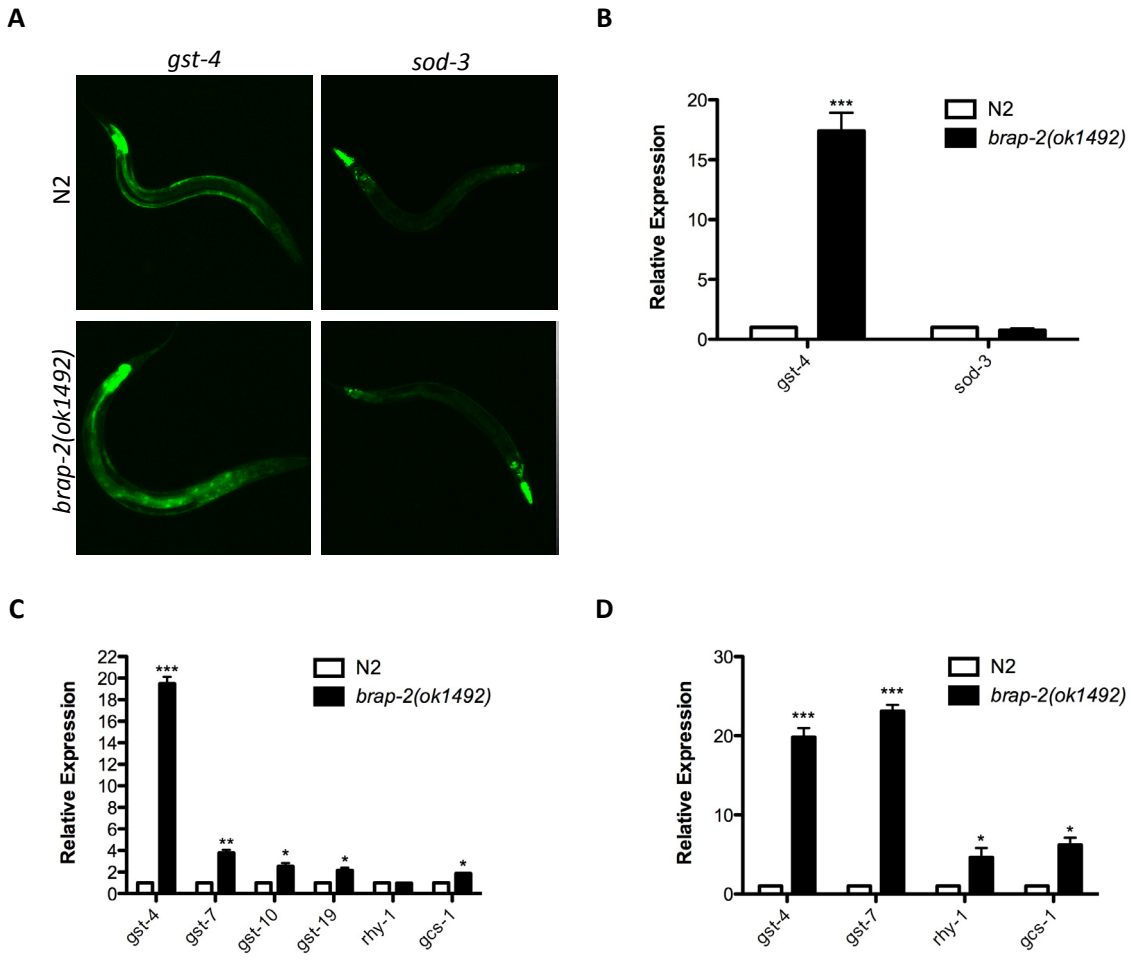

Figure 2.1 *gst-4* expression is increased in *brap-2(ok1492)* mutants.

(A) *brap-2(ok1492)* mutants display increased *gst-4p*::GFP expression in the body wall muscle and intestine, but *sod-3p::*GFP expression is not affected. **(B)** Expression of the SKN-1 target *gst-4* and the DAF-16 target *sod-3* were measured by qRT-PCR in *brap-2(ok1492)* mutants and wild type animals. **(C)** SKN-1 target genes (*gst-4*, *gst-7*, *gst-10*, *gst-19, rhy-1 and gcs-1*) mRNA levels were compared in *brap-2* mutant and wild type animals, *gst-4* shows the most significant increase among the 6 phase II genes. **(D)** The relative pre-mRNA expression level of *gst-4, gst-7, rhy-1* and *gcs-1* was measured by qPCR in N2 and *brap-2(ok1492)*. Statistical analyses was derived from one-way ANOVA with Tukey's test; $p<0.001$***, $p<0.01$**, $p<0.05$*.

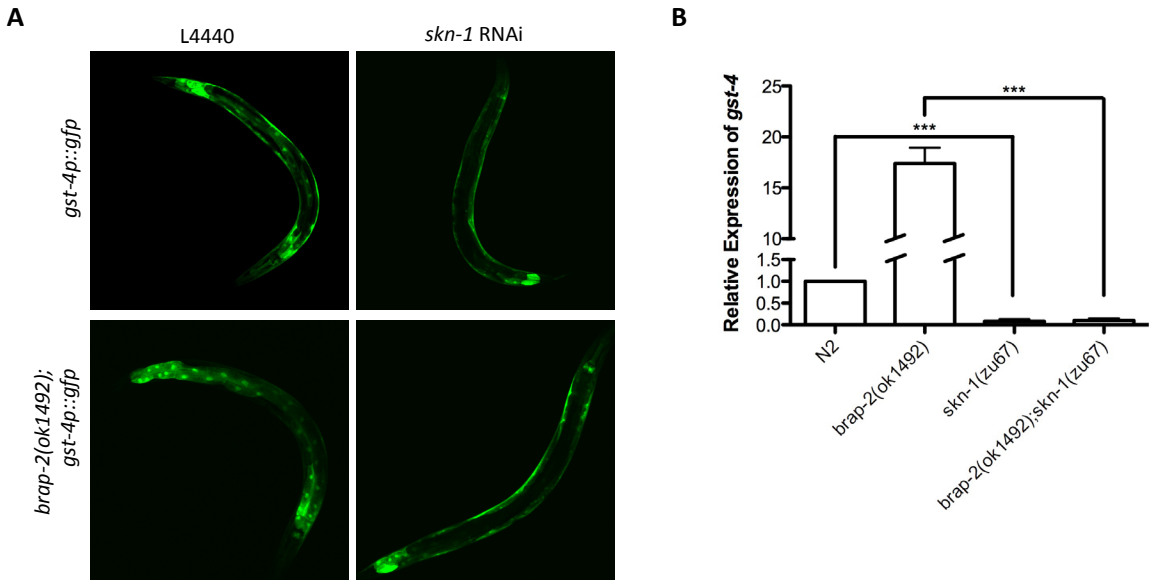

Figure 2.2 Enhanced *gst-4* expression in *brap-2(ok1492)* requires SKN-1.

(A) Synchronized *gst-4p::gfp* and *brap-2(ok1492);gst-4p::gfp* worms were grown on bacteria carrying control (L4440) or *skn-1* RNAi inducing plasmids. *skn-1* knockdown decreases *gst-4p::*GFP expression in wild type and *brap-2(ok1492)* mutants L4 animals. **(B)** *gst-4* mRNA levels were quantified by qRT-PCR. Values were compared to *act-1* endogenous control and relative to N2. The double mutant *brap-2(ok1492);skn-1(zu67)* showed a reduction of *gst-4* mRNA expression. Statistical analyses was derived from one-way ANOVA with Tukey test; $p<0.001$***.

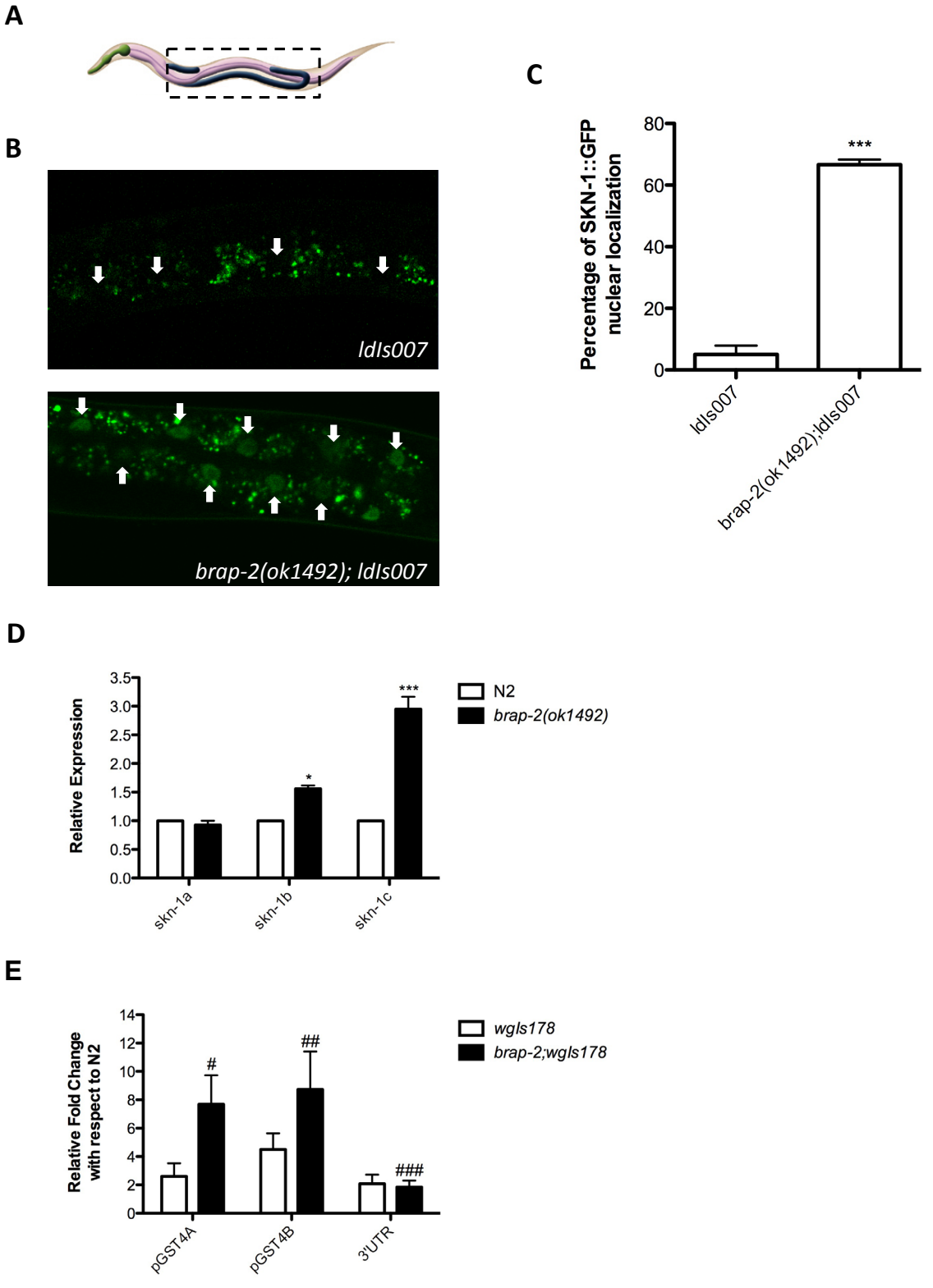

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Figure 2.3 Increased SKN-1 intestinal nuclear localization and binding to the *gst-4* promoter in *brap-2(ok1492)*.

(A) Representation indicating the intestinal region analyzed in an L4 *C. elegans*. Diagram adapted and modified from Wormbase. **(B)** Localization of *skn-1::gfp* fusion protein in the intestine of wild type (N2) (top) and *brap-2(ok1492)* (bottom) worms, demonstrating increased nuclear accumulation of SKN-1::GFP in *brap-2(ok1492)* animals. The white arrows represent the locations of the intestinal nuclei. **(C)** SKN-1::GFP localization in *brap-2(ok1492)* animals was quantified and compared to wild type. While 5% of wild type worms shows nuclear subcellular localization of SKN-1::GFP within intestinal cells, 67% of *brap-2(ok1492)* showed nuclear localization. Three independent experiments was performed, with n=20 per trial, statistical analysis was done using Student's t-Test; $p<0.001$*** vs *ldIs007*. **(D)** Quantification of *skn-1a*, *skn-1b* and *skn-1c* mRNA transcript levels using qRT-PCR. Results demonstrated increase *skn-1b* and *skn-1c* expression in *brap-2(ok1492)*. Statistical analysis was performed using one-way ANOVA with Tukey's test; $p<0.001$***, $p<0.05$* vs N2. **(E)** ChIP-qPCR analysis of SKN-1 binding at two *gst-4* promoter regions in *wgIs178* and *brap-2(ok1492);wgIs178*. Relative fold change is calculated with respect to N2 (absence of the GFP antigen). Data represented the mean of four independent trials and error bars represent standard error of the mean. Statistical analysis was derived using one-way ANOVA with Tukey's test; p values are as follow: $p=0.065^{\#}$, $p=0.19^{\#}$ and $p=0.78^{\#\#\#}$.

2.4.2 Regulation of SKN-1 by BRAP-2 involves the ERK/MAPK pathway

Ras signaling through the Raf/MEK/ERK kinase cascade is believed to be a major signaling pathway during metazoan development, controlling many different biological processes. Upon cell stimulation, receptor tyrosine kinases recruit and activate Ras GTPase to initiate the MAPK pathway. Published data characterizes Brap2/IMP as a Ras effector that negatively regulates MAPK activation, where IMP depletion results in an increase in MAPK signaling [35]. It has previously been reported that MPK-1/ERK phosphorylates SKN-1 to regulate its target genes and longevity. Since MAPK expression has been associated with the regulation of Brap2/IMP and SKN-1 [10, 35], we hypothesized that the increase in phosphorylated MAPK activity in *brap-2(ok1492)* may regulate the transcription factor SKN-1.

Previously, Brap2/IMP has been shown to be a Ras interacting protein that directly binds to H-Ras in a GTP-dependent manner [35]. To determine if this interaction is evolutionarily conserved in *C. elegans*, co-transfection followed by Western blot analysis was performed to observe this potential upstream protein-protein interaction. We found that BRAP-2 interacts only with the constitutively active form of Ras LET-60(G12V) (Figure 2.4A). The same report also revealed that mammalian KSR1 interacts with Brap2/IMP. By stimulating the auto-ubiquitination of Brap2/IMP, Ras relieves the inhibition on KSR, and allows KSR to translocate to the plasma membrane and mediate the formation of RAF/MEK/ERK complex. There are two forms of KSR in *C. elegans* – KSR-1 and KSR-2. Our Western blot analysis demonstrates that BRAP-2 interacts specifically with KSR-2 (Figure 2.4B). Since the loss of *brap-2* increases the translocation of SKN-1 into the nucleus, we hypothesized that the loss of BRAP-2 will

also increase MAPK signaling. To show that the MAPK pathway is stimulated in the absence of BRAP-2, antibody staining against activated MAPK was performed and whole worm fluorescence levels were quantified. We found that the p-MAPK expression increases in *brap-2(ok1492)* 3-fold in comparison to the wild type (Figure 2.5A). To support this, we first looked at the *mpk-1* transcript levels in *brap-2(ok1492)*, and we were able to show a 1.5-fold increased of mRNA, indicating *brap-2* mutant up-regulates the transcription of *mpk-1* (Figure 2.5B). Furthermore, Western blot using worm lysates was also performed. *brap-2(ok1492)* induced a 1.33-fold and1.5-fold increase in endogenous MAPK and p-MAPK expressions at the protein level, respectively (Figure 2.5C,D). These results indicate that loss of BRAP-2 induces MAPK expression and this increased activity may play a role in BRAP-2/SKN-1 stress response regulation.

Since SKN-1 phosphorylation by MPK-1 enhances its activity, if MAPK activity were to be inhibited in *brap-2(ok1492)*, we would expect a decrease in *gst-4* expression. In *C. elegans*, the ERK/MAPK pathway includes MEK-1/2. To knock down MEK-1/2 activity, we used the MEK inhibitor U0126. When MEK-1/2 activity was depleted we observed a reduction in both *gst-4p::gfp* expression (Figure 2.6A) and *gst-4* mRNA levels (Figure 2.6B), indicating that the higher activity of the MAPK signaling in *brap-2(ok1492)* animals is responsible for increased SKN-1 transcriptional activity. Taken together, these findings suggest that in *C. elegans*, BRAP-2 may serve as a potential inhibitor of KSR in the ERK/MAPK pathway for the downstream regulation of detoxification genes during oxidative stress. Since *brap-2(ok1492)* promotes *gst-4* expression, we furthered our investigation by performing a genetic suppression screen in

order to identify novel genes and/or protein components associated with *brap-2(ok1492)* that are necessary for the activation of SKN-1.

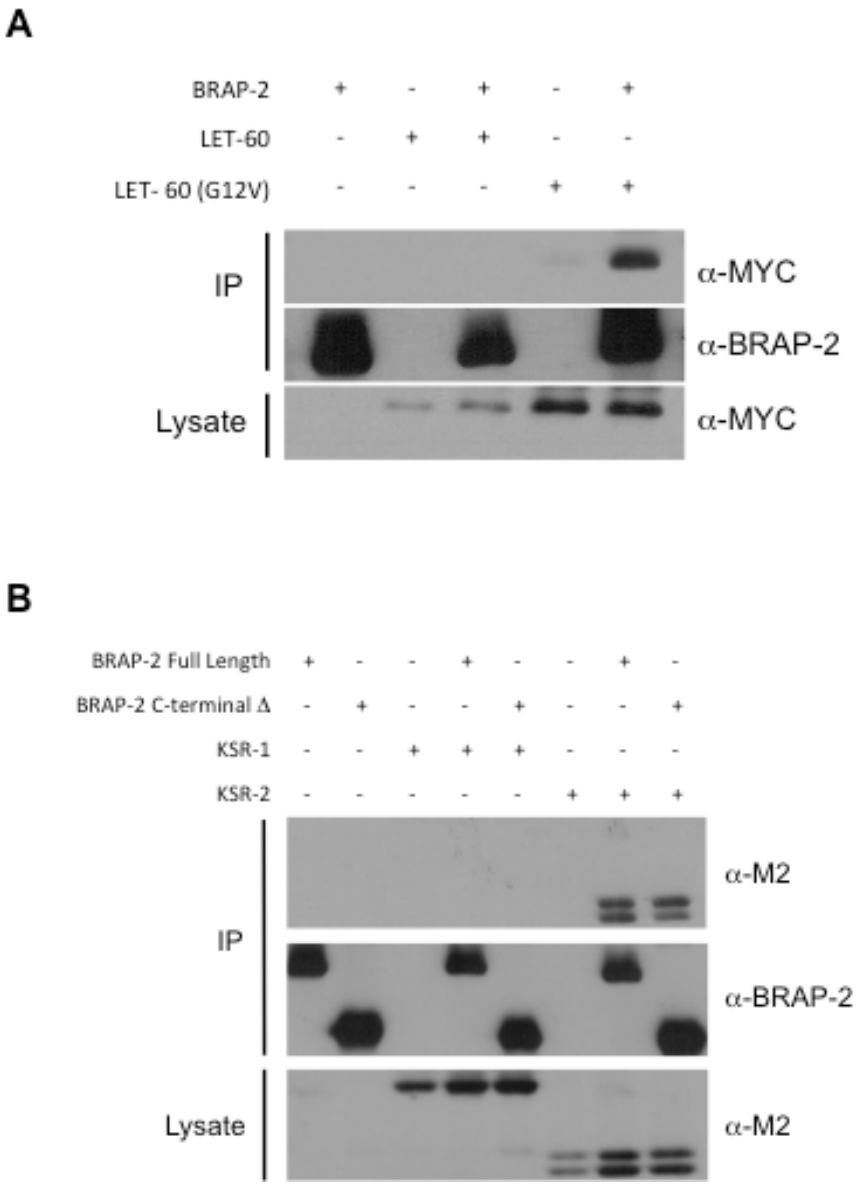

Figure 2.4 *C. elegans* BRAP-2 is an effector protein for LET-60 and interacts with KSR-2.

A) GST pull-down assay was used to show the interaction between GST::BRAP-2 and MYC::LET-60 or LET-60 containing the G12V mutation (gain-of-function LET-60). Western blot result reveals that BRAP-2 interacts with activated LET-60 *in vitro*. **(B)** The full-length and truncated form (containing the BRAP-2 and Ring Finger domain; C-terminal Δ) of BRAP-2 fused to GST tag were used to co-transfect with 3Flag::KSR-1 or 3Flag::KSR-2. Western blot results indicate that BRAP-2 full length and C-terminal Δ interact specifically to KSR-2 *in vitro*.

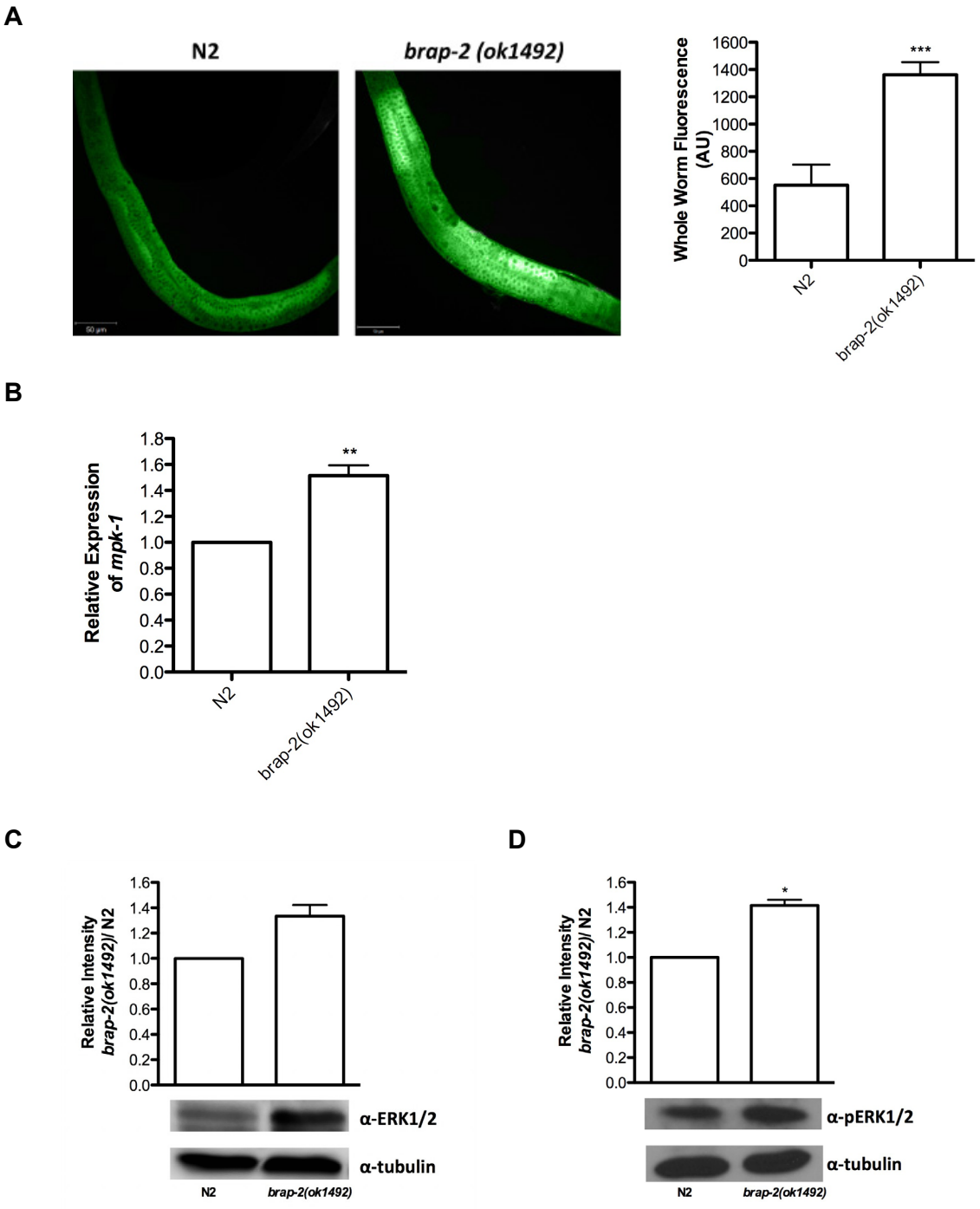

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Figure 2.5 *C. elegans* BRAP-2 regulates the MAP kinase pathway.

(A) Whole worm antibody staining was performed using N2 and *brap-2(ok1492)* worms with α–pMAPK antibody. The fluorescence intensity was quantified using ImageJ [http://rsbweb.nih.gov/ij/] software, by manually tracing around each worm to measure the mean pixel density (mean pixel density of the worm area minus pixel density of the background). For N2 and *brap-2(ok1492)* 15 worms (L4 stage) were analyzed per strain. An increased p-MAPK signalling detected in *brap-2(ok1492)* animals. Error bars represent the standard error of the mean, $p<0.001$*** vs N2. **(B)** RNA was extracted from synchronized L4 worms and the *mpk-1* mRNA levels were quantified using qRT-PCR. The *brap-2(ok1492)* mutant displayed a 1.5-fold increase in *mpk-1* transcript level. **(C-D)** Whole worm lysates Western Blot was performed by loading 50 µg of total protein lysates, the **(C)** ERK-1/2 and **(D)** pERK1/2 bands intensity was measured and normalized to their respective α-tubulin protein levels using ImageJ software. Data represented the mean of three independent trails. Western blot with α-pMAPK antibodies shows increased endogenous pMAPK protein in *brap-2(ok1492)* worm lysates in comparison to wild type. All statistical analyses were performed using Student's t-Test; $p<0.001$*** vs N2, $p<0.01$** vs N2, $p<0.05$* vs N2.

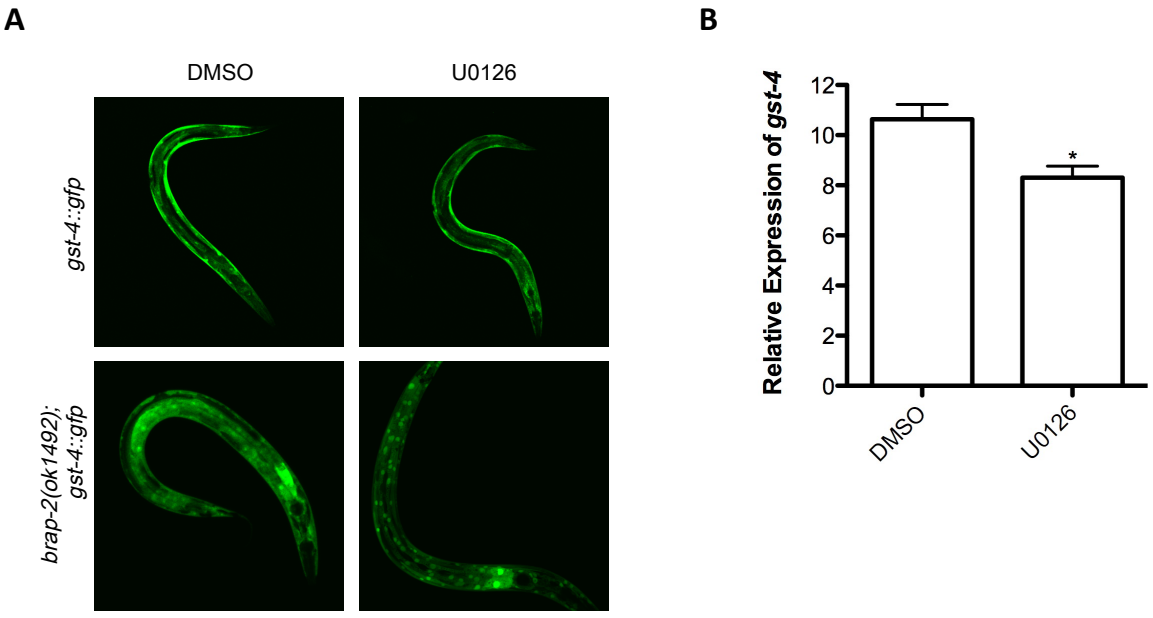

Figure 2.6 Increased *gst-4* expression in *brap-2(ok1492)* requires MEK-1/2.

(A) Synchronized *gst-4p::gfp* and *brap-2(ok1492); gst-4p::gfp* worms were grown on NGM plates containing control DMSO or 20 μM MEK inhibitor U0126. GFP expression was examined when animals reached the L4 stage. *brap-2(ok1492)* mutants show decreased *gst-4p::gfp* expression in the intestine. **(B)** *gst-4* mRNA expression in DMSO and drug treated worms quantified by qRT-PCR. Values were normalized to actin (*act-1*) endogenous control. The U0126 treated animals shows a decrease in *gst-4* mRNA when MAPK activity is inhibited. Statistical analysis was carried out using Student's t-test; $p<0.05$* vs DMSO control.

2.4.3 The ELT-3 GATA transcription factor is an activator of *gst-4* expression

Transcription factors are integral components of gene regulatory networks, serving as key factors at the center of gene activation or repression. The regulation of gene expression could be a product of the interactions between multiple transcription factors. Therefore, identifying genes encoding transcription factors that work in combination with our target gene of interest will help to solidify our understanding of the complex regulatory network involved in oxidative stress response. *brap-2(ok1492)* activates a signaling cascade that enhances the SKN-1 detoxification pathway, an increase that can be visualized with enhanced *gst-4::gfp* expression. Thus, the *brap-2(ok1492);gst-4p::gfp* strain was used as the basis for our RNAi screen to test for candidate genes that suppress *gst-4p::gfp* expression. Out of 913 tested clones, we have identified 20 candidate genes that, when knocked down, reduce *gst-4* expression in *brap-2* (Table 2). Among the candidates, *elt-3* was chosen as a transcription factor or co-activator that possibly interacts with SKN-1 to promote this biological effect, on the basis that *elt-3* is a potential regulator for aging. Through fluorescence microscopy, we observed that *brap-2(ok1492)* worms treated with *elt-3* RNAi displayed reduced levels of *gst-4p::gfp* in the intestine compared to N2 (Figure 2.7A). To further confirm this, we quantified mRNA levels in *brap-2(ok1492);elt-3(vp1)* double mutants and found a greater than 60% reduction in *gst-4* (Figure 2.7B). Both results indicate that *elt-3* is a potential regulator for the induction of *gst-4* expression.

After defining the importance of ELT-3 in the expression of phase II detoxification genes, we were interested in determining whether ELT-3 can co-activate and thus promote SKN-1 biological activity. In order to verify that ELT-3 is required for SKN-1 activation of *gst-4* under stress, we first tested the *gst-4* induction level after

arsenite or paraquat treatment in *elt-3* mutants. In response to stress, the mRNA level of *gst-4* increased in the wild type, and a significant decreased in expressions were observed in *elt-3(vp1)*, indicating that ELT-3 is partially needed to induce SKN-1 targets upon oxidative stress (Figure 2.8A,B). Furthermore, three other SKN-1 targets (*gcs-1*, *gst-7* and *gst-10*) were also studied and did not show a significant decrease of the targets upon exposure to stress in *elt-3(vp1)* (Figure S2), indicating the regulation is specific to *gst-4*. We have previously demonstrated an enhanced *skn-1b* and *skn-1c* mRNA *in brap-2(ok1492)*, therefore to determine if ELT-3 is required for enhanced *skn-1c* expression in *brap-2(ok1492)*, qRT-PCR in the *brap-2(ok1492);elt-3(vp1)* double mutant, displayed a suppression of *skn-1c* mRNA levels in comparison to *brap-2(ok1492)* (Figure S2.3). This implies that *elt-3* is necessary for the increased activation of SKN-1 when BRAP-2 is depleted.

Moreover, we also tested whether a direct interaction of SKN-1 and ELT-3 exists on the *gst-4* promoter. To monitor the potential for an alteration in *gst-4* gene expression *in vitro*, a luciferase reporter construct fused to the *gst-4* promoter sequence was created and transfected into the HEK293T cells along with SKN-1 and/or ELT-3. Although a moderate increase in *gst-4* promoter expression was seen with either SKN-1 or ELT-3 alone, a much larger increase in expression was detected in the presence of both SKN-1 and ELT-3. This indicates that together, these transcription factors activate expression of the *gst-4* promoter synergistically (Figure 2.8C). Furthermore, we co-transfected HEK293T cells with tagged SKN-1 and/or ELT-3. Following co-immunoprecipitation and Western blotting, an interaction between SKN-1 and ELT-3 was detected, suggesting that these two transcription factors heterodimerize (Figure 2.8D). Therefore, the data

presented here indicates that ELT-3 is not only a transcription factor for hypodermis development, but also has a role in regulating phase II genes and the oxidative stress response. These observations will help provide insight into how these two relatively unrelated proteins may function together in response to oxidative stress in *C. elegans*.

We also determined the expression pattern and cellular localization of ELT-3 *in vivo*. The transgenic strain *elt-3::gfp* was used and we were able to detect *elt-3* in hypodermal cells and in posterior intestinal nuclei in both the wild type and *brap-2(ok1492)* (Figure 2.9). Thus, unlike SKN-1, ELT-3 subcellular localization appears independent of BRAP-2 and MPK-1 activity.

Table 2. A list of candidate *brap-2(ok1492);gst-4::gfp* suppressor genes (transcription factors).

Gene ID	*C. elegans* gene name	Vertebrate homolog Protein	Mutant alleles available	Function/ Expression
C33D3.1	*elt-2*	GATA family	Yes	Cardiac and endoderm development
F34D10.5	*lin-48*	C2H2-type Zinc Finger Protein	Yes	Hindgut, mail tail and excretory duct cell development
K02B9.4	*elt-3*	GATA family	Yes	Expressed in hypodermal cells
F58A4.7	*hlh-11*	bHLH/ AP4	Yes	-----
F15C11.1	*sem-4*	Sal-like protein	Yes	Neuronal, mesodermal and vulval cells development
F43C11.7	-----	Uncharacterized conserved protein	No	Predicted E3 Ubiquitin Ligase
Y47D3B.7	*sbp-1*	Sterol regulatory element binding protein	Yes	Lipid metabolism
T01B10.4	*nhr-14*	Estrogen receptor	Yes	-----
F44C4.2	*nhr-37*	Hormone receptor	Yes	-----
K10C3.6	*nhr-49*	Hepatocyte nuclear factor 4 family	Yes	Fat regulator and lifespan
F21H11.3	*tbx-2*	T-box transcription factor	Yes	-----
Y65B4BR.5	-----	-----	No	-----

Gene ID	*C. elegans* gene name	Vertebrate homolog Protein	Mutant alleles available	Function/ Expression
R07H5.10	*zip-6*	bZIP transcription factor	No	-----
K03C7.2	*fkh-9*	Forkhead/HNF3 family	Yes	Expressed in the nervous system
T19E7.2	*skn-1*	Nuclear factor erythroid 2-related factor	Yes	Pharyngeal, muscle and intestinal cells development; oxidative stress response during postembryonic development
F14F3.1	*vab-3*	Pax-6 Paired Domain	Yes	Epithelial and neuronal development.
T26A8.4	-----	-----	Yes	Germ cell and vulva development
F58A4.11	*gei-13*	BED finger domain	No	Required for normal body shape, cuticle synthesis and locomotion
T06GT6.5	-----	-----	No	-----
F59B10.1	*pqn-47*	C11orf9	Yes	Vulva development, locomotion, cuticular integrity

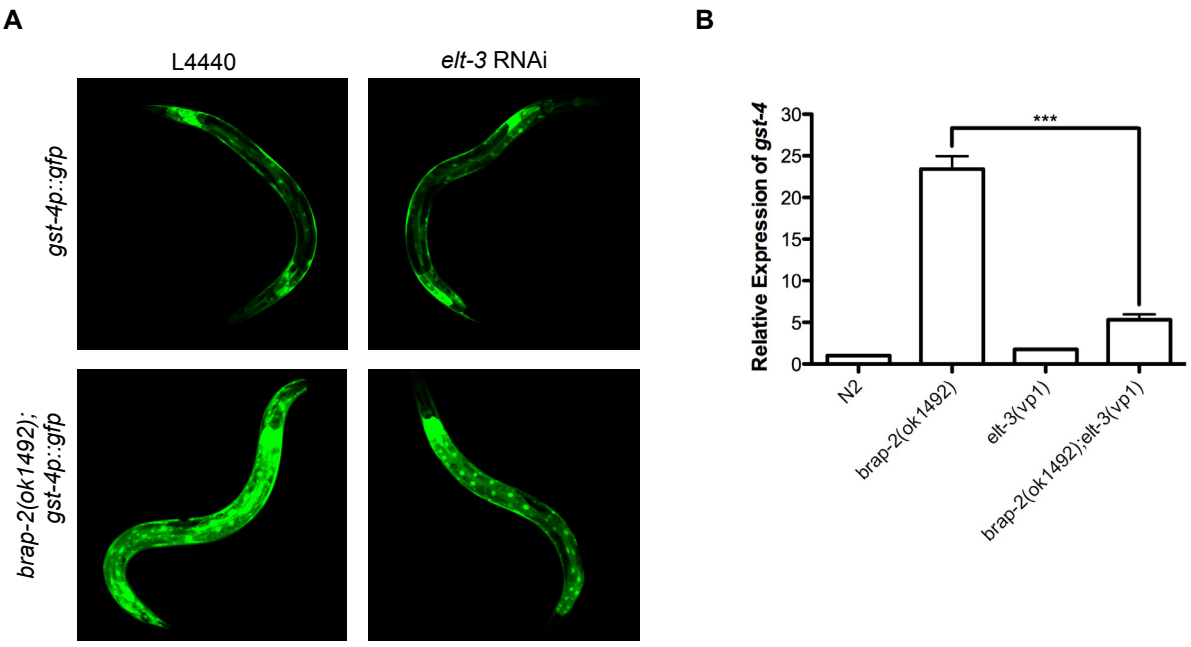

Figure 2.7 Loss of *elt-3* reduced *gst-4* expression in *brap-2(ok1492)* mutants.

(A) Synchronized *gst-4p::gfp* and *brap-2(ok1492); gst-4p::gfp* worms were grown on control (L4440) or *elt-3* RNAi plates. GFP expression was visualized at the L4 stage. *brap-2(ok1492)* worms show reduced *gst-4p::gfp* expression when *elt-3* is knocked down. **(B)** qRT-PCR of gst-4 mRNA in various strains. The double mutant *brap-2(ok1492);elt-3(vp1)* shows a 70% reduction in *gst-4* mRNA expression in comparison to *brap-2(ok1492)*. Statistical analysis was carried out using ANOVA with Tukey's test; $p<0.001$***.

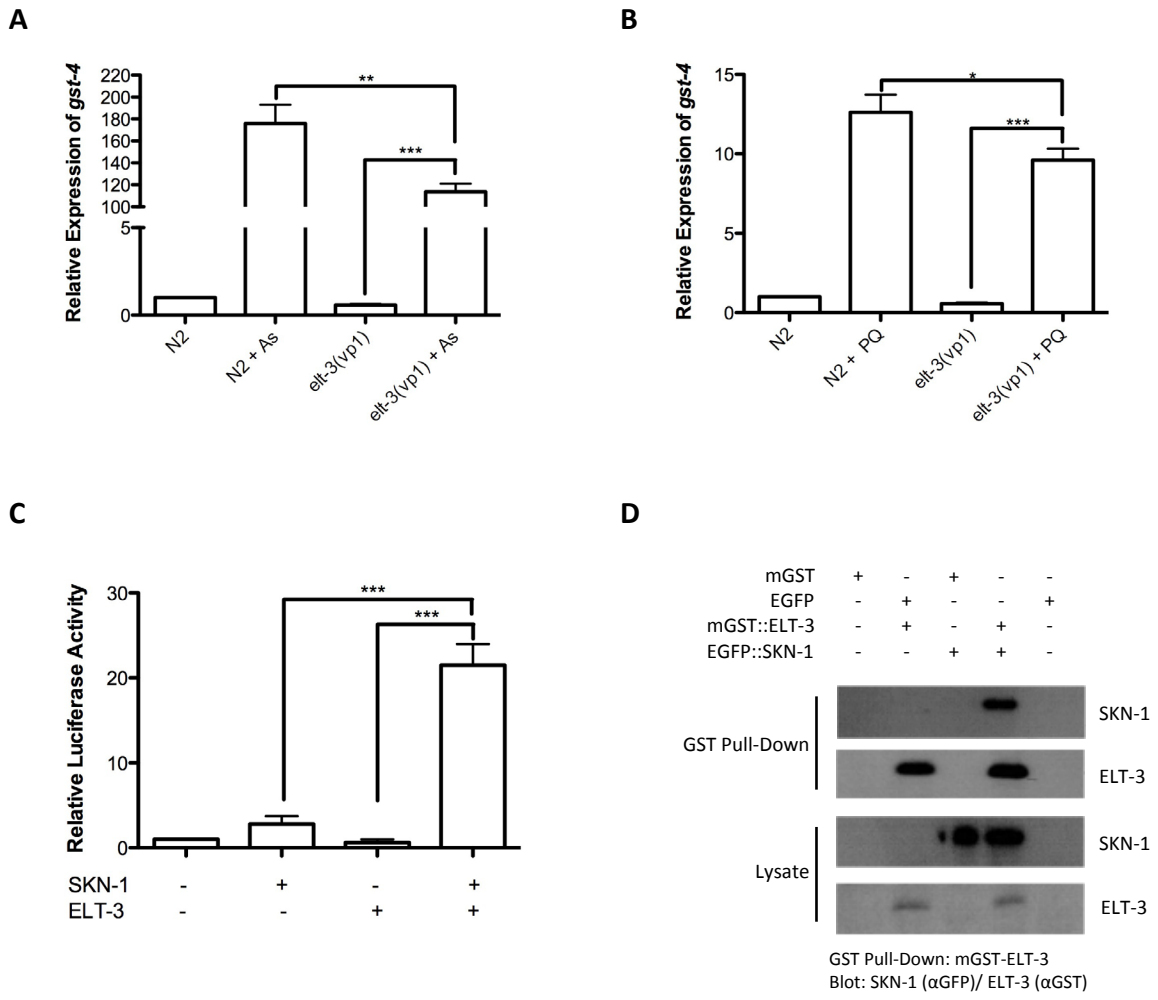

Figure 2.8 ELT-3 is partially required for *gst-4* expression during oxidative stress and synergistically activates expression from *gst-4* promoter through interaction with SKN-1.

(A,B) Synchronized worms were collected and treated with **(A)** 5 mM sodium arsenite or **(B)** 100 mM paraquat for 2 hours followed by RNA isolation. qRT-PCR was performed to measure the *gst-4* mRNA transcript levels. Results indicated ELT-3 is essential for *gst-4* expression during oxidative stress. **(C)** Luciferase assay was performed and the transcriptional activity was determined by the activation of the *gst-4* promoter-luciferase reporter vector. Maximum activation occurs upon co-expression of both ELT-3 and SKN-1 constructs. **(D)** A GST pull down assay was performed using anti-GFP antibody on Western blot. The assay reveals that the EGFP::SKN-1 and mGST::ELT-3 interact and hetero-dimerize *in vitro*. qRT-PCR statistical analyses were conducted using one-way ANOVA with Tukey's test; $p<0.05$*, $p<0.01$**, $p<0.001$***.

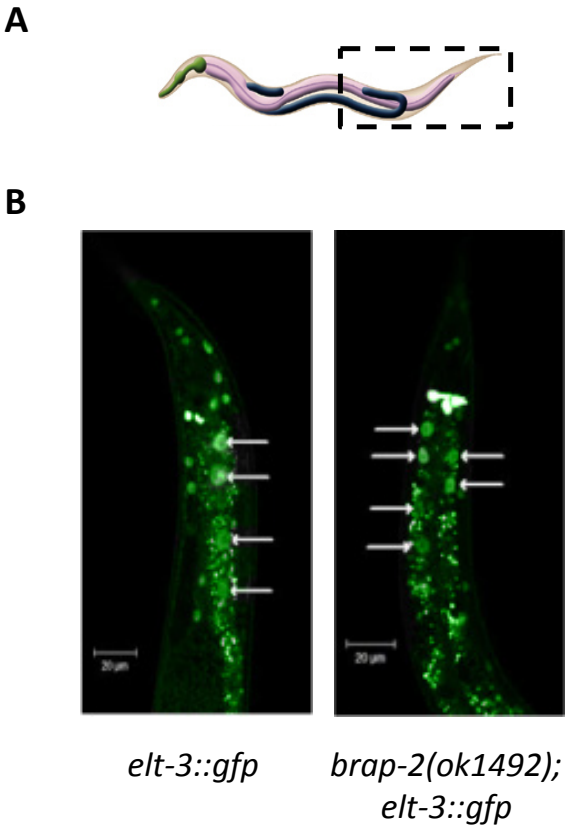

Figure 2.9 ELT-3 is expressed in the posterior intestinal nuclei

(A) Representation indicating the tail region analyzed in an L4 *C. elegans*. Diagram adapted and modified from Wormbase. **(B)** The transgenic worms containing *elt-3::gfp* construct was used to visualized the localization of ELT-3 in wild type (left) and *brap-2(ok1492)* mutants (right). ELT-3 is expressed in the nuclei in the posterior region of the intestine of L4 stage worm (white arrows).

2.4.4 ELT-3 is required for SKN-1 dependent enhancement of lifespan

SKN-1 overexpression promotes longevity in *C. elegans*. In addition to published data implicating the transcription factor ELT-3 in the regulation of aging, we have shown that ELT-3 is needed for *gst-4* expression. Thus we sought to determine whether ELT-3 is required to co-activate the SKN-1 regulated pathway for lifespan extension. To test this, we obtained a SKN-1 overexpressing transgenic strain and knocked down *elt-3* by RNAi and performed a lifespan assay. This study of longevity supported our hypothesis that depletion of *elt-3* in an over-expressing SKN-1 strain does in fact lead to a decrease in lifespan. *ldIs008* (L4440) (16.68 +/- 0.79 days) have a significantly longer lifespan than *ldIs008* (*elt-3* RNAi) (11.08+/-0.45 days) (Figure 2.10). Therefore, this indicates the enhanced lifespan observed in transgenic animals over-expressing SKN-1 is dependent on functional ELT-3.

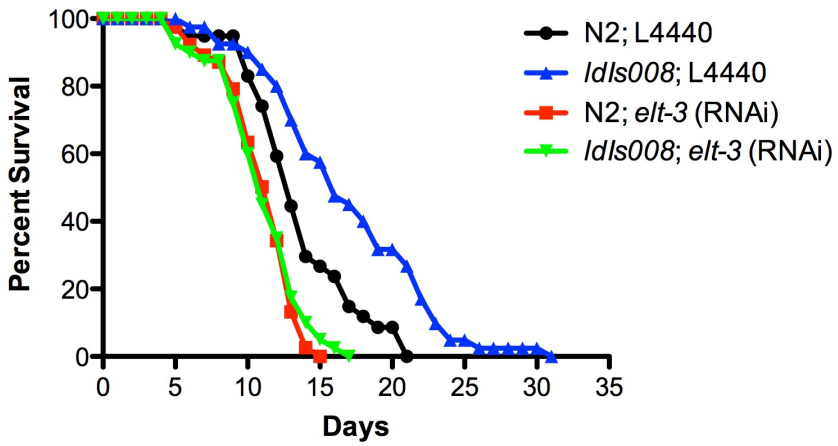

Figure 2.10 Extended lifespan in overexpressing SKN-1 requires ELT-3

Survival curves of N2 and worms containing the SKN-1 transgene (*ldIs008*) fed on control bacteria (L4440) or bacteria expressing *elt-3* RNAi from L1 worms at 20°C. While *ldIs007*; L4440 has a significantly longer lifespan than N2; L4440, mean lifespans are 16.7±0.79 days and 13.6±0.62 days respectively, n=40. No extension of lifespan was observed in overexpressing SKN-1 in *elt-3* RNAi (*ldIs008* mean = 11.1±0.45 days compare to N2 mean = 11.1±0.38 days, n=40). Three independent experiments was performed, data represents one trial.

2.5 Discussion

The work presented here suggests a new role for BRAP-2 in response to oxidative stress through the transcription factor SKN-1. Using *C. elegans* as a model organism, we have shown that the *brap-2(ok1492)* deletion mutant enhances the expression of the phase II detoxification gene, *gst-4*. Additionally, this strain was used to perform a transcription factor RNAi screen, which identified SKN-1 and ELT-3 as potential candidates that regulate the expression of *gst-4*. Investigation through the use of both genetic and molecular approaches, have demonstrated that both the SKN-1 and ELT-3 response to oxidative stress is mediated through the MAPK pathway.

Like mammals, nematodes have well-developed lines of defense for protection against toxic compounds [36]. The *C. elegans* genome encodes a wide range of stress genes, providing systematic stress response and detoxification strategies. Determining which detoxification gene that is specifically expressed in *brap-2(ok1492)* would help to narrow the scope of potential regulation pathways that should be investigated. We report that this deletion of *brap-2* displays a high level of the phase II detoxification gene *gst-4* but not *sod-3*. *C. elegans gst-4* has been shown to detoxify both endogenously and exogenously derived toxic compounds, where their mRNA levels were up-regulated upon exposure to the drug paraquat [37]. Koon and Kubiseski reported that *brap-2* mutant worms demonstrated larval arrest and lethality when exposed to both hydrogen peroxide and paraquat, while the wild type was not affected [24]. As such, an increased *gst-4* expression in *brap-2(ok1492)* suggests a novel pathway of regulation involved in detoxification response.

The transcription factor SKN-1 is regarded as a master regulator for the expression of phase II detoxification genes. Thus our goal is to investigate whether BRAP-2 can regulate SKN-1 and its target genes, as well as to identify the corresponding signaling mechanism. Here, we have presented evidence to suggest that the regulation of *gst-4* by *brap-2* is in fact dependent on the transcription factor SKN-1. Our results show that SKN-1 is highly expressed and detected in intestinal nuclei in *brap-2(ok1492)* worms, suggesting that the absence of functional BRAP-2 affects SKN-1 localization. Additionally, our finding that decreased *gst-4* mRNA expression in *brap-2(ok1492)*;*skn-1(zu67)* double mutant animals, indicates that BRAP-2 influences the stress response pathway. Further evidence, including qRT-PCR and ChIP, detected an increased *skn-1c* mRNA expression and an enrichment of SKN-1 at *gst-4* promoter region respectively, suggesting that the enhanced *gst-4* expression as seen in *brap-2(ok1492)* is caused by an increased SKN-1 binding at *gst-4* promoter element and amplified transcriptional activity.

Brap2/IMP has been identified as an ON/OFF switch in the Ras signaling pathway. Brap2 can either: (1) bind to the KSR scaffold protein and prevent it from translocating to the cell membrane to promote MEK activation and decrease the amplitude of the MAPK response signaling by inhibiting the formation of a complex between RAF and MEK in quiescence cells, or (2) trigger self-degradation and autoubiquitination when RAS is activated through the binding to RasGTP and modulates the RAF-MEK-ERK kinase cascade. The mammalian MAPK signaling pathway has a homolog counterpart conserved in *C. elegans*, and we were able to show physical interactions between BRAP-2/KSR-2 and BRAP-2/LET-60(G12V). Therefore our

observations suggest BRAP-2 regulation of the Ras signaling pathway in *C. elegans* is somewhat conserved to the mammalian system.

Furthermore in *C. elegans*, it has been postulated that several protein kinase pathways are responsible for regulating SKN-1 in response to oxidative stress. Previous research examined the p38 MAPK, DAF-2 insulin-like and extracellular receptor regulated kinase pathways as potential regulators and studies were carried out looking at changes to the direct phosphorylation of SKN-1, which promotes stress gene activation. In the absence of upstream kinases, SKN-1 phosphorylation is altered, leading to decreased target gene expression. A recent finding has shown that the *C. elegans* ERK cascade promotes longevity through the transcription factors SKN-1 and DAF-16, where MPK-1 directly phosphorylates SKN-1 for increased SKN-1 nuclear accumulation. Given our proposed model where BRAP-2 negatively regulates SKN-1 activity, we looked at whether MEK-2, a kinase upstream of SKN-1 and MPK-1, is required for this regulation. Our observation that reduced *gst-4* expression caused by the inhibition of MEK-1/2 in *brap-2(ok1492)* using the inhibitor U0126, provides further evidence of regulation mediated by the Ras signaling pathway. These findings in *C. elegans* would be of interest for re-examining the current models of stress response, and possibly, the potential for its biological significance in oxidative stress to be conserved in its mammalian counterpart.

Using *brap-2(ok1492);gst-4p::gfp* expressing transgenic animals as the basis of our genetic screen, we have revealed 20 possible regulators of *gst-4* expression. From these candidates, *elt-3* was identified as potential regulator for *gst-4* regulation. *elt-3* was initially identified as part of the GATA transcription factor family in *C. elegans*, and is expressed in the embryonic epidermis during development [38-40]. It has recently been

reported that *elt-3* is vital to the regulation of aging, where it is responsible for changing the expression of specific genes such as *sod-3,* involved in somatic aging [41]. Furthermore, it was also revealed that *elt-3* is regulated by the DAF-2 insulin-like signaling pathway, where *elt-3* loss-of-function reverses lifespan extension caused by mutant *daf-2*. However, another report refuted this, claiming that there is no direct evidence for this interaction, which demonstrates that *elt-3* directly controls intestinal genes associated with aging in *C. elegans* [42-45]. The potential for an indirect influence of intestinal gene expression cannot be ruled out. Our results show that partial *gst-4* suppression can be caused by the loss of *elt-3* in the absence of *brap-2*. We were also able to provide evidence that mutant *elt-3* suppressed the induction of *gst-4* following exposure to arsenite or paraquat. ELT-3 is also required for enhanced *skn-1c* expression in *brap-2(ok1492)*. In addition, we were able to show that SKN-1 and ELT-3 hetero-dimerize to enhance transcriptional activity of phase II target genes through pull-down and Luciferase assay respectively, which indicates that ELT-3 may be involved in the regulation of this stress signaling pathway, along with other still unidentified regulators to facilitate stress signal transduction. These results were consistent with our hypothesis whereby, ELT-3 plays an important role in co-activating SKN-1 in response to stress.

The transcription factor Nrf2 is a regulator of the major pathways that control detoxification and oxidative stress responses in humans [46]. Highly conserved among species, defective Nrf2 regulation is commonly found in human diseases such as cancer and Parkinson's disease. For this reason, Nrf2 is used as a candidate target in designing therapeutic agents for attenuation of oxidative stress. The molecular identity and role of Brap2/IMP in MAPK in mammalian cells, through response to external stimuli, provide

us with a novel-regulating mechanism in the Ras signaling pathway. To date, we have defined *C. elegans* BRAP-2 as a negative regulator of SKN-1, where *brap-2(ok1492)* caused the localization of SKN-1 to intestinal nuclei, and enhanced *gst-4* expression in response to stress.

Oxidative stress is critical in many cancer and neurodegenerative diseases. Currently there are no reports describing a direct connection between BRAP2 and Nrf2 in the mammalian system. The work presented here provides insight into BRAP-2 and its role in the oxidative stress response. We believe that using *C. elegans* to study the coordination of ERK/MAPK signaling through regulators such as BRAP-2, and components of the Ras signaling pathway enable us to gain a better understanding of diseases caused by stress, as well as identify new targets for future therapies. The study of *C. elegans* mutants lacking BRAP-2 regulation have been shown to be highly sensitive to oxidative stress, providing us with a valuable genetic tool to reveal a possible new role for BRAP-2/IMP in the regulation of the SKN-1/ELT-3 complex in response to oxidative stress through the ERK/MAPK pathway (Figure 2.11).

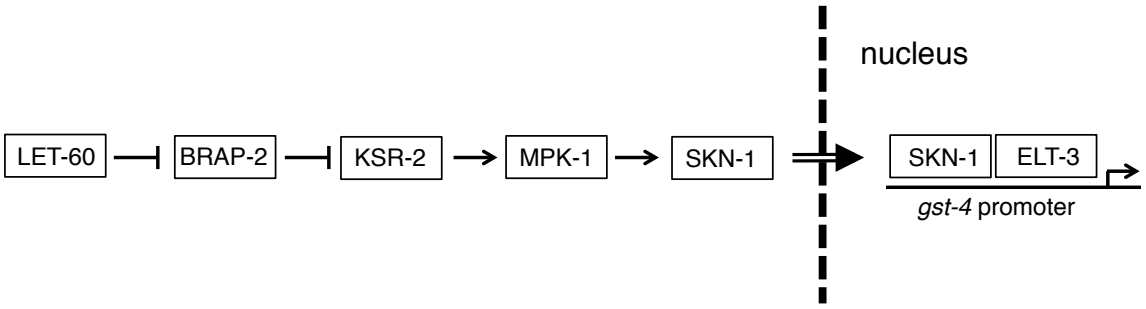

Figure 2.11 Proposed model of *C. elegans* BRAP-2's role in the Ras/MAP kinase pathway.

The BRAP-2 expression in the cytoplasm results in the inhibition of nuclear localization of SKN-1. When LET-60/Ras is activated, it binds to BRAP-2 and relieves its inhibition of KSR-2. KSR-2 then activates MPK-1, which in turn phosphorylates SKN-1, promoting SKN-1 nuclear translocation. In the nucleus, SKN-1 binds with ELT-3 and induces *gst-4* expression. Single line arrows represent activation, bar headed lines represents inhibition and double line arrow represents change in localization.

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2.7 Supplemental Figures

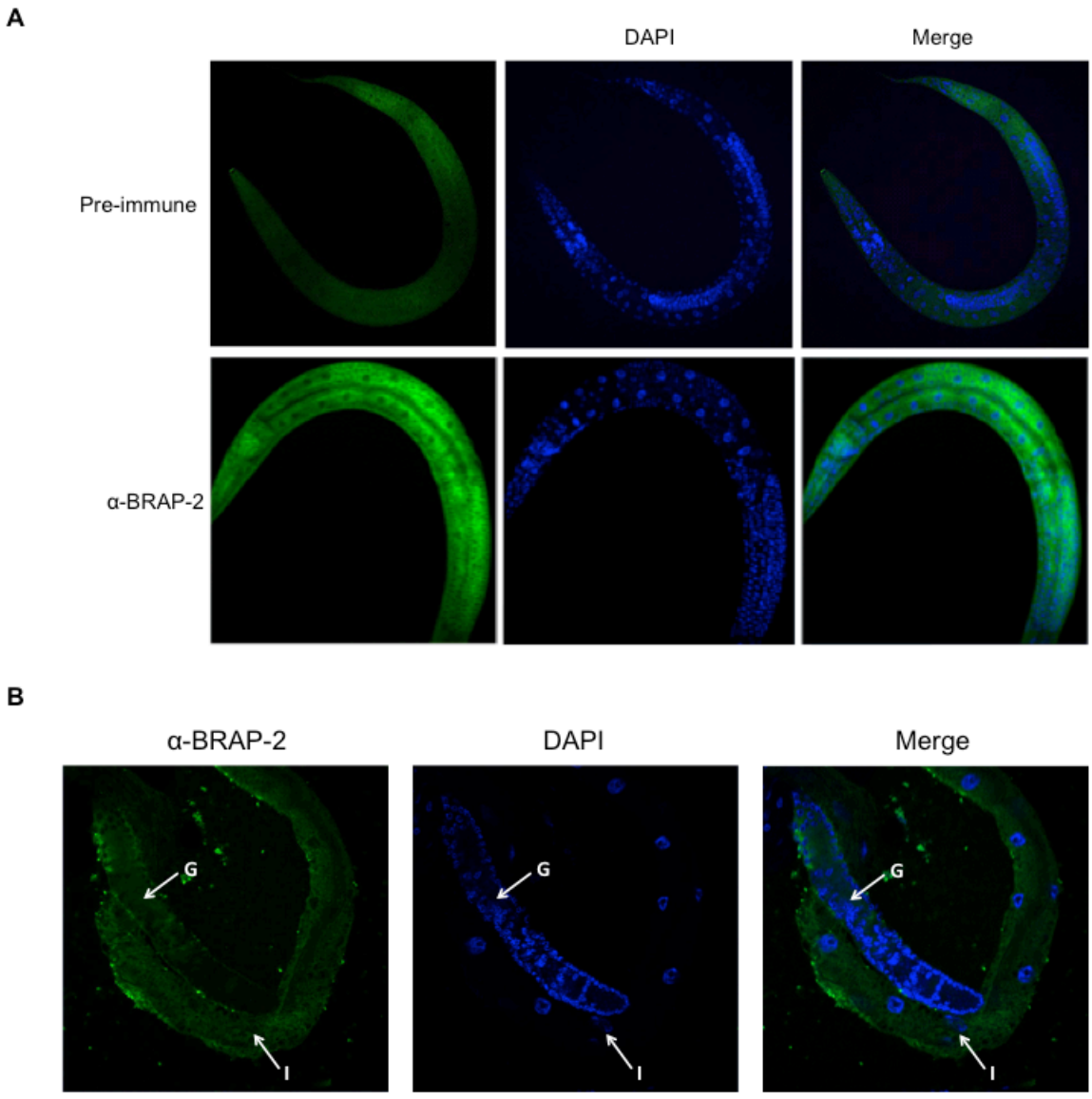

Figure S2.1 BRAP-2 is expressed in the intestine and the germ line tissues.

BRAP-2 is expressed in varies tissues. **(A)** Whole worm antibody staining probing with α-BRAP-2 reveals that BRAP-2 is visible in the intestine and the germ line in wild type L4 animals. **(B)** Gonad dissection was performed followed by probing with α-BRAP-2 antibody, where the antibody staining illustrated BRAP-2 is expressing in the intestine (I) and gonad (G).

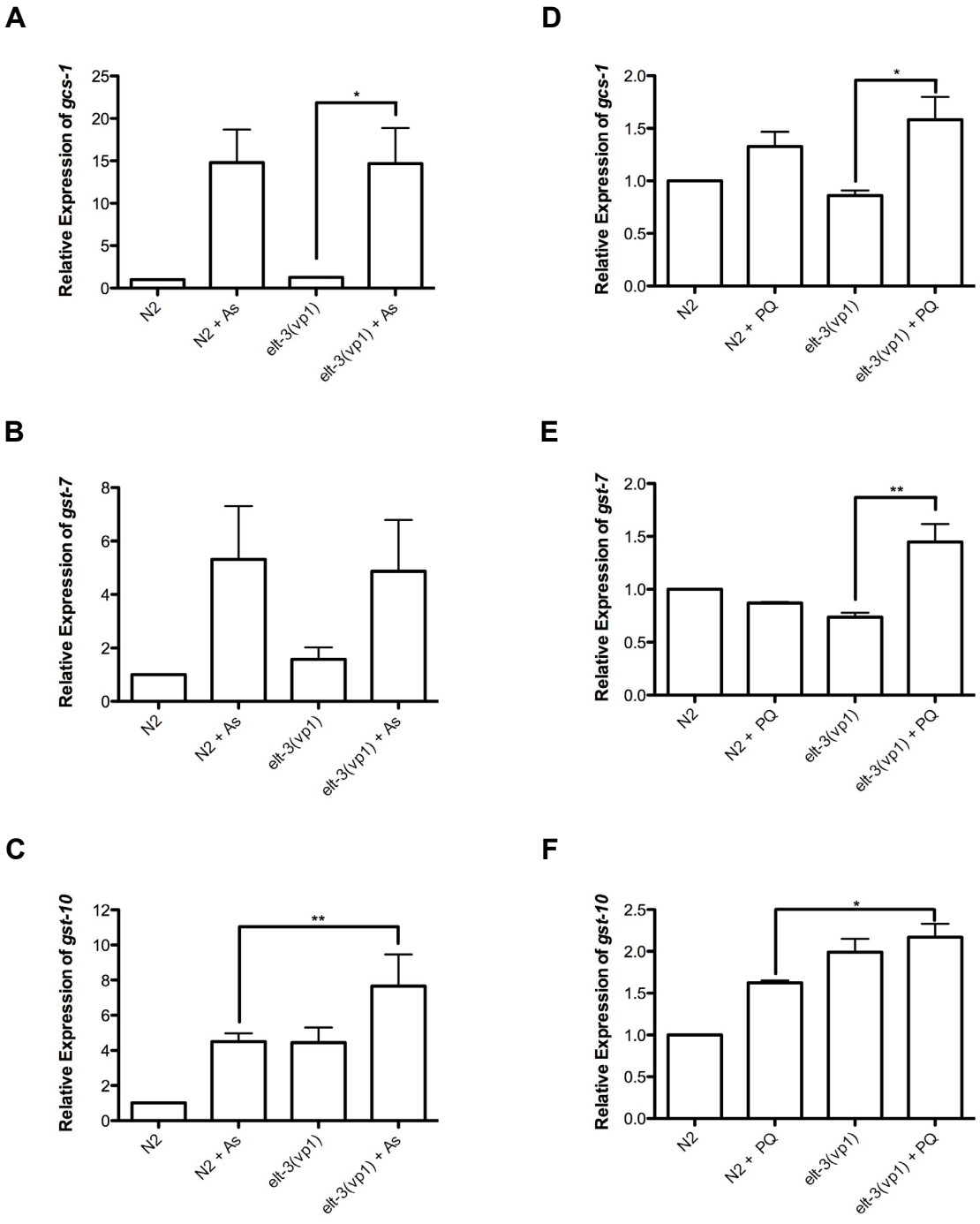

Figure S2.2 ELT-3 is not required for enhanced *gcs-1*, *gst-7* or *gst-10* expressions upon exposure to oxidative stress reagents.

Synchronized worms were collected and treated with **(A-C)** 5 mM sodium arsenite or **(D-F)** 100 mM paraquat for 2 hours followed by RNA isolation. qRT-PCR was performed and the SKN-1 targets *gcs-1*, *gst-7* and *gst-10* mRNA transcript levels were measured. Statistical analyses were conducted using one-way ANOVA with Tukey's test; $p<0.05$*, $p<0.01$**.

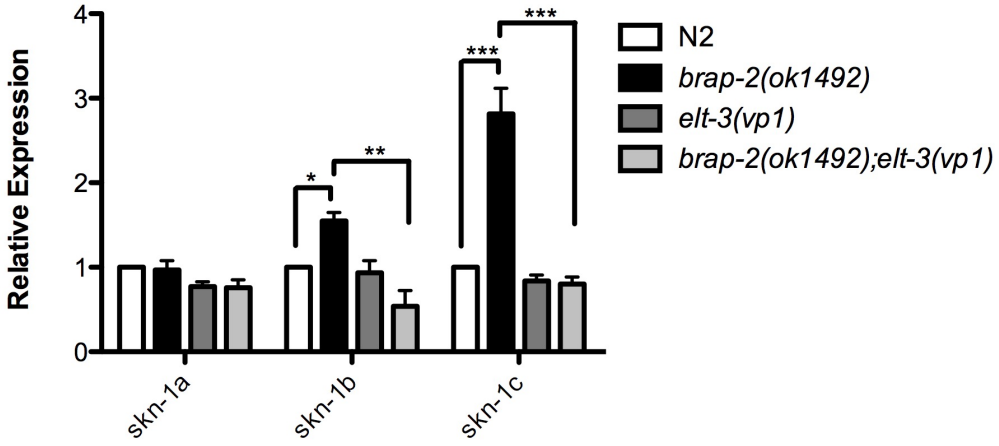

Figure S2.3 ELT-3 is required for enhanced *skn-1* expression in *brap-2(ok1492)* mutants

Various worm strains were grown on NGM plates followed by RNA isolation and quantification of *skn-1a*, *skn-1b* and *skn-1c* mRNA transcript levels using qRT-PCR. The assay indicated ELT-3 is required for *skn-1b* and *skn-1c* expression in *brap-2(ok1492)*. Statistical analyses were carried out using one-way ANOVA with Tukey's test; $p<0.05$*, $p<0.01$**, $p<0.001$***.

Chapter 3

NHR-49 is required for the SKN-1 dependent oxidative stress response in *Caenorhabditis elegans*

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AUTHORS CONTRIBUTIONS

NHR-49 is required for the SKN-1 dependent oxidative stress response in *Caenorhabditis elegans*

Queenie Hu, Dayana R. D'Amora, Lesley T. MacNeil, Marian Walhout and Terrance J. Kubiseski

Manuscript in preparation

All figures and the data presented in this chapter are mostly my own efforts with the following exception. Lesley T. MacNeil and Marian Walhout performed the RNAi screen as shown in Table 2. Dayana R. D'Amora assisted in designing Luciferase assay presented in Figures 3.4 and 3.5B

Terrance J. Kubiseski, Dayana R. D'Amora and I are responsible for drafting the manuscript.

3.1 Summary

The overproduction of reactive oxygen species (ROS) in cells can lead to the development of deleterious diseases associated with aging. Thus in order to survive, tightly regulated detoxification mechanisms have been developed in organisms to defend against oxidative stress. The human transcription factor Nrf2 is a well-documented, master regulator of detoxification genes. The *C. elegans* SKN-1 (ortholog of Nrf2) is a widely studied transcription factor with many essential roles including, organismal development, longevity and stress resistance. Our lab has previously shown that *C. elegans* BRAP-2 (Brca-1 associated binding protein 2) has a role in up regulating phase II genes such as *gst-4*, by increasing the nuclear localization of SKN-1 through the Ras signaling pathway. To further investigate this BRAP-2/SKN-1 mediated pathway regulation, a transcription factor RNAi screen was conducted to explore potential activators required to induce *gst-4* in *brap-2(ok1492)*. Among 20 positive candidates, the screen revealed that the lipid metabolism regulator NHR-49/PPARα is necessary to promote this biological effect. Here, we provide evidence that knockdown of *nhr-49* suppresses enhanced *gst-4* expression caused by a mutation in *brap-2*. The NHR-49 mediator, MDT-15, also has an important role in this regulation. We also demonstrated that *nhr-49* and *mdt-15* are required to express SKN-1 target genes upon exposure to chemicals such as paraquat and sodium arsenite. Lastly, lifespan analyses indicate that functional NHR-49 is crucial for longevity in worms overexpressing SKN-1. Therefore the regulation of BRAP-2, NHR-49 and SKN-1 are essential for the activation of phase II genes. These findings demonstrate a new role for the fatty acid metabolism regulator NHR-49 as a stress gene regulator. Using *C. elegans,* a simple and yet highly conserved model, has provided us with a better understanding of new transcriptional and cellular

signaling mechanisms involved in stress responses and longevity that can be applied to higher organisms.

3.2 Introduction

In nature, cells may encounter both exogenous and endogenous stressors that could alter normal physiological processes. One such form of stress is reactive oxygen species (ROS), which have a potent ability to threaten cell survival. Indeed, it is well documented in literature that the imbalance between ROS and protective detoxification enzymes can lead to extensive oxidative damage to macromolecules such as DNA, lipids and proteins [1, 2]. Therefore to protect cells against oxidative stress, organisms have developed lines of defense in order to cope with changes in levels of ROS to maintain homeostasis. The regulation of detoxification genes frequently involves complex transcriptional regulatory networks, and as a result the potential for cross-talk between stress signaling pathways and their targets increases. The induction of detoxification genes could be a by-product of interactions between two or more transcription factors [3]. Thus, in order to understand the genetic regulatory network involved in maintaining cellular integrity it is vital to identify these factors that regulate genes that promote survival.

Like mammals, the nematode *C. elegans* has well-defined stress defense systems for protection from toxic compounds [4]. These signaling pathways and their regulation share evolutionary conservation with their mammalian counterparts [5]. Thus *C. elegans* offers us an ideal model in dissecting stress genes and their regulatory network. In recent years, increased attention has been paid to the conserved transcription factors, DAF-16/FOXO and SKN-1/Nrf2 in *C. elegans*, due to their associated roles in regulating transcription in response to extended lifespan and oxidative stress [6-9]. These factors have been shown to regulate the transcriptional activities of essential detoxification genes

such as *sod-3* and *gst-4* respectively, for resistance against oxidative stress [10-12]. Although the signaling pathways and mechanisms that control the nuclear localization of both transcription factors have been revealed, the oxidative stressors that activate these specific pathways and distinct target genes remain poorly understood. Therefore, defining the specificity of transcriptional regulation is needed to reiterate the notion that cellular stress response signaling is a cooperative and yet complex network.

The mammalian BRAP2 was first identified as a potential Brca1 binding protein that recognizes its nuclear location signal motif, and may prevent Brca1 from translocating to the nucleus [13]. Further studies have suggested that Brap2 is a Ras-responsive E3 ubiquitin ligase for ubiquitination, a crucial posttranslational modification for protein degradation [14]. In addition, it also functions as a modulator in Ras signaling pathways, facilitating activation of the MAP kinase cascade upon cell stimulation [15-17]. Recent work in *C. ele*gans has implicated BRAP-2 in the regulation of stress pathways, where animals with loss of functional *brap-2* display hypersensitivity when exposed to hydrogen peroxide or paraquat, leading to lethality or developmental arrest early in larval development [18]. Furthermore, we recently found that BRAP-2 negatively regulates the transcription factors SKN-1/ELT-3 through the Ras signaling pathway for the induction of the phase II detoxification gene *gst-4*. An initial RNAi screen shows that NHR-49 (nuclear hormone receptor), a transcription factor with a role in lipid synthesis and potentially for stress gene regulation, suppresses *gst-4* expression in *brap-2(ok1492)*. Therefore it will be of our interest to pursue the role of NHR-49 in aging and stress response.

NHRs are a group of ligand-activated transcription factors that can regulate many physiological processes such as metabolism or development in diverse metazoan. Early work on the *C. elegans* NHR-49 transcription factor has defined it as key component of the "fasting response" that leads to fatty acid metabolism for both basal or starvation state. Van Gilst *et al.* proposed *nhr-49* could promote fat breakdown or mobilization of fat for energy in response to food availability (Figure 1.5) [19-21]. Therefore loss of *nhr-49* causes an increase in the worm's body fat and stimulates an impaired nutritional response. Additionally, *nhr-49* mutants exhibit a dramatically shortened lifespan compared to the wild type [22]. In fact, similar studies indicated that short lifespan in these mutants is caused by an imbalance in lipid composition leading to lipo-toxicity in animals (an increase in saturated to unsaturated fat ratio) [23]. Although there is a correlation between the accumulation of fat and longevity, the molecular basis of this phenomenon is not well understood. The influence of NHR-49 in fatty acid metabolism is executed in conjunction with a mediator subunit known as MDT-15 [24]. Studies in *C. elegans* demonstrated that the loss of *mdt-15* fails to regulate *nhr-49*-dependent fasting response genes. Recent work done by Goh *et al.* suggest MDT-15 interacts with the master regulator SKN-1 to facilitate oxidative metabolism and promote lifespan in an *nhr-49* independent manner [25]. However, in a recent RNAi screen we performed showed that NHR-49 is able to regulate phase II detoxification genes in response to *brap-2(ok1492)* mutation. A new area of interest is to study the connection between fat metabolism and extended life span [26]. Therefore whether the fat regulator NHR-49 and the stress regulator SKN-1 function in a combinatorial fashion or through parallel pathway to promote the induction of oxidative stress genes remains to be explored.

Here we report that the transcription factor NHR-49 and its mediator MDT-15 are essential for phase II detoxification response, and this regulation is dependent on SKN-1. We also provide evidence to suggest that functional NHR-49 is required in the BRAP-2/SKN-1 detoxification pathway. Furthermore, we demonstrate that lifespan extension caused by SKN-1 over-expression requires NHR-49. Ultimately, we were able to provide evidence for a novel pathway of NHR-49/SKN-1 regulation, where oxidative stress induction in *brap-2(ok1492)* activates the Ras/MAPK pathway leading to increased SKN-1 phosphorylation by MPK-1 and nuclear import. Here, activated SKN-1 is able to bind to NHR-49/MDT-15 in the nucleus and this transcription factor complex induces *gst-4* expression. These observations have contributed to uncovering a novel pathway for the prevention of oxidative stress and aging. The elucidation of SKN-1 co-activators for the control of this transcriptional regulation is insightful, as it not only provides critical information in mapping the complex regulatory network for stress genes regulation, but may also assist in the development of anti-stress drugs for future treatment of diseases.

3.3 Experimental Procedures

3.3.1 *C. elegans* Strains

All *C. elegans* strains were maintained as described by Brenner [27]. Worm strains were provided by the *Caenorhabditis* Genetics Center (CGC, University of Minnesota). Several strains were kind gifts and acknowledged accordingly. Double mutant strains were generated according to standard protocols Strains used in this study were as follows: Bristol strain N2, *dvIs19* (CL2166). *ldIs008* (LD1250) (gift from Keith Blackwell lab), *brap-2(ok1492)* (YF15), *nhr-49(ok2165)* (YF127), *brap-2(ok1492);nhr-49(ok2165)* (YF126), *mdt-15(tm2182)* (XA7702) and *brap-2(ok1492);mdt-15(tm2182)* (YF131), *nhr-49(nr2041)* (STE68), *nhr-49(et7)* (STE108), *nhr-49(et8)* (STE109), *nhr-49(et13)* (STE110) (STE108, STE109 and STE110 are gifts from Stefan Taubert lab). Unless otherwise specified, all experiments were performed at 20°C.

3.3.2 RNAi Treatment

RNAi assay was performed by feeding worm strains with *E. coli* HT115 (DE3), transformed with the bacterial plasmid pL4440 control or transcribed double stranded RNA (dsRNA) homologous to the target gene cloned into pL4440. The dsRNA was grown on nematode growth medium (NGM) containing 0.4 mM IPTG, 100 µg/mL ampicillin and 12.5 µg/mL tetracyclin. Synchronized worms were grown on these RNAi plates and collected when animals reached L4 stage, followed by *gfp* expressions analysis using confocal microscope or RNA isolation.

3.3.3 Phenotypic Analysis and Fluorescence microscopy

Live L4 *gst-4p::gfp* expressing worms were picked and anesthetized using 25 mM Levamisole (Sigma L9756) and mounted on 2% agarose pad. Images of fluorescent

worms were taken using a Zeiss LSM 700 confocal laser-scanning microscope with Zen 2010 Software®.

3.3.4 Drug Treatment

For each strain, synchronized worms were grown on NGM plates and were collected at L4 stage using M9 buffer. Sodium arsenite (Sigma #35000) or paraquat (Sigma #856177) were diluted in M9 buffer to a final concentration of 5 mM and 100 mM, respectively. Collected worms were treated in each drug at room temperature for 2 hours followed by RNA isolation and qRT-PCR. Each experiment was completed in triplicate.

3.3.5 RNA Isolation and Quantitative Real Time PCR

Mixed-stage worms were collected and washed with three times with M9 buffer. Total RNA was extracted with TRI Reagent (Sigma #93289) according to the manufacturer's protocol, with some modifications. The pelleted RNA was recovered with RNase-free water and residual DNA in the sample was digested using the DNase Free DNA removal kit (Ambion AM1906). Total RNA concentration was measured using Fisher Thermo NanoDrop2000 and 0.5 µg of RNA was used to produce cDNA by reverse transcription using RNA to cDNA kit (Applied Biosystem #4387406) following the manufacturer's protocol. Quantitative real-time PCR was conducted to measure the designated mRNA transcript level using SYBR green premix (Clontech #693676) and the Qiagen Rotor-gene Q system. qRT-PCR data were derived from 3 independent replicates and were analyzed using the comparative method (ΔΔCt). Results were graphed and the relative expression of each strain was compared to N2. The endogenous control used for normalization was *act-1* unless otherwise specified.

3.3.6 Luciferase Assay

Human Embryonic Kidney (HEK) 293T cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. Cells were transfected with the Firefly promoter plasmid (0.25 μg), the designated transcription factor plasmids (0.25 μg) and the pRL-TK Renilla internal control (0.03 μg) in a 12-well plate. Cells were collected after 48 hours of transfection and Luciferase assay was performed with the Promega Dual-Glo Luciferase Assay System Kit (E2920) according to the manufacturer's protocol. Each transfection was performed in duplicate and the samples were then averaged. The Firefly and Renilla luminescence signals were detected using the BioTek Synergy H4 plate reader. Three independent experiments were performed, where values were normalized relative to the empty vectors control.

3.3.7 Lifespan Analysis

Lifespan assay was performed as previously described in Wilkinson *et al.* [28]. The long-lived strain *ldIs008* and N2 were synchronized and raised at 20°C on RNAi plates containing the designated RNAi bacteria. For each strain, 40 worms were picked and put on L4440 (control) or *nhr-49* RNAi seeded plates. Worms were transferred to fresh RNAi plates (away from progeny) and survival was scored every day in response to prodding. Statistical analysis was performed using the online survival analysis program OASIS® [29].

3.3.8 Statistical Analysis

Graphs presented here were generated using GraphPad Prism 5 (GraphPad Software Inc®). Unless otherwise specified, the statistical analysis was performed using one-way ANOVA with Tukey's test at 95% confidence interval.

3.4 Results

3.4.1 The transcription factor NHR-49 and its mediator subunit MDT-15 are required to regulate *gst-4* in *brap-2(ok1492).*

Previously, we have shown that *C. elegans* BRAP-2 is required to regulate the transcription factor SKN-1 to induce phase II detoxification genes. To further the study of this gene regulatory network, a transcription factor RNAi screen was conducted to investigate potential regulators that may be essential to up regulate *gst-4* in *brap-2(ok1492)* mutant worms. Our screen identified 20 RNAi clones that substantially decreased *gfp* expression in *brap-2(ok1492)*, and NHR-49 was one such positive candidate. Thus, we first validated the result of this screen by performing an RNAi assay to knockdown *nhr-49*. L4 *brap-2(ok1492)* carrying the *gst-4p::gfp* transgene was fed on *nhr-49* RNAi and the intestinal GFP expression was examined. The *brap-2(ok1492);gst-4p::gfp* worms treated in *nhr-49* RNAi showed a weak *gst-4p::gfp* expression compared to the controls (Figure 3.1A). To confirm that the GFP expression observed following the RNAi assay was not an artifact, a *brap-2(ok1492);nhr-49(ok2165)* double mutant was generated and the levels of *gst-4* were quantified using qRT-PCR. Results indicated an 80% reduction of *gst-4* mRNA in the double mutant (Figure 3.1B). Taken together, this indicates that *nhr-49* is essential for promoting *gst-4*.

The NHR-49 transcription factor requires the mediator MDT-15 to modulate downstream gene expression and lipid composition [24]. Furthermore, recent studies show that MDT-15 contributes to detoxification gene induction, where MDT-15 is shown to be a SKN-1 interacting partner and is required in response to oxidative stress [23, 25, 30]. Therefore, to determine if MDT-15 is also needed to up-regulate *gst-4* expression in *brap-2(ok1492)*, a *mdt-15* RNAi assay was performed to knockdown *mdt-15* in *brap-*

2(ok1492);gst-4p::gfp. Additionally, the double mutant *brap-2(ok1492);mdt-15(tm2182)* was generated and *gst-4* mRNA levels were also measured using qRT-PCR. As predicted, loss of *mdt-15* showed a reduction in *gst-4* levels (Figure 3.1A and 3.1C), indicating that MDT-15 also plays a significant role in *gst-4* regulation.

The function of NHR-49 has been associated to lipid regulation and storage, and limited studies have shown a direct relation of *nhr-49* to stress gene regulation. Therefore it would be beneficial to further the study of NHR-49 in this context. Since we have shown that *nhr-49* is required for the expression of the phase II gene *gst-4*, we hypothesized an increase in *gst-4* transcript levels would be seen with an overexpression of *nhr-49* (Figure 3.2A). *gst-4* mRNA levels in gain-of-function *nhr-49* strains were quantified and a close to 2-fold increase of *gst-4* is seen in these worms (Figure 3.2B). Furthermore, we also wanted to determine if NHR-49 dependent phase II gene induction requires SKN-1. Strains overexpressing *nhr-49* were fed with *skn-1* RNAi and then *gst-4* mRNA levels were measured using qRT-PCR. The depletion of *skn-1* caused a decrease in *gst-4* expression when compared to the untreated RNAi control (Figure 3.2C). Taken together, the evidence presented here suggests that together NHR-49 and SKN-1 are required to promote the induction of SKN-1 target genes.

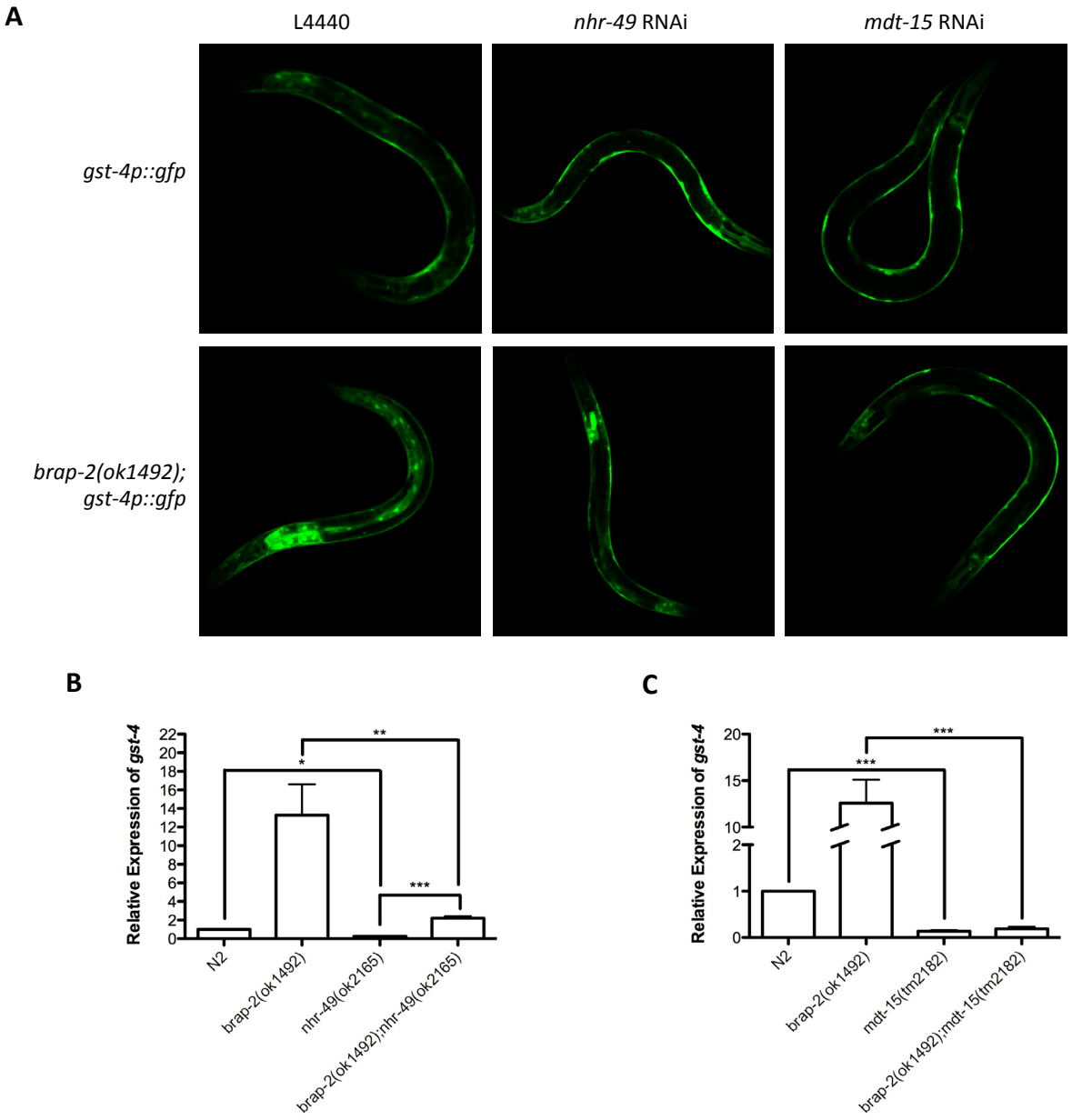

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Figure 3.1. *nhr-49* and *mdt-15* are essential for enhanced *gst-4* expression.

(A) *gst-4p::gfp* and *brap-2(ok1492);gst-4p::gfp* worms were treated with *nhr-49* or *mdt-15* RNAi followed by examination of the GFP expression using confocal microscopy. Representative GFP images show *brap-2(ok1492);gst-4p::gfp* worms grown in both *nhr-49* or *mdt-15* RNAi causes a reduction of *gst-4p::gfp* expression in the intestine. Twenty worms were examined and the images depict one worm. **(B, C)** Following RNA extraction, *gst-4* mRNA levels were quantified with qRT-PCR. Relative *gst-4* expression is reduced in both **(B)** *brap-2(ok1492);nhr-49(ok2165)* and **(C)** *brap-2(ok1492);mdt-15(tm2181)*; $p<0.05$*, $p<0.01$**, $p<0.001$***.

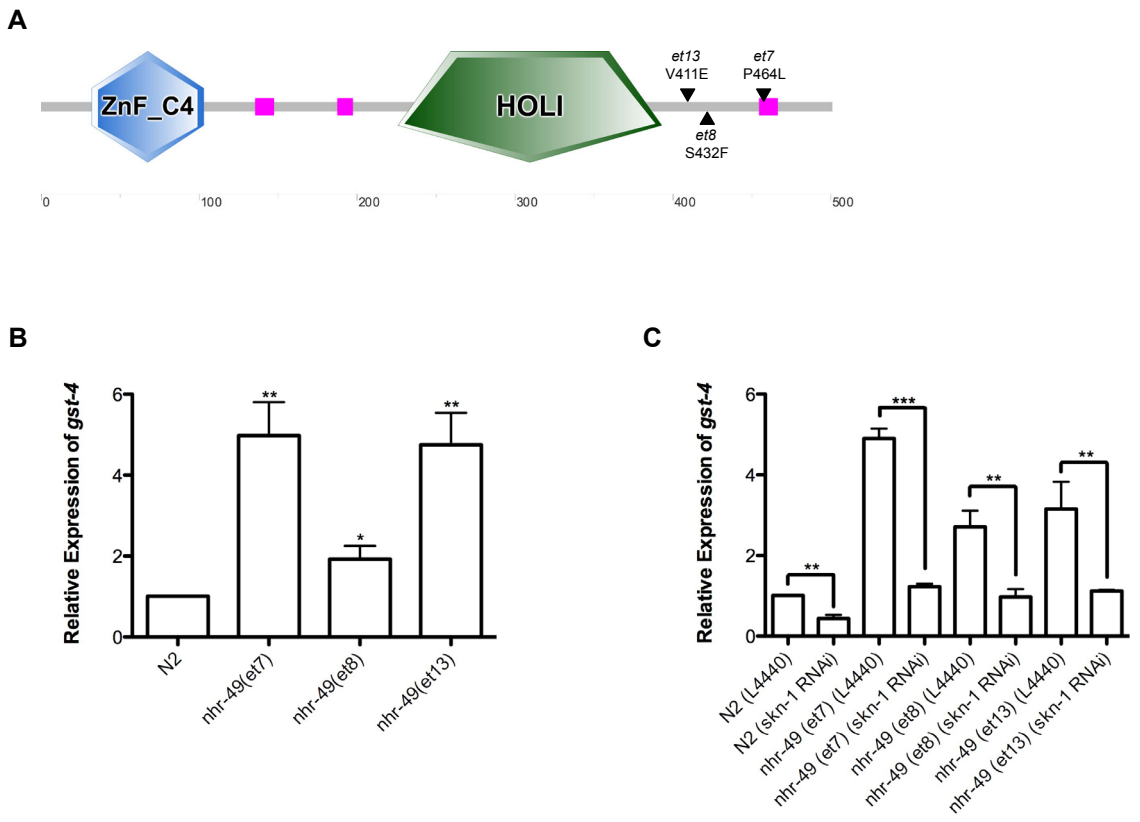

Figure 3.2. *skn-1* is required to regulate *gst-4* expression in gain-of-function *nhr-49* worms.

(A) A schematic representation depicting the major domains of NHR-49. The full length *C. elegans* NHR-49 (501aa) contains a Zinc Finger domain (ZnF_C4, residues 32-103) and a ligand binding domain of hormone receptors (HOLI, residues 226-393). The positions of point mutations that result in three gain-of-function *nhr-49* strains are indicated with arrowheads. The figure was generated using EMBL SMART database (http://smart.embl-heidelberg.de/). **(B-C)** RNA was extracted from synchronized L4 worms followed by quantification of *gst-4* transcript levels using qRT-PCR. **(B)** The three gain-of-function *nhr-49* strains were used to examine *gst-4* mRNA expression; results display an increase in *gst-4* mRNA by at least 2-fold. **(C)** The *nhr-49* gain-of-function strains were treated with control (L4440) or *skn-1* RNAi inducing plasmids and *gst-4* mRNA levels were quantified. A reduction of *gst-4* was seen with *skn-1* knockdown compared to strains fed on the L4440 control; $p<0.01$**, $p<0.05$* vs N2 in **(B)**; $p<0.001$***, $p<0.01$** vs L4440 control in **(C)**.

3.4.2 *skn-1*, *mdt-15* and *nhr-49* are all essential to induce expression of *gst-4* in response to sodium arsenite and paraquat.

In *C. elegans*, oxidative stress can be induced using sodium arsenite or paraquat, both of which have been shown to significantly increase phase II detoxification enzymes in wild type worms [10]. Previous studies have already suggested that *skn-1* and *mdt-15* are required to up regulate phase II detoxification genes upon arsenite induction [25]. Hence, we are also interested to investigate if NHR-49 is essential for the transcriptional regulation to stress response genes. Here we grew synchronized wild type worms (N2 or *dvIs19*) and knocked down *nhr-49*, *mdt-15* or *skn-1* using RNAi, followed by exposure to 5 mM sodium arsenite or 100 mM paraquat for 2 hours. GFP expression in *dvIs19* worms were examined using confocal microscope (Figure 3.3A). The wild type worms show an increase of *gst-4p::gfp* expression within intestinal nuclei upon exposure to arsenite or paraquat. This fluorescence is reduced following *nhr-49*, *mdt-15* and *skn-1* RNAi. To further demonstrate *nhr-49* is required to induce oxidative stress genes, synchronized N2 worms were fed on *nhr-49* RNAi followed by drug treatment. qRT-PCR was then performed to quantify levels of *gst-4*. As seen in Figure 3.3B and 3.3C, similar results were observed with qRT-PCR as were observed in the *gst-4p:gfp* expressional phenotype. Furthermore, to verify the results of the RNAi assay the *gst-4* transcript levels were also quantified in the *nhr-49(ok2165)* and *nhr-49(nr2041)* mutant strains (Figure S3.1A). As predicted, both *nhr-49* mutants display a reduction of *gst-4* mRNA when exposed to sodium arsenite or paraquat (Figure S3.1 B,C). Taken together, these results indicate a dramatic decrease of *gst-4* mRNA with knockdown of *nhr-49*, *mdt-15* or *skn-1*, demonstrating the importance of these transcription factors and their mediator in the regulation of oxidative stress genes upon exposure to stress inducing drugs.

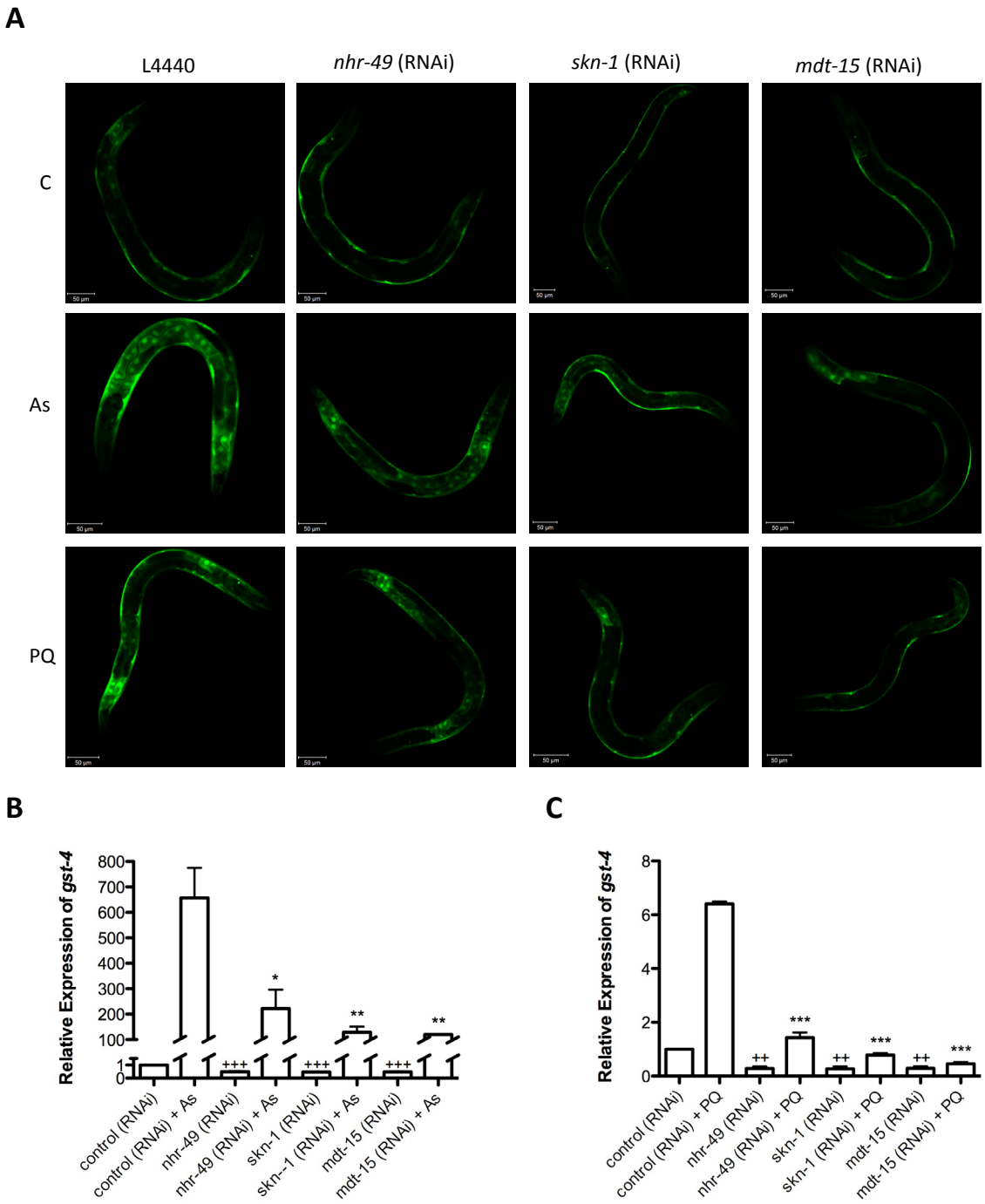

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Figure 3.3. *skn-1*, *mdt-15* and *nhr-49* are essential to induce arsenite and paraquat responsive gene *gst-4* in L4 worms

(A) Synchronized *gst-4p::gfp* worms were grown in the control (L4440), *skn-1* RNAi, *nhr-49* RNAi or *mdt-15* RNAi followed by exposure to M9 buffer (C), 5 mM sodium arsenite (As) or 100mM paraquat (PQ) for 2 hours at L4 stage. Worms were recovered on NGM plates for 1 hour and the GFP expression was examined using confocal microscop. Results show a reduction in GFP levels in RNAi treated worms. Twenty worms were examined and figures depict one worm. **(B-C)** Synchronized L4 stage worms were collected after RNAi exposure and drug treatment followed by RNA extraction, and *gst-4* mRNA transcript levels were quantified using qRT-PCR. Values are relative to the control (RNAi) and normalized to the endogenous control *act-1*. The knock down of either *nhr-49*, *skn-1* or *mdt-15* exhibit a decrease in *gst-4* mRNA expression after **(B)** Arsenite or **(C)** paraquat treatment in comparison to the N2 drug-treated control; $p<0.001^{+++}$, $p<0.01^{++}$ vs untreated control, $p<0.001$*** vs drug treated control in **(B-C)**.

3.4.3 Increase in *gst-4* transcriptional activity caused by NHR-49 and MDT-15 is dependent on SKN-1

The binding of transcription factors to specific DNA regions is vital for dissecting the complexities of the stress induced transcriptional regulatory network and the quantitative level of gene expression of their targets. Thus, identifying the sites that transcription factors bind in order to cause genes to "turn on and off" is of upmost importance. The modENCODE Consortium has been working on one such project, ChIP-Seq an ongoing exploration of transcription factor DNA binding sites on promoter regions throughout the *C. elegans* genome [31]. Although modENCODE has identified at least 70,000 transcription factor binding sites in the worm genome, a direct binding for NHR-49 on the *gst-4* promoter was not predicted [32]. Therefore we sought to determine how *nhr-49* could regulate stress genes without a direct transcription factor-DNA interaction. Since SKN-1 is already known to interact with and bind to the *gst-4* promoter, we hypothesized the possibility of NHR-49 may function as a co-activator of SKN-1 mediated gene regulation. To test this, we created a luciferase reporter construct containing the *gst-4* promoter sequence, and co-transfected it into HEK293T cells along with SKN-1 and/or NHR-49 plasmids and conducted a Luciferase assay. In the presence of SKN-1 a 10-fold increase in *gst-4* promoter activity is observed. While, only a moderate increase in promoter activity is produced with NHR-49 alone. However, when both SKN-1 and NHR-49 are present, a substantial increase in *gst-4* transcriptional activity is detected. Previous studies have proposed that the mediator MDT-15 is an integral component of the NHR-49 and SKN-1 regulated pathways [24, 25]. Therefore we asked whether the presence of MDT-15 would also enhance *gst-4* transcription. As seen in Figure 3.4, co-transfection of SKN-1 with MDT-15 displays a 10-fold increase in

gst-4 transcriptional activity, while no change was observed in the presence of NHR-49 and MDT-15. Taken together, these results suggest that NHR-49 and MDT-15 redundantly promote SKN-1 activation of the *gst-4* promoter.

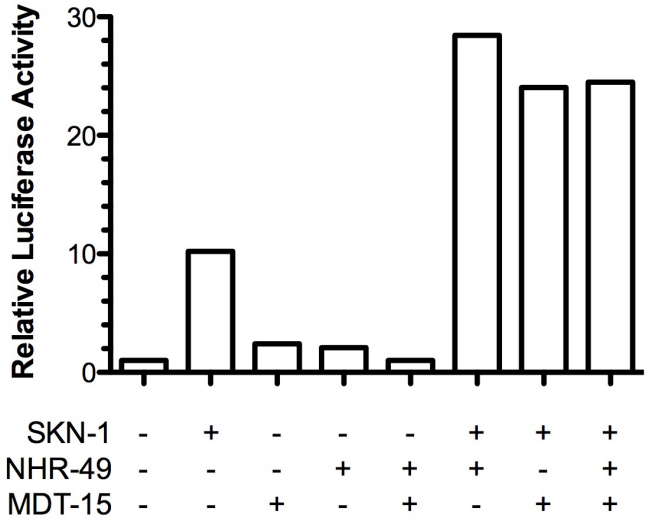

Figure 3.4. The regulation of *gst-4* by SKN-1 is dependent on the transcription factor NHR-49 or the mediator MDT-15.

A Luciferase assay was performed and the transcriptional activity was determined by quantifying the activation of the *gst-4* promoter-luciferase reporter vector. Results demonstrate at least a 2-fold increase of luciferase signal in the presence of NHR-49 and/or MDT-15 along with SKN-1, indicating they are required with SKN-1 to co-activate *gst-4* transcriptional activity. Three independent experiments were performed and results represent one trial.

3.4.4 NHR-49 and MDT-15 are required to activate *skn-1c* transcriptional activity

Previously, we provided evidence that *brap-2(ok1492)* possesses enhanced *skn-1b* and *skn-1c* mRNA levels (i.e. two *skn-1* isoforms that are required for caloric restriction and stress resistance), where this up-regulation is essential for *gst-4* expression for detoxification. In Figure 3.1B, we show that *nhr-49* is also required to induce *gst-4* in *brap-2(ok1492)*. Therefore, we hypothesized that *nhr-49* expression would be up-regulated in *brap-2(ok1492)* and in overexpressing *skn-1* worms. The *nhr-49* mRNA transcript was quantified using qRT-PCR in *brap-2(ok1492)* and *ldIs008* (overexpressing *skn-1* strain), and a 2-fold increase in the levels of *nhr-49* was observed in both strains (Figure S3.2A,B). This indicates that the activation of the BRAP-2 mediated stress response pathway will induce *nhr-49* expression.

Furthermore, we also measured the mRNA levels of *skn-1c* in *nhr-49* deletion strains. As shown in Figure 3.5A, a 2-fold increase of *skn-1c* was observed in *brap-2(ok1492)*, indicating there is a potential for accumulated SKN-1 to initiate detoxification gene induction. However, the loss of *nhr-49* in *brap-2(ok1492)* suppressed *skn-1c* transcripts, restoring it to wild type levels. This implies that *nhr-49* may play a vital part in inducing the production of *skn-1c* transcripts.

Therefore we hypothesized that NHR-49 or the SKN-1/MDT-15/NHR-49 complex can provoke a positive feedback mechanism through direct binding of the *skn-1c* promoter and enhance its transcriptional activity. The modENCODE project has detected an enrichment of SKN-1 at the *skn-1c* promoter region, but could not detect any direct binding of NHR-49, therefore to show that *nhr-49* or *mdt-15* are required to co-activate this transcriptional regulation, a Luciferase reporter construct fused with the *skn-1c* promoter sequence was created and a Luciferase assay was performed. As shown in

Figure 3.5B, the presence of either NHR-49 or MDT-15 with SKN-1 causes a 2-fold increase in *skn-1c* transcription. Taken together, these results suggest that functional NHR-49 is required to co-activate SKN-1 for enhanced *gst-4* expression in *brap-2(ok1492)*.

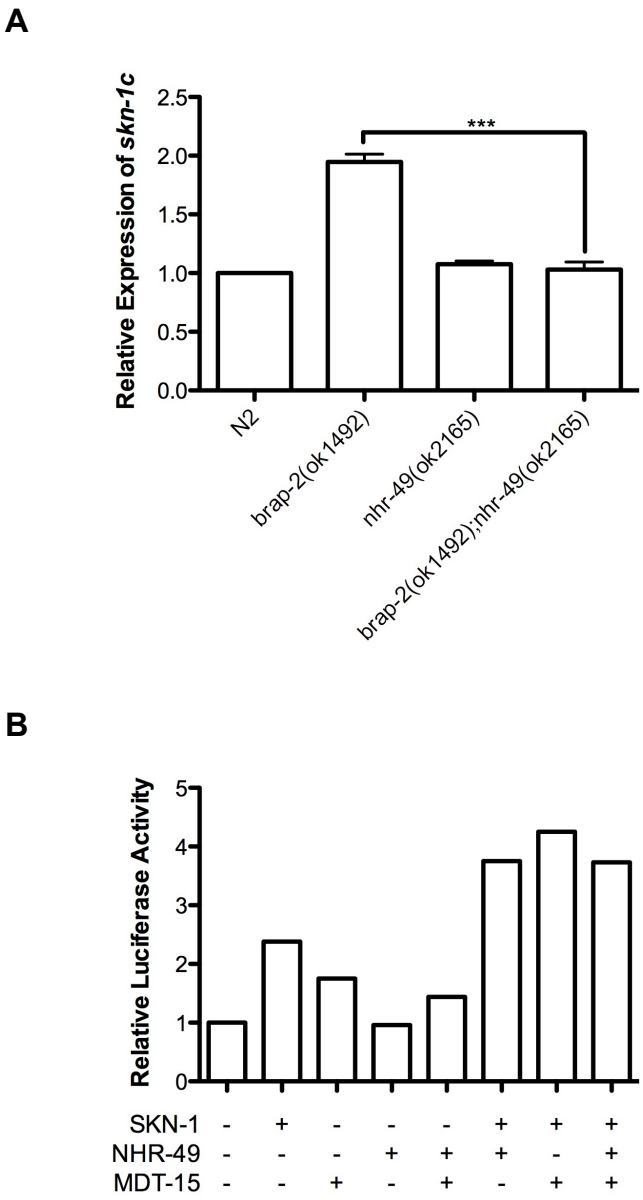

Figure 3.5. Functional NHR-49 or MDT-15 are required to promote *skn-1c* transcriptional activity.

(A) Relative *skn-1c* mRNA expression was quantified in *nhr-49(ok2165)* and *brap-2(ok1492);nhr-49(ok2165)* mutant strains using qRT-PCR. The worms displayed a 50% reduction of *skn-1c* in the double mutant compared to *brap-2(ok1492)*, $p<0.001$*** **(B)** Luciferase assay was performed and the transcriptional activity was determined by activation of the *skn-1c* promoter-luciferase reporter vector. The results demonstrate SKN-1 requires a co-activator or mediator to maximize transcription. Three independent experiments were performed, and the result represents one trial.

3.4.5 NHR-49 is required for extended lifespan in overexpressing SKN-1

SKN-1 activity is essential to induce oxidative stress genes as well as longevity response [8, 33]. It is shown that overexpressing SKN-1 promotes longevity in *C. elegans*. Impairing *nhr-49* activity also displayed shortened lifespan [19, 20]. Thus, to determine if NHR-49 is required to co-activate with SKN-1 for lifespan extension effect, we use RNAi to knock down *nhr-49* in overexpressing SKN-1 strain LD1250 and conducted a lifespan assay. As shown in Figure 3, *ldIs008*;L4440 (16.68±0.79) has a significantly longer mean lifespan than N2:L4440 (13.62±0.62), while no difference was observed between *ldIs008*;*nhr-49*(RNAi) (10.15±0.39) and N2;*nhr-49*(RNAi) (9.62±0.34). Therefore, longevity assay did support our hypothesis that the extended lifespan in over expressing SKN-1 is dependent on functional NHR-49.

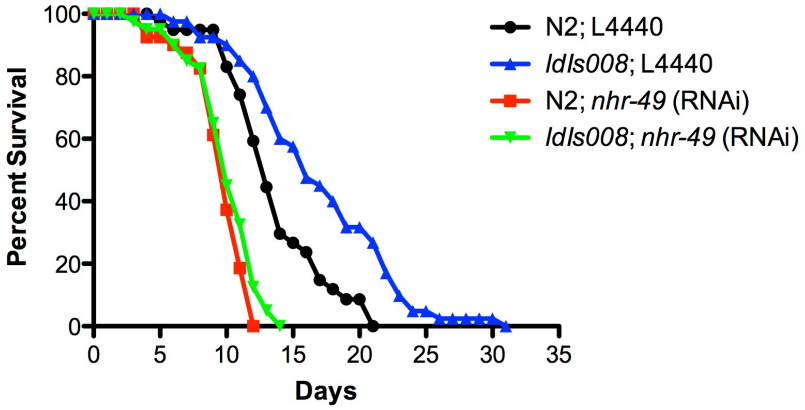

Figure 3.6. Extended lifespan in worms overexpressing SKN-1 requires NHR-49.

Survival curves of N2 and worms containing the SKN-1 transgene (*ldIs008*), fed on the control (L4440) or bacteria expressing *nhr-49* RNAi beginning at the L1 stage at 20°C. *ldIs008*;L4440 has a significantly longer lifespan than N2;L4440 with mean lifespans of 16.7±0.79 days and 13.6±0.62 days, respectively (N=40). No lifespan extension was observed in overexpressing SKN-1 worms when fed with *nhr-49* RNAi (*ldIs008* mean=10.1±0.45 days compared to N2 mean=9.6±0.38 days, N=40). Three independent experiments were performed, and the result represents one trial.

3.5 Discussion

3.5.1 Summary

ROS can have a significant biological role, serving as signaling molecules to activate pathways of the innate immune response [34]. On the other hand, a gross accumulation of these highly volatile derivatives disrupts homeostasis and causes extensive cellular damage [35]. The regulation of oxidative stress response transcription factors and their downstream detoxification genes play a vital role in balancing cellular homeostasis. Thus it is important to define the intricacies of stress gene regulation to better understand the protective mechanisms that maintain cellular physiology and structural integrity. In this study, we further investigated the role of the Ras responsive protein BRAP-2 and the transcription factor SKN-1 in the *C. elegans* oxidative stress response. Here we show that NHR-49 (PPARα ortholog) and its mediator subunit MDT-15 (MED15 ortholog) are essential to regulate SKN-1 dependent stress genes in *brap-2(ok1492)*. Notably, a functional NHR-49 and MDT-15 are crucial in responding to stress genes upon exposure to paraquat or sodium arsenite. With the use of various assays, we have found that NHR-49 and MDT-15 may co-activate SKN-1 in a combinatorial fashion for enhanced induction of *gst-4* and *skn-1c*, and these co-regulators are also necessary for lifespan extension. By investigating mRNA expression of designated target genes in null mutants, we were able to provide evidence that NHR-49 not only participates in fat metabolism, but is also a key player in oxidative stress response.

3.5.2 The introduction of NHR-49 as a new stress response regulator opens new avenues for the study of the oxidative stress response

Previously we have reported the transcription factor SKN-1/Nrf2 is essential in up regulating *gst-4* in the *ok1492* deletion mutant of *brap-2*. However, all known oxidative stress response pathways in *C. elegans*, including ERK/MAPK, act specifically on SKN-1 [36, 37]. Working knowledge of other specific transcription factors that work in combination with SKN-1 is unavailable. Their identification would serve to broaden our understanding of stress gene regulation and allow us to fully elucidate the converging signaling pathways involved in this response. To explore the idea of new co-activators we have conducted a transcription factor specific RNAi screen to identify new regulators involved in the BRAP-2/SKN-1 pathway. The screen revealed that NHR-49 is strongly required for the induction of *gst-4p::gfp* in *brap-2(ok1492)*. Furthermore, lifespan analysis also indicated that NHR-49 may potentially hetero-dimerize with SKN-1 to promote longevity. Initially, the focus of NHR-49 research in *C. elegans* explored its role in regulating fatty acid metabolism. In addition, a recent study suggested NHR-49 also helps to promote lifespan in animals lacking a germline by controlling lipid metabolic pathways [38, 39]. Although loss of *nhr-49* shows hypersensitivity to various stress inducing molecules including paraquat, no studies have implicated NHR-49 in the oxidative stress response. The transcriptional regulation of stress genes is complex. Therefore identification of NHR-49 as a potential stress regulator will help to determine its novel role in promoting longevity, a physiological process that is not only affected by lipid homeostasis, but also impacted by mobilization of the oxidative stress response.

3.5.3 NHR-49 and MDT-15 regulate phase II detoxification genes, and contribute to a positive feedback loop that promotes SKN-1C production

Similar to humans, the regulation of lipid metabolism by NHR-49 in *C. elegans* requires a mediator subunit, a co-factor that enables the enhancement of transcription initiation. Several studies showed NHR-49 interacts with MDT-15/MED15 for the downstream activation of fatty acid metabolism gene expression and involved in nutritional response [23, 24, 30]. It has recently been reported that MDT-15 executes the oxidative stress response in conjunction with SKN-1 in *C. elegans* [25]. For this reason, we wished to determine if MDT-15 is also required to regulate *gst-4* in *brap-2(ok1492)*. Our observation showed a dramatic reduction of *gst-4* expression in *brap-2(ok1492);mdt-15(tm2182)* double mutants. Since MDT-15 has a central role in regulating NHR-49 and SKN-1, it is possible the increase in *gst-4* seen in *brap-2(ok1492)* caused by NHR-49 acting on SKN-1 is also dependent on MDT-15.

Although we showed that the loss of *nhr-49* suppressed the induction of *gst-4* and other phase II detoxification genes, there is no evidence indicating NHR-49 has a direct TF-DNA interaction on the *gst-4* promoter, nor can the MDT-15/NHR-49 interaction increase the transcription of stress genes. Hence understanding how NHR-49 regulates *gst-4* transcription was unknown. To answer this question we performed a Luciferase assay and surprisingly, we were able to present that co-transfection of SKN-1/NHR-49 or SKN-1/MDT-15 amplifies *gst-4* transcriptional activity. However, the transcription level did not change in the presence of NHR-49 and MDT-15 alone, implying the interaction between NHR-49/MDT-15 is possibly acting as an enhancer and available to bind to a second transcription factor to facilitate stress signal transduction. Particularly, our data is consistent to the work done by Goh *et al*., where we were able to show that the presence

of MDT-15 is required to regulate phase II genes and increase *gst-4* transcription synergistically. This further explains that MDT-15 indeed positively co-regulates SKN-1 through a direct protein-protein interaction. Despite this, we do not see a greater increase of *gst-4* transcription in the presence of SKN-1/MDT-15/NHR-49, suggesting that MDT-15 and NHR-49 are redundant.

The regulation of gene expression is a complex circuit, and cells must often employ structural positive and negative feedback mechanisms to carefully alter transcription while at the same time coping with changes in the intracellular environment. We observed that *skn-1c* mRNA transcripts were restored to wild type levels in the *brap-2(ok1492);nhr-49(ok2165)* double mutant strain, implying that *nhr-49* could possibly have a role in influencing *skn-1c* activity. Previous modENCODE projects have shown that SKN-1 but not NHR-49 or MDT-15 can directly bind to the *skn-1c* promoter. Thus we hypothesized that SKN-1 might also have a critical role in aiding NHR-49 to promote and activate *skn-1c* transcription. To test this, a Luciferase assay was performed and maximal *skn-1c* transcriptional activity was detected when NHR-49 or its mediator MDT-15 is present with SKN-1. Accordingly, these findings support the observation that low *skn-1c* mRNA seen in *brap-2(ok1492);nhr-49(ok2165)*, and we believe the SKN-1/MDT-15/NHR-49 complex is able to create a positive feedback mechanism that ensures SKN-1C is continuously produced in response to oxidative stress for the further downstream amplification of *gst-4* (Figure 3.7).

3.5.4 A new role for BRAP-2 in stress response and aging

The idea that ROS accumulation overtime leads to aging and cell death is widely regarded and has been extensively studied in various models [35, 40]. However, the

induction of cytoprotective genes relies on an intricate and tightly regulated system of transcription. Although we have previously shown strong evidence that BRAP-2 has a crucial role in the regulation of phase II detoxification genes through SKN-1, this only represents a very small piece of the puzzle in a complex network. Continued effort towards the study of BRAP-2 and its downstream regulators will ultimately aid in modeling the complex signaling pathways involved in detoxification initiation and regulation. Taken together, our data provides insight into BRAP-2 and its role in the oxidative stress response, where NHR-49 influences aging and stress response activation through co-regulation with SKN-1, an activation that requires a functional MDT-15. We were not able to detect direct binding of SKN-1 and NHR-49. However, we have shown that NHR-49 is required to co-ordinate with SKN-1 to induce the expression of stress genes. Recent literature has reported that MDT-15 can interact with both SKN-1 and NHR-49 independently. It will be of interest to determine whether MDT-15 may act as a bridge, linking SKN-1 and NHR-49 to form a "transcription factor complex" to induce *gst-4* in *brap-2(ok1492)*. It is also notable to mention there is an increase interest to study the correlation of fat metabolism and oxidative stress response. The role of MDT-15 and NHR-49 has a great impact on fatty acid regulation; whether alternating the animal lipid composition would activate BRAP-2 and its downstream transcription factors will be remained to be explored. Here we have established a new role for NHR-49 as a stress gene regulator. Our work here provides a solid framework for the continued study of stress genes and the ways in which they are regulated to maintain cell integrity and prevent damage caused by ROS.

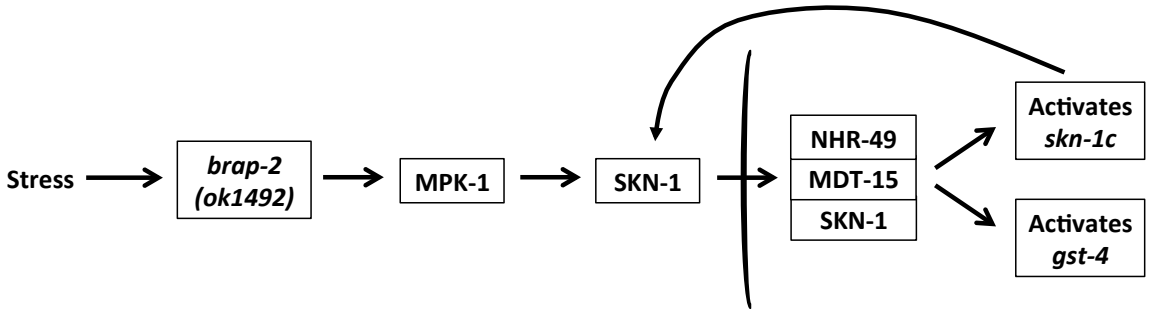

Figure 3.7. Proposed novel model of NHR-49 in the regulation of phase II detoxification gene *gst-4*.

The induction of oxidative stress in *brap-2(ok1492)* mutant worms activates ERK through the Ras signaling pathway (as previously shown in Chapter 2). Activated MPK-1 can then phosphorylate SKN-1 for nuclear translocation, which binds to NHR-49/MDT-15 in the nucleus. This transcription factor complex induces *gst-4* expression. This complex can also create positive feedback through *skn-1c* promoter binding to up regulate its transcription, thereby enhancing the SKN-1C response to oxidative stress and its target genes.

3.6 Reference

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3.7 Supplemental Figures

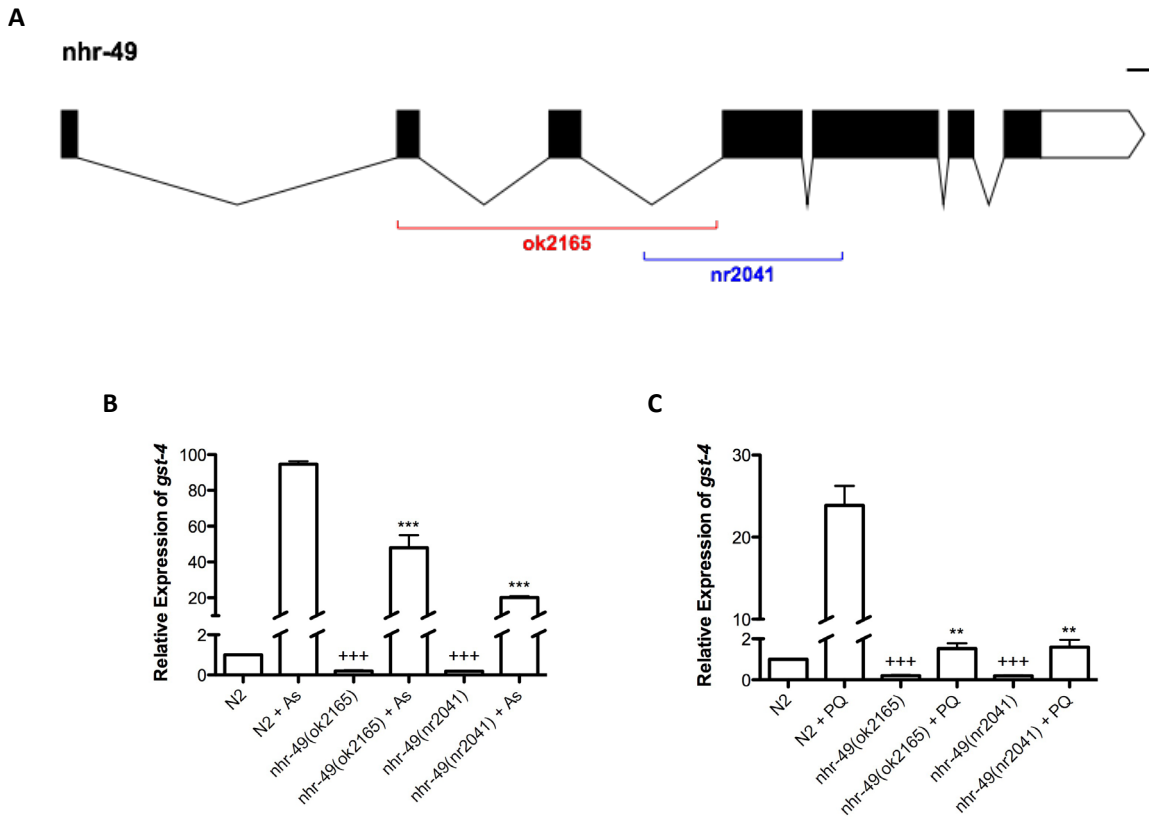

Figure S3.1. *nhr-49* is required to induce *gst-4* upon exposure to sodium arsenite (As) and paraquat (PQ).

(A) A schematic diagram representing the genomic structure of *C. elegans nhr-49*, which is located on chromosome I and contains 7 exons, with a transcript length of 4928 bp. The length of the coding sequence is 1506 bp and translates into a 501aa protein. The allele of the first mutant strain *ok2165* has a 1437 bp deletion (shown in red: 1513-2949), and the second mutant allele *nr2041* has a 893 bp deletion in the region of 2622-3514 (shown in blue). The alignment was done by BlastN search and the figure was generated using the online program Exon-Intron Graphic Maker (http://wormweb.org/exonintron). **(B-C)** Synchronized N2, *nhr-49(ok2165)* and *nhr-49(nr2041)* worms were collected at the L4 stage and exposed to **(B)** 5 mM sodium arsenite and **(C)** 100 mM paraquat for 2 hours. RNA was extracted and the levels of *gst-4* mRNA was quantified using qRT-PCR. Results demonstrate that *nhr-49* is required to induce *gst-4* upon exposure to both arsenite and paraquat. All qRT-PCR experiments were performed in three independent trials, and error bars represent the standard error of the mean, $p<0.001$+++ vs N2; $p<0.001$***, $p<0.01$** vs drug treated N2.

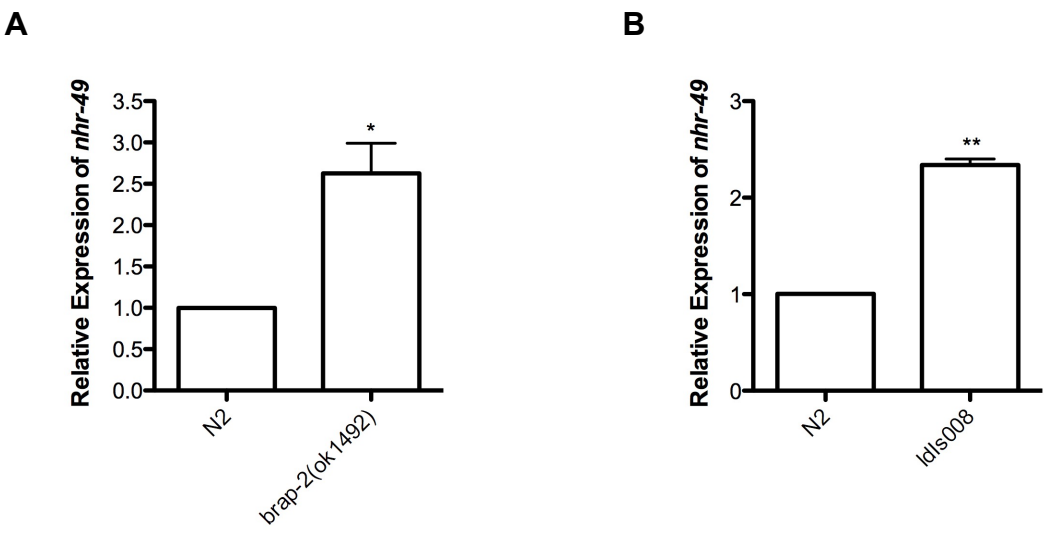

Figure S3.2. *brap-2(ok1492)* and gain-of-function *skn-1* (*ldIs008*) strains show an increase in *nhr-49* mRNA expression.

nhr-49 mRNA levels were quantified using qRT-PCR in **(A)** *brap-2(ok1492)* mutant and **(B)** *ldIs008* at the L4 stage. Results show a 2.5-fold increase of *nhr-49* expression in both *brap-2(ok1492)* and *ldIs008* in comparison to N2. Statistical analysis was carried out using paired t-test; $p<0.01$**, $p<0.05$* vs N2.

Chapter 4

GENERAL DISCUSSION

4.1 Identifying the role of BRAP-2 provides a link between the Ras signaling pathway and stress gene network in *C. elegans*

Our lab has been interested in studying the Ras effector protein known as BRAP2/IMP. The proteins of the Ras pathway have been intensively studied, as they have a critical role in receptor mediated transduction that is necessary for transcription, cell division, differentiation and actin dynamics [1]. Mutations in any of these proteins could cause detrimental effects such as tumour progression, thus the understanding of the regulation of these complex molecules is even more necessary [2]. The mammalian BRAP2 has been proposed to regulate the assembly of the mitogenic signaling complex in response to Ras signaling, indicating the importance of BRAP2 as a molecular switch in activating the signal cascade [3]. The nematode *C. elegans* has a BRAP2 homologue denoted as BRAP-2. A recent publication by our lab demonstrated that *C. elegans* BRAP-2 is necessary for preventing an inappropriate response to elevated levels of ROS [4], therefore we were interested in defining the mechanism and role of BRAP-2 in response to oxidative stress conditions.

The work presented here provides us with novel information on the regulation of stress genes. Utilizing *C. elegans* as a model, how BRAP-2 can regulate the transcription factor (TF) SKN-1 and its target genes has been investigated, and the corresponding signaling pathways have also been identified. Our RNAi screen revealed 20 potential new regulators or co-activators of SKN-1, and this was exciting as it is the first time some of these TFs were identified as having a significant role in regulating stress genes in *C.*

elegans. Whether these candidates regulate SKN-1 target genes in a SKN-1-dependent or SKN-1-independent manner, it will provide clues for the mechanism of BRAP-2/SKN-1 regulation, and also benefit future clinical studies if these pathways are conserved. In this project, we have incorporated genetics and biochemical techniques as well as *in vitro* and *in vivo* analyses, for our goal of identifying the mechanism and new roles of select TFs in the BRAP-2/SKN-1 pathway.

The induction of detoxification genes is often associated with a complex transcriptional network. While *C. elegans* provides an ideal system to study genetic interactions and signaling pathways of interest, there is always cross talk between stress pathways and the regulation of their target genes. SKN-1 induction of its targets is under the regulatory control of multiple signaling cascades [5]. As such, it is important to emphasize that unexpected mechanisms might also be involved that could affect one another. Nevertheless, our findings appear to be promising in support of our proposed hypotheses. The significance of these findings have been deliberated in Chapters 2 and 3. In this chapter, I will focus on discussing this investigation's limitations and what the focus of future research to expand further on this topic should be.

4.2 BRAP-2/KSR and BRAP-2/LET-60 interactions highlight new roles for Ras signaling and MAPK pathways in *C. elegans*

The results reported illustrate that the mutation of *C. elegans* BRAP-2 has a role in influencing the expression and localization of SKN-1/Nrf2 as well as its target genes through the Ras signaling pathway. In *C. elegans*, activation of the Ras pathway requires both Ras and the scaffold protein KSR [6]. The worm genome encodes three Ras homologs - LET-60, RAS-1 and RAS-2. Among the three homologs, LET-60/Ras is well characterized and known to stimulate the MAPK cascade consisting LIN-45/Raf, MEK-

2/MEK and MPK-1/ERK for various biological events such as vulval development and oogenesis [7, 8]. Using western blot analysis, we were able to provide evidence that suggests BRAP-2 is able to physically interact with activated LET-60/Ras and KSR-2/KSR. A recent study has shown that the MAPK cascade could also phosphorylate SKN-1 for its re-localization from the cytoplasm to the nucleus [9], and this is supported by our findings in which *brap-2(ok1492)* worms possess increased SKN-1 localization in the intestinal nuclei. Additionally, these mutant worms also displayed increased p-MAPK expression in the intestine and an increase in overall protein levels. Another interesting observation made in our investigation was that the depletion of MEK-2 in *brap-2* mutants worms significantly decreased the *gst-4* mRNA, highlighting the importance of BRAP-2 for MAPK activity. The above results support the idea that increased MAPK causes the translocation of SKN-1 and alters the expressions of its target genes. These findings suggest that the *C. elegans* BRAP-2 regulation of SKN-1 through the Ras and MAPK cascade could be highly conserved.

BRAP-2 has been characterized as a Ras effector protein that binds to LET-60/Ras and KSR-2/KSR, but it is also known to function as an E3 ligase for ubiquitination [10]. Structural analysis of *C. elegans* BRAP-2 shows that it contains 4 recognizable domains (BRAP2 domain, RING domain, Zinc Finger domain and a coiled-coil region), with the RING and Zinc Finger domains conferring functional roles of ubiquitin ligase activity [4]. Although we were able to provide evidence suggesting that the N-terminal region consisting of the BRAP-2 and RING finger domains could in fact bind to KSR-2, it is not enough to emphasize the importance of BRAP-2 for its ubiquitination activity, there is a chance KSR-2 could also be recruited to the BRAP-2 Zinc Finger, therefore further western blot analysis is required to determine the exact

location for LET-60 and KSR-2 binding on BRAP-2. Furthermore, experiments such as abolishing the function of the RING domain by site-directed mutagenesis mutating Cys260 and/or Cys264 to Alanine and testing for the rescue of the observed phenotype will be of great importance [11].

4.3 SKN-1 and ELT-3 work together to regulate detoxification genes

The *C. elegans* stress response network is associated with many signaling pathways, and little is known of their activation and subsequent regulation. Therefore, following the identification of BRAP-2's mechanism for the regulation of SKN-1, we were interested in further clarifying how this mechanism works. Utilizing RNAi in combination with *brap-2(ok1492);gst-4p::gfp* worms was a valuable tool to determine possible candidates that were capable of SKN-1 co-regulation for enhanced *gst-4* expression and promotion of lifespan. It was fascinating that the screen revealed 20 potential candidates out of 960 transcription factors that are encoded in the *C. elegans* genome. For some of these genes it is the first time that they have been identified as having a role in the oxidative stress response. Among the candidates ELT-3 was selected for this study, as previous work done by Budovskaya et al. showed that this hypodermis expressing TF also plays a role in regulating adult lifespan [12]. Unfortunately, their work was criticized for providing insufficient evidence that the ELT-3 is expressed to a significant enough level in the worm intestine to directly control intestinal genes for lifespan extension [13-16]. However, it is possible indirect influences of intestinal gene expression cannot be ruled out. Therefore we further extended our understanding of *elt-3* in response to oxidative stress and its regulation of lifespan through BRAP-2.

Accordingly, we investigated the significance of ELT-3 and SKN-1 regulation through genetic and biochemical assays. One aspect that we were particularly interested

in pursing is whether ELT-3 is capable of enhancing *gst-4* expression and increase lifespan along with SKN-1. As expected, although our findings did not show any obvious *elt-3* expression within intestinal nuclei, our genetic analysis provided evidence that increased *skn-1c* mRNA levels in *brap-2(ok1492)* requires *elt-3*, as well *elt-3* is necessary for *gst-4* induction. Biochemical experiments were also performed to further verify SKN-1/ELT-3 regulation. It was surprising that SKN-1 and ELT-3 physically interact, and we believe they hetero-dimerize to enhance transcriptional activity of phase II target genes, and increase lifespan. With this data, we have mapped a potential pathway for BRAP-2 regulation of SKN-1 and ELT-3 for oxidative stress response and extended lifespan.

Another aspect of this project is to identify new activators and identify the mechanism through which BRAP-2 is exerting its regulatory effect on SKN-1. As mentioned previously, the eukaryotic GATA factors are also known as "pioneer transcription factors", which act as important initiators in chromatin remodeling. We showed *elt-3* is required for enhanced *gst-4*, but no ELT-3 DNA binding enrichment site on the *gst-4* promoter in the wild type or *brap-2* was detected. The expression pattern of *elt-3* was also observed and we detected it in hypodermal and posterior intestinal nuclei in both wild type and *brap-2* worms (see Figure 2.9). Thus it is possible that ELT-3 subcellular localization is independent of MAPK activity. Although the GATA TFs are evolutionary conserved across species, currently there are no reports describing the *C. elegans* GATA factors function as "pioneer factors". Accordingly, we are pursuing ChIP analysis to determine if *brap-2* is important to facilitate this transcriptional regulation. Should we successfully show that ELT-3 is a pioneer TF, the regulation identified here in *C. elegans* will be important and useful for future longevity studies in higher organisms, and ultimately will show that they have a protective role against stress conditions.

Following the identification of this novel SKN-1/ELT-3 pathway for promoting lifespan, it will be of great interest to extrapolate these observations to gain a view of the "larger picture" and its application in mammalian systems. Currently, we have turned our attention to investigating whether this interaction and transcriptional ability is also applicable to human homologs. However, the human genome encodes 6 GATA factors and there is no conclusive answers showing the exact mammalian counterpart to ELT-3. Therefore we predicted GATA3 most closely resembled our gene of interest and used it for study [17-19]. We are pursuing "pull-down" and Luciferase assays to determine Nrf2/GATA interaction and their transcriptional regulation of Nrf2 target genes. Our prediction of this interaction can potentially be the first step for moving towards mammalian studies for the characterization of our described mechanism that is highly conserved in nematodes and mammals.

4.4 NHR-49 has a crucial role not only in lipid metabolism, but also in stress response that is dependent on SKN-1

The gene regulatory network that is able to exert avoidance mechanisms to prevent stress is complicated, and discovering unknown pathways is still under investigation. As mentioned previously, the RNAi screen identified 20 potential candidates for SKN-1 co-regulation, we have presented sufficient evidence to unravel how ELT-3 could regulate stress genes and lifespan with SKN-1. However, it is clear that the regulation of SKN-1 is remarkably complex, and there is still a long way ahead to fully grasp the scope of the relationship between BRAP-2 and SKN-1 in response to oxidative stress. Further exploration is necessary to gain more valuable ideas and a more focused direction for future studies on this pathway.

Another exciting observation of this project, was the identification of a second positive TF that is required for BRAP-2/SKN-1 regulation, NHR-49/PPARα. Previous literature reported that the function of NHR-49 is linked to lipid regulation and fat storage [20-22]. As far as we know, there is no evidence implicating NHR-49 in oxidative stress responses. Since our RNAi screen indicated NHR-49 could co-regulate stress genes with SKN-1, one aim of this study was to determine if NHR-49 is responsible for inducing stress genes along with SKN-1 and promote lifespan in *brap-2(ok1492)*. Our findings were able to demonstrate that the loss of *nhr-49* suppresses *gst-4* expression in *brap-2(ok1492)*. This indicates the importance of NHR-49 in co-activating SKN-1 for the enhancement of SKN-1 target gene expression. One interesting observation from our results was that the NHR-49 mediator MDT-15 is required with SKN-1 for this regulation. This is consistent with previous work done by Goh *et al.* where they showed MDT-15 can physically interact with SKN-1C and it is required for oxidative stress responses [23, 24].

In addition, Pang *et al.* revealed MDT-15 is required as the cofactor for SKN-1-mediated lipid metabolism. The authors speculated the loss of *mdt-15* through RNAi suppresses *skn-1* reporter activation [25]. Surprisingly, our results showed that the loss of MDT-15 binding partner *nhr-49* in *brap-2(ok1492)* mutant restores *skn-1c* mRNA transcripts to the same level as wild type worms, implying *nhr-49* has a role in *skn-1c* regulation. We also discovered NHR-49 and/or MDT-15 are required to co-activate with SKN-1 for promoting *skn-1c* transcriptional activity. Through experimental analyses, our results define a novel pathway where the SKN-1/MDT-15/NHR-49 complex regulates stress response through a positive feedback mechanism to ensure the continued production of SKN-1C. Previous studies have reported the mediator MDT-15 can also

interact with NHR-49 for lipid synthesis [26]. Therefore, to answer whether MDT-15 acts as a bridge to link NHR-49 and SKN-1 and function together for gene regulation, further biochemical studies such as pull-down assays or luciferase assay will be necessary.

In this study we have provided a novel link between the mediator MDT-15 and the 2 TFs SKN-1 and NHR-49. It is important to mention a new area of study showed that SKN-1 responds to stressors not only restricted to oxidative stress, but also in metabolic stress. Thus it will be of interest for researchers to explore the relationship between fat metabolism and stress response for longevity [27, 28]. It is possible that altering diet and metabolism could lead to a change that induces a state of oxidative stress. A recent study utilized a germline-deficient strain known as *glp-1* (abnormal germline proliferation), and reported elevated stress resistance, a longer lifespan, and an abnormal accumulation of lipids [29, 30]. However, how these phenotypes are related to one another was not reported. Steinbaugh *et al.* further studied the phenomena and their findings indicated the excessive amounts of oleic acid (an unsaturated fatty acid) found in the yolk of *glp-1* mutants can act as a signal that causes the activation of SKN-1, leading to an increase in SKN-1 target genes associated with altering worm's stress resistance, longevity, and lipid metabolism [31, 32]. Nonetheless, their work has been focused on elucidating whether the absence of the gonad could send out additional signals to influence lipid accumulation and lifespan, while the presence of the germline affecting these same biological processes was not addressed. Hence additional work needs be done to resolve this question. Interestingly, oleic acid can also promote transcriptional activation of NHR-49 [33]. Given that MDT-15 has a central role in regulating fatty acid β-oxidation genes through NHR-49 or SKN-1 independently [34], further attention should be paid to how these proteins cooperate to maintain lipid and stress homeostasis,

as well as to determine the core metabolic pathway for NHR-49/MDT-15/SKN-1 in regulating stress genes.

4.5 Potential significance for BRAP-2 oxidative stress regulation in *C. elegans* and mammals

The discovery that a *C. elegans brap-2* mutation activates the Ras signaling cascade and causes an increase in the initiation of SKN-1 detoxification pathway is exciting, as this is the first time BRAP-2 has been shown to not only be a cytoplasmic retention protein as suggested in previous studies, but it also has a vital role in up regulating stress related genes. Additionally, we were able to show ELT-3 and NHR-49, are both necessary for SKN-1 co-regulation for enhanced SKN-1 target gene expression, as well for lifespan extension. With its high degree of gene conservation, studies using *C. elegans* revealed the function and interaction of vital signaling pathways. This work presented here suggests a new role for BRAP-2 in response to oxidative stress through SKN-1.

Outside of the worm model, substantial studies suggest that BRAP2 has diverse roles such as monocyte differentiation and testis development [35, 36]. Currently, there are no reports describing a direct connection between BRAP2 and Nrf2 in mammalian systems. BRAP2/IMP has shown to be a key regulator in the Ras signaling pathway, thus its presence or absence can be a factor in determining the expression levels of the Ras cascade and downstream events [37-39]. A recent study by DeNicola *et al.* suggested that overexpressing Ras signaling through the oncogene K-Ras can increase the transcription of Nrf2 and reduced the intracellular ROS levels [40]. However, K-Ras could also induce tumourigenesis when Nrf2 is depleted due to an imbalance in the redox state of cells. Therefore in light of our results reporting that *brap-2* knockout worms show increased

skn-1 and phase II gene expression, we are pursuing siRNA experiments using the mouse cell line NIH3T3 as described by DeNicola *et al.* and increasing Ras signaling by knocking down BRAP2, to determine whether it will alter the expression of Nrf2 and its target genes, as well as whether it will change the protein levels of GATA3 and HNF4α, the homologs of *C. elegans* ELT-3 and NHR-49, respectively. Whether BRAP2 is capable of regulating phase II detoxification genes, future mammalian studies should focus on utilizing human cell lines to verify that our described model of regulation is conserved.

4.6 Future studies should focus on the role of BRAP-2 in worm development

Although the above work and discussion implicates BRAP-2 is vital for the regulation of SKN-1 and expression of phase II genes, there are still several questions that remain regarding the role of BRAP-2 in other physiological contexts. It is visible how little we understand the functions and biological significance of BRAP-2, and extensive work needs to be done in order to increase our fundamental knowledge of this protein. It will be of interest to first look at where exactly BRAP-2 is expressed. Despite an *in situ* staining done to investigate the expression of BRAP-2 in the entire organism, this is not sufficient to indicate its endogenous expression, as the fixed preparations could produce artifacts in the analysis. Furthermore, some tissues or cells such as neurons may have minimal expression that cannot be detected by antibodies. Through the use of transgenes, BRAP-2 constructs containing a GFP translational reporter can be generated, this allow us to visualize *brap-2* gene expression patterns and cellular autonomy in live animals, which could also assist us in future genome wide or high throughput gene expression projects.

While identifying the expression pattern of certain proteins in animals contributes important information, such as providing its function in developmental processes, it also enables researchers to predict its role in improper cellular function. A previous publication from our lab mentioned that the *brap-2(ok1492)* mutant is a slow growing strain in comparison to wild type, as well it appeared to have a smaller body size [4]. This will provide valuable novel findings for roles of BRAP-2 in *C. elegans* that span more than the oxidative stress response.

4.7 Future work will shed light on the significance of the stress genes network and other functional roles

Although this project has provided strong evidence that BRAP-2 is involved in the stress signaling to regulate SKN-1, the data presented here only represents a minor piece of this complicated network and more effort needs to be put towards gaining a better understanding of BRAP-2 in these complex processes. Therefore our objectives should continue focus on the study of novel regulatory components of the SKN-1/Nrf2 detoxification pathway that were identified in the RNAi screen in order to decode the stress signaling pathways that have not been investigated.

To further determine the relationship between BRAP-2 and SKN-1 regulation in *C. elegans*, the remaining candidates identified from the RNAi screen should be validated and studied. An initial RNAi assay validation has been done and indicated other potential candidates such as *fkh-9*, *vab-3* and *sem-4* showing similar *gst-4* reduction in *brap-2(ok1492)*, where these genes are already known to have functional roles in neuronal or vulval cell development [41-43]. It will be worthwhile to conduct double mutant and qPCR analysis to see if these candidates are able to suppress the enhanced *gst-4* expression induced by *brap-2(ok1492)*. Unpublished results from our lab indicate that the

candidate *sem-4* has a vital role in the oxidative stress regulation and lifespan extension. SKN-1 requires the activity of TFs and co-activators to co-ordinate the oxidative stress response, thus elucidating the transcriptional regulatory network could provide a solid framework for determining the mechanisms in stress response that limit cellular damage. Identification of these candidates through RNAi was exciting as they would also be of clinical significance and provide potential targets for future therapeutics.

The publication of the completely sequenced genome of *C. elegans* and mapped protein-protein interactions was integral for the investigation of biological processes [44]. Despite a distinct relationship to humans, so far approximately 40% of *C. elegans* proteins were found to share biological similarities with humans, and many important signaling pathways present in humans have a homologous counterpart conserved in *C. elegans* [45, 46]. Therefore conducting a Y2H screen utilizing BRAP-2 as the bait protein to identify unique interacting partners may offer further insight into BRAP-2 function. It is expected that BRAP-2 will bind to LET-60/Ras and KSR-2/KSR as shown in Chapter 2 (Figure 2.4), but it will be interesting to identify other novel interacting partners that BRAP-2 can bind with. Although using Y2H is a powerful tool to identify unique protein targets of BRAP-2, one caveat with the Y2H approach, is the potential for a high incidence of false positives. As such, an affinity purification/ mass spectrometry approach using transgenic worms would also be a more viable option to identify candidate proteins that bind to BRAP-2. These new binding partners may perhaps provide clues for new signaling pathways where BRAP-2 can regulate or have other functional roles, for example as a scaffold protein suggested by Fatima and colleagues [47]. The downstream signaling pathways of BRAP-2 are likely to be conserved among species and its precise mechanisms are awaiting resolution. Therefore, identifying a greater subset of proteins

that interact with BRAP-2 in *C. elegans* will be of upmost importance to understand BRAP-2 and its biological significance.

The Ras signaling pathway has been implicated in diverse biological responses such as DNA damage, and can mediate pro-survival, pro-apoptotic responses and cell cycle arrest. BRAP2 was initially found to bind to the nuclear localization signal motif of the tumour suppressor gene BRCA1 [48]. It was proposed that this binding could prevent BRCA1 from translocating to the nucleus and activating gene transcription and DNA repair. As shown in the *in situ* antibody staining of wild type worms, in addition to the intestine, another tissue that exhibited BRAP-2 expression is in the gonad, and thus warrants further investigation into its significance in the germline. The worm gonad represents an important organ for egg production and fertilization. Previous studies have confirmed the involvement of *C. elegans brc-1* in DNA repair. Loss of *brc-1* could lead to increased germline apoptosis [49]. As such, in addition to exploring the role of BRAP-2 in response to oxidative stress, we are also interested in investigating BRAP-2 biological role in mediating DNA damage.

Knowledge of BRAP2 is limited. Although there are an increasing number of studies showing the importance of Brap2/IMP in mammals and these studies provide a biological role for BRAP-2 in different tissues. There are few publications to support the findings of this particular study in the regulation of detoxification pathways, and thus a great deal of work remains to be done to elucidate the function of BRAP-2 in *C. elegans*. The continuing effort devoted towards this field of study will ultimately help us to gain a better understanding of the complex process involved in the Ras signaling pathway and its biological responses to different activities. Identifying new cellular signaling cascades are of paramount importance for building a foundation for scientific knowledge, and for

the information it can bestow to clinical research. The elucidations of signaling pathways betters our understanding of these regulatory processes and associated diseases to provide strategies for their treatment. The work presented here in my dissertation on BRAP-2 only represents a small piece of a larger puzzle regarding its properties and role in stress pathway regulation. Further work done by our lab, or other researchers will provide a more well-rounded understanding of the function and biological significance of BRAP2/IMP in signal transduction and cellular health.

4.8 References

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Appendix

Table 3. List of Worm Strains. Worm strains were obtained from the *Caenorhabditis* Genetic Center unless otherwise specified. Double mutants were created according to the standard single-worm PCR protocol.

Strains	Description
N2	Wild Type
CF1553	*muIs84 [(pAD76)sod-3p::GFP+rol-6]*
CL2166	*dvIs19 [(pAF15) gst-4p::GFP::NLS]*
DR1785	*mIn1[mIs14 dpy-10(e128)/unc-4(e120)] II*
EU1	*skn-1(zu67) IV/nT1 [unc-?(n754) let-?] (IV;V)*
JG1	*elt-3(vp1) X* **(Gift from McGhee Lab)**
JG5	*vpIs1[elt-3::GFP+lin-15(+)]*
LD001	*ldIs007 [skn-1b/c::GFP+rol-6(su1006)]*
LD1250	*ldIs008* **(Gift from Blackwell Lab)**
OP178	*unc-119(ed3) III; wgIs178 [skn-1::TY1::EGFP::3xFLAG + unc-119(+)]*
STE68	*nhr-49(nr2041) I*
STE108	*nhr-49(et7) I* **(Gift from Taubert Lab)**
STE109	*nhr-49(et8) I* **(Gift from Taubert Lab)**
STE110	*nhr-49(et13) I* **(Gift from Taubert Lab)**
YF15	*brap-2(ok1492)] backcross 3x*
YF67	*brap-2(ok1492) II; dvIs19 [(pAF15) gst-4p::GFP::NLS]*
YF68	*brap-2(ok1492) II; ldIs007 [skn-1b/c::GFP+rol-6(su1006)]*
YF71	*brap-2(ok1492) II; tqEx34*
YF104	*brap-2(ok1492) II/mIn1[mIs14 dpy-10(e128)] II*
YF112	*brap-2(ok1492) II; vpIs1[elt-3::GFP+lin-15(+)]*
YF114	*brap-2(ok1492) II; elt-3(vp1) X*
YF121	*brap-2(ok1492) II; elt-3(vp1) X; dvIs19 [(pAF15) gst-4p::GFP::NLS]*
YF126	*brap-2(ok1492) II; skn-1(zu67) IV/nT1 [unc-?(n754) let-?] (IV;V)*
YF127	*brap-2(ok1492) II; muIs84 [(pAD76)sod-3p::GFP+rol-6]*

Strains	Description
YF131	*brap-2(ok1492) II; mdt-15(tm2182) III*
YF165	*brap-2(ok1492) II; unc-119(ed3) III; wgIs178 [skn-1::TY1::EGFP::3xFLAG + unc-119(+)]*
XA7702	*mdt-15(tm2182) III*

Table 4. List of forward and reverse primers for SW-PCR

Name	Tm	Sequence
ok14925	57.62°C	5’ GAGTGTATTCGAGTTTGATTCCC 3’
ok1492N23	56.31°C	5’ TTTGTTCTGCCTAGGAATAAGTG 3’
olok14925	61.45°C	5’ GTCAGCACCGAAAATGTGTCAG 3’
ok14923B	56.73°C	5’ CAGACAACGTCGAATGATCTC 3’
elt-3 vp1 For	58.54°C	5’ CCAGCCTGTGACACTCCC 3’
elt-3 vp1 Rev	56.88°C	5’ CGCATTGATAGAGGCCTTC 3’
elt-3 Rev	59.13°C	5’ CAAGTTTCAGTCGTGGGAGG 3’
nhr-49 N2 For	55.24°C	5’ GCCCCGTTAACTCAGAATT 3’
nhr-49 N2 Rev	53.70°C	5’ ATTTGCGATCTTCGGTATC 3’
nhr-49 ok2165 Rev	52.47°C	5’ CAAGTTTGTCCTTCTAGCAGT 3’
mdt-15 N2 For	53.53°C	5’ ACATACTTGATCCACTCATCG 3’
mdt-15 tm2182 For	52.94°C	5’ TTGTTGACTGGTGATGTCAT 3’
mdt-15 Rev	52.37°C	5’ GATCCTTCAGCACATATGTATC 3’

Table 5. List of forward and reverse primers for qRT-PCR

Name	Tm	Sequence
qACT-1 For	56.3°C	5’ GTGGTATGGGACAGAAGA 3’
qACT-1 Rev	64.5°C	5’ GCTTCAGTGAGGAGGACTGG 3’
qGST-4 For	64.4°C	5’ TGCTCAATGTGCCTTACGAG 3’
qGST-4 Rev	64°C	5’ AGTTTTTCCAGCGAGTCCAA 3’
qGST-7 For	64.3°C	5’ AATTCGTGGAGCTGGAGAGA 3’
qGST-7 Rev	64.5°C	5’ CAGCAACCGAGTTGACTTGA 3’
qGST-10 For	64.3°C	5’ ATTCGAAGACATTCGGTTCG 3’
qGST-10 Rev	64.5°C	5’ AACATGTCGAGGAAGGTTGC 3’
qGST-19 For	64.3°C	5’ GATTTGCTGGGAAAACTCCA 3’
qGST-19 Rev	63.9°C	5’ TTCCAGCAGACCATTGAGAA 3’
qGCS-1 For	64.2°C	5’ CCAATCGATTCCTTTGGAGA 3’
qGCS-1 Rev	64.3°C	5’ GCTACTTCCGGGAATGTGAA 3’
qRHY-1 For	64.1°C	5’ CCAAGACACTCTTCCGTTCC 3’
qRHY-1 Rev	64.4°C	5’ TCGGATTTGTTGGACGGTAT 3’
qSOD-3 For	64.3°C	5’ GGATGGTGGAGAACCTTCAA 3’
qSOD-3 Rev	64.4°C	5’ AAGGATCCTGGTTTGCACAG 3’
qSKN-1A For	68.1°C	5’ AAGCGTGGACGTCAATCCAA 3’
qSKN-1A Rev	68.7°C	5’ ACCTTGTTCTTTCCGCGTCG 3’
qSKN-1B For	64.7°C	5’ GGACGTCAACAGCAGACTCA 3’
qSKN-1B Rev	64.3°C	5’ CGTGGAGATTCCGAAGAGAG 3’
qSKN-1C For	67.1°C	5’ TACTCACCGAGCATCCACCA 3’
qSKN-1C Rev	67°C	5’ TGATCAGCAGGAGCCACTTG 3’
qFMO-2 For	64.5°C	5’ GGAACAAGCGTGTTGCTGT 3’
qFMO-2 Rev	63.9°C	5’ GCCATAGAGAAGACCATGTCG 3’
qMPK-1 For	69.3°C	5’ CCATTATTCCTTGCAGCCGCT 3’
qMPK-1 Rev	63.5°C	5’ CACATAATCGTATCGACATCGAG 3’
premRNA gst-4 For	64.5°C	5’ TTTTGATGCTCGTGCTCTTG 3’
premRNA gst-4 Rev	64.3°C	5’ CAAATGGAGTCGCTGGAAAT 3’
premRNA gst-7 For	64.8°C	5’ GCCAAGTGAGTTTGGGATTG 3’
premRNA gst-7 Rev	64.1°C	5’ CGGCTTACATCTCGGTTCTC 3’

Name	Tm	Sequence
premRNA gcs-1 For	63.9°C	5’ AAAACCGATCATGCCATTTC 3’
premRNA gcs-1 Rev	64.3°C	5’ TGGCATCGAAAAATTGTGAA 3’
premRNA rhy-1 For	64.9°C	5’ GTCATCATCGCCACACACTC 3’
premRNA rhy-1 Rev	64.7°C	5’ TGCAAGTCTGCACGTTCTTC 3’
ChIP pGST-4A For	64.5°C	5’ GCTACTTGGATAACCAGCTCCA 3’
ChIP pGST-4A Rev	62.4°C	5’ TTGAATCGATGTATCAATTGTTTTT 3’
ChIP pGST-4B For	62°C	5’ AAAACTCAATTAGTGGAAAATTTGAA 3’
ChIP pGST-4B Rev	63.1°C	5’ TTTTCCCAGTTCTTGTCTATCTCA 3’
ChIP 3’UTR For	65.4°C	5’ ACGCTCCCAGAGAGCGTAA 3’
ChIP 3’UTR Rev	64.2°C	5’ CTGCCACTTTCTGTCACTGC 3’