TRANSCRIPTIONAL REGULATORS OF OXIDATIVE STRESS RESPONSES IN
THE NEMATODE *CAENORHABDITIS ELEGANS*

by

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Abstract

Reactive oxygen species are chemically reactive molecules that are crucial for many cellular functions, but their buildup can cause toxic damage, otherwise known as oxidative stress. Oxidative stress is thought to cause or exacerbate many diseases. To defend themselves against oxidative stress, cells mount sophisticated defenses to remove ROS and repair damage caused by ROS. In particular, sequence-specific DNA binding transcription factors induce the expression of cytoprotective enzymes upon stress. In the model organism *Caenorhabditis elegans*, the transcription factor SKN-1 is considered a “master regulator” that is required to activate many cytoprotective and antioxidant genes, and is critical for resistance to oxidative stress. However, little is known about whether and how SKN-1 interacts with transcriptional coregulators, essential factors that help specify transcriptional responses. Moreover, although evidence exists for SKN-1 independent oxidative stress responses, the responsible transcription factors are unknown. In this thesis, I identified a subunit of the Mediator transcriptional coregulator complex, MDT-15, as a coregulator for *skn-1*-dependent oxidative stress responses. This role is independent of a previously identified role for MDT-15 in lipid metabolism. Additionally, I found that *mdt-15* is also required for *skn-1*-independent oxidative stress responses. Using a candidate reverse genetic screen, I identified an MDT-15-interacting transcription factor, the nuclear hormone receptor NHR-49, as a regulator of a SKN-1-independent oxidative stress response. Interestingly, some NHR-49-dependent stress response genes were also upregulated in fasting and in long-lived germline-less mutants, indicating a shared response in all three conditions. In summary, this thesis provides the first description of MDT-15 as
a coregulator of SKN-1 and identifies a new role for NHR-49 in the oxidative stress response. SKN-1, NHR-49, and MDT-15 are all conserved in humans, and the human orthologs of SKN-1 and NHR-49 also interact with the Mediator complex. Thus, my work offers therapeutic implications for diseases in which oxidative stress plays a role, such as cancer, metabolic diseases, and other age-related diseases.
Preface

Chapter 1

Portions of Chapter 1 have been published as a Survey and Summary review article in the journal Nucleic Acids Research. Grants, J.M., Goh, G.Y.S., Taubert, S. ‘The Mediator complex of C. elegans: insights into the developmental and physiological roles of a conserved transcriptional coregulator’ (Grants et al., 2015). As co-first author, I contributed substantially to planning, writing and editing the review.

Chapter 2

Chapter 2 has been published in the journal Aging Cell. Goh, G.Y.S., Martelli, K.L., Parhar, K.S., Kwong, A.W.L., Wong, M.A., Mah, A., Hou, N.S. and Taubert, S. ‘The conserved Mediator subunit MDT-15 is required for oxidative stress responses in Caenorhabditis elegans’ (Goh et al., 2014). As first author, I performed and/or supervised all experiments presented in this thesis (except gene expression analysis of RNAi samples in Figures 2.2A, 2.3A-B, 2.5, and 2.8A-B, conducted by K.S. Parhar and S. Taubert). I analyzed and interpreted the data, contributed substantially to writing the manuscript and was responsible for all final figures.

Chapter 3

Chapter 3 is part of a manuscript in preparation for submission to a peer-reviewed journal. Goh, G.Y.S., Winter, J.J., Lai, R., Lee, K., Veal, E.A., Taubert, S. ‘Regulation of a stress response program by the Nuclear Hormone Receptor NHR-49 in C. elegans’. As co-first
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Veal, University of Newcastle upon Tyne, U.K.). I analyzed and interpreted the data,
contributed to writing the manuscript and was responsible for all final figures.
Additionally, Fig. 3.3A was part of a paper published in the journal PLoS One. Lee, K.,
Goh, G.Y.S., Wong, M.A., Klassen, T.L., Taubert, S. ‘Gain-of-Function Alleles in
\textit{Caenorhabditis elegans} Nuclear Hormone Receptor \textit{nhr-49} Are Functionally Distinct’ (Lee
et al., 2016). This experiment was performed and data analyzed by K. Lee and S. Taubert.

\textbf{Chapter 6}

Portions of Chapter 6 (Materials and Methods) pertaining to experiments conducted in
Chapter 2 have been published in the journal Aging Cell. Goh, G.Y.S., Martelli, K.L.,
Parhar, K.S., Kwong, A.W.L., Wong, M.A., Mah, A., Hou, N.S. and Taubert, S. ‘The
conserved Mediator subunit MDT-15 is required for oxidative stress responses in
\textit{Caenorhabditis elegans}’ (Goh et al., 2014). Portions of this chapter pertaining to
experiments conducted in Chapter 3 are part of a manuscript in preparation for submission
S. ‘Regulation of a stress response program by the Nuclear Hormone Receptor NHR-49 in
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<table>
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<tbody>
<tr>
<td>8-oxo dG</td>
<td>8-oxo-2’deoxyguanosine</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant response element</td>
</tr>
<tr>
<td>ATBC</td>
<td>Alpha-tocopherol and Beta-carotene Study</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BLI-3</td>
<td>Blistered cuticle-3 (C. elegans)</td>
</tr>
<tr>
<td>bZIP</td>
<td>Basic leucine zipper domain</td>
</tr>
<tr>
<td>CARET</td>
<td>β-carotene and Retinol Efficacy Trial</td>
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<tr>
<td>CDK8</td>
<td>Cyclin dependent kinase 8</td>
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<tr>
<td>CNC</td>
<td>Cap ‘n’ Collar transcription factor</td>
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<tr>
<td>CR</td>
<td>Calorie restriction</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
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<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAF</td>
<td>Abnormal dauer formation gene (C. elegans)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>FAT</td>
<td>Fatty acid desaturase (C. elegans)</td>
</tr>
<tr>
<td>FMO</td>
<td>Flavin-containing monooxygenase</td>
</tr>
<tr>
<td>FOXO</td>
<td>Forkhead box O transcription factor</td>
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<td>GCL</td>
<td>Glutamate-cysteine ligase</td>
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<tr>
<td>Gcn4</td>
<td>General control protein 4 (S. cerevisiae)</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GLP-1</td>
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<td>GOF</td>
<td>Gain of function</td>
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<td>GSK-3</td>
<td>Glycogen synthase kinase-3 (C. elegans)</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>GTF</td>
<td>General transcription factor</td>
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<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HLH-30</td>
<td>Helix-loop-helix 30 transcription factor (C. elegans)</td>
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<tr>
<td>HNF4α</td>
<td>Hepatocyte nuclear factor 4α</td>
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<td>HO-1</td>
<td>Heme oxygenase 1</td>
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<td>IIS</td>
<td>Insulin/insulin growth factor-like signaling pathway</td>
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<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>LOF</td>
<td>Loss of function</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MED/MDT</td>
<td>Mediator subunit</td>
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<tr>
<td>Msn2</td>
<td>Multicopy suppressor of SNF1 mutation 2 (S. cerevisiae)</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHR</td>
<td>Nuclear hormone receptor</td>
</tr>
<tr>
<td>NQO1</td>
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<tr>
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<td>Nuclear factor (erythroid-derived 2)-like 2</td>
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<td>O$_2^-$</td>
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<td>Positive transcription elongation factor b</td>
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<td>Phosphoglycerate mutase family member 5</td>
</tr>
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<td>Pol II</td>
<td>RNA Polymerase II</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SEC</td>
<td>Super elongation complex</td>
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<td>Sterol regulatory element binding protein</td>
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<td>TAD</td>
<td>Transactivation domain</td>
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<tr>
<td>tBOOH</td>
<td>Tert-butyl hydroperoxide</td>
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<td>Transforming growth factor beta</td>
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<td>Target of rapamycin</td>
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For my family
Chapter 1: Introduction

1.1 Reactive oxygen species in biology

Oxygen is vital to most life forms on earth, due to its role as a terminal electron acceptor in the mitochondrial electron transport chain as part of aerobic respiration. The evolutionary success of aerobic respiration is because it generates adenosine triphosphate (ATP) more efficiently than anaerobic respiration; for example, in fermentation, 2 molecules of ATP are made per molecule of glucose compared to 38 molecules of ATP in aerobic respiration. However, high levels of oxygen are toxic to most organisms, including aerobes. This is due to the formation of oxygen-derived free radicals, which contain one or more unpaired electrons and are thus highly reactive. Examples of such free radicals include superoxide (O$_2^-$) and hydroxyl radicals (OH'). Moreover, some oxygen-containing molecules are highly reactive even without an unpaired electron, for example hydrogen peroxide (H$_2$O$_2$). Collectively, reactive oxygen-derived free radical species and non-free radicals are classified as reactive oxygen species (ROS). Additionally, reactive nitrogen, chlorine and other species also exist; therefore, some authors use the term ‘reactive species’ instead of ROS (Halliwell and Gutteridge, 2015).

The first person to suggest that ROS are produced in vivo was Denham Harman, who proposed that cumulative damage resulting from free radicals produced by respiratory enzymes that utilize oxygen causes aging (Harman, 1956). Superoxide dismutase was discovered approximately a decade later; along with the earlier discoveries of catalase and peroxidases, this provided evidence that both free radical and non-free radical ROS are indeed present in vivo, at physiologically relevant concentrations (Finkel and Holbrook, 2000; Imlay, 2013; McCord and Fridovich, 1969). Numerous enzymes that
neutralize various ROS and their by-products have since been discovered in a wide range of organisms, leading to the view that control of ROS levels is critical for cellular and organismal homeostasis (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2015; Harris, 1992; Matés et al., 1999).

The mitochondrial electron transport chain (ETC) is thought to be the primary source of ROS in vivo in eukaryotes. Electron leakage from the ETC, particularly at Complex I and Complex III (Lenaz, 2001; Turrens, 1997), leads to the formation of superoxide. Initial estimates suggested that 1-2% of all oxygen reduced by the ETC is converted to superoxide; however, as this estimate was derived from isolated mitochondria under oxygen concentrations much higher than those found intracellularly, the actual amount in vivo is likely to be lower (Boveris and Chance, 1973; Turrens, 1997). ROS can also be produced by exogenous sources, for instance oxidative xenobiotics such as paraquat and asbestos, exposure to hyperoxic conditions, and infection by some pathogens (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2015).

ROS cause damage due to their unpaired electron(s), which makes them highly unstable and reactive. They are prone to either donating an electron to or taking an electron from non-radical molecules, thus initiating and potentiating chain reactions (Halliwell, 1991). In this way, ROS react with many cellular macromolecules, including lipids, proteins and DNA. For example, hydroxyl radicals react with fatty acid side chains of membrane phospholipids, leading to lipid peroxidation, which can disrupt membrane integrity (Halliwell, 1991). Similarly, hydroxyl radicals can modify DNA bases, for example in the formation of 8-oxo-2’deoxyguanosine (8-oxo dG) from guanosine. If
these DNA lesions are not properly repaired, they can increase the risk of cancer (Cooke et al., 2003).

Despite the potential for ROS to cause damage, they also play a number of vital roles in vivo. It is therefore necessary for cells to maintain tight control over ROS levels. Below, I summarize the physiological and pathophysiological roles of ROS, mechanisms by which ROS levels are regulated and the ways in which cells can minimize damage caused by ROS.

1.1.1 Reactive oxygen species in health and disease

Due to their ability to react with and modify a wide range of cellular macromolecules, the roles of ROS in biological systems have been intensively studied. ROS have been implicated in a large number of diseases. The roles of ROS in some major disease types are reviewed below. However, more recently, ROS have also been identified as important signaling molecules and may be critical to promote human health.

1.1.1.1 Oxidative stress in disease

Oxidative stress can be defined as “a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential damage” (Halliwell and Gutteridge, 2015; Sies, 1991). Currently, there is no quantitative definition of oxidative stress. Measuring free radicals in vivo, such as by electron spin resonance, is technically challenging due to the high reactivity of free radicals (Palmieri and Sblendorio, 2007). Instead, oxidative stress is often measured using a combination of techniques, including biomarkers that measure oxidative damage, for example peroxidized lipids, 8-oxo dG,
and protein carbonylation levels (Cooke et al., 2003; Halliwell and Gutteridge, 2015; Suzuki et al., 2010); an increase in the expression or activity of antioxidant genes (Kohen and Nyska, 2016); and visual examination of pathology (Kohen and Nyska, 2016).

Recently, it has also become possible to directly measure levels of some ROS in experimental settings using genetically encoded fluorescent reporters (Belousov et al., 2006; Morgan et al., 2011). A large number of diseases are associated with high levels of oxidative stress, as measured by these markers; however, the significance of elevated oxidative stress levels in these diseases (i.e. whether they are important for pathology and/or in any way causal) is for the most part unknown (Andersen, 2004; Dalle-Donne et al., 2003; Halliwell, 1991). Despite this, many studies have been conducted on the molecular roles for ROS in these disease contexts, and in some cases intervention trials with antioxidants have been performed or are ongoing. Here, I briefly summarize the evidence for roles of ROS in three major disease classes: cancer, metabolic disease, and neurodegenerative disease, as well as in aging, the original field that sparked widespread interest in ROS in biomedicine. This information is also summarized in Table 1.1.

1.1.1.1 Cancer

ROS have been linked to cancer for several decades. As early as 1984, it was shown that exposure of mouse fibroblasts to ROS can lead to their transformation (Zimmerman and Cerutti, 1984). As described in Section 1.1, ROS-mediated damage to DNA can lead to mutagenesis. Cancer is in effect a genetic disease caused by mutations that lead to uncontrolled cell proliferation and growth (Vogelstein and Kinzler, 2004). Most cancers have loss-of-function mutations in tumour suppressor genes e.g. p53, and/or gain-of-
function mutations in oncogenes such as Ras (Futreal et al., 2004; Vogelstein and Kinzler, 2004). While random DNA damage by ROS may cause mutations in one or more of these genes, leading to carcinogenesis, ROS also play other important roles in the development of cancer. For example, low levels of oxidative stress have pro-proliferative effects on fibroblasts in culture (Davies, 1999; Wiese et al., 1995), and ROS can act as signaling molecules in pathways that drive growth and proliferation such as the epidermal growth factor (EGF) signaling pathway (Bae et al., 1997).

To determine whether oxidative stress drives cancer initiation and/or progression, several groups have generated knockout mice lacking antioxidant enzymes in order to study whether these mice have increased tumour incidence. Mice lacking CuZn superoxide dismutase (CuZnSOD), which is found in most cellular compartments and is a major superoxide scavenger, develop normally but show increased incidence of liver nodular hyperplasia or hepatocellular carcinoma later in life, concurrent with increased oxidative stress biomarker levels (Elchuri et al., 2005). Mice carrying a homozygous deletion in Mn superoxide dismutase (MnSOD), which acts in the mitochondria, die within the first 1-3 weeks of life (Lebovitz et al., 1996; Li et al., 1995), due to cardiac abnormalities and, in the former study, severe neurodegeneration. However, mice heterozygous for MnSOD develop normally, but show higher incidence of a number of cancers later in life (Van Remmen et al., 2003). These mice also show increased levels of oxidative stress. Similarly, mice lacking glutathione peroxidases 1 and 2, which reduce hydrogen peroxide and other organic peroxides, show a higher incidence of intestinal cancer upon infection with Helicobacter bacteria (Chu et al., 2004).
Based on these data, it would be reasonable to hypothesize that antioxidants might have anti-cancer effects. However, the vast majority of all human trials conducted to date have either shown no effect of antioxidants in cancer prevention, or in some cases have shown an increase in cancer incidence upon antioxidant supplementation (Block et al., 2007; Goodman et al., 2011). For instance, two randomized controlled trials, the Beta-Carotene and Retinol Efficacy Trial (CARET) and the alpha-tocopherol and beta-carotene (ATBC) study were both terminated early due to an increase in lung cancer incidence (28% and 16% respectively) and deaths (17% and 8% respectively) in participants receiving β-carotene, an antioxidant and precursor of vitamin A (Albanes et al., 1995; Goodman et al., 2011; Omenn et al., 1996; The Alpha-tocopherol Beta Carotene Cancer Prevention Study Group, 1994). These studies were predominantly conducted in populations at high risk of cancer, *i.e.* cigarette smokers and workers with substantial exposure to asbestos (Goodman et al., 2011). One exception is the Linxian study, a large study conducted in rural China from 1986-1991, participants who received a combination of micronutrients, including β-carotene, selenium, and vitamin E (usually given as α-tocopherol), showed a decrease in stomach cancer risk (Blot et al., 1993). However, this population was deficient in several micronutrients and had one of the highest incidences of stomach and esophageal cancers in the world (Blot et al., 1993). This highlights the importance of study group selection in any particular antioxidant trial. In support of this, the CARET trial noted an inverse correlation between baseline serum β-carotene levels and lung cancer incidence (Omenn et al., 1996). Other issues also prevent systematic meta-analysis of clinical trials, including lack of consistency in types and dosages of antioxidants tested, lack of sufficient numbers of participants, and lack of
randomized controlled trials (Block et al., 2007; Goodman et al., 2011). Additionally, the length of intervention and choice of endpoints also affect trial outcomes (Goodman et al., 2011). Despite these challenges, the available evidence suggests that antioxidant supplementation does not prevent cancer formation, and may even accelerate it in high-risk populations.

Why would antioxidants increase cancer incidence? Many tumours must overcome high levels of oxidative stress due to their high levels of energy production, which generates large amounts of ROS (Szatrowski and Nathan, 1991; Trachootham et al., 2006). Additionally, detachment from the cellular matrix is also associated with high oxidative stress (Schafer et al., 2009), making overcoming oxidative stress a requirement for metastatic cells. Furthermore, radiotherapy and some chemotherapy treatments act by increasing ROS production (Gorrini et al., 2013). Mutations or metabolic changes that cause increased resistance to oxidative stress, for example by stabilizing cytoprotective transcriptional regulators or upregulating metabolic pathways that regenerate reduced glutathione and thioredoxin occur in many tumours and can be associated with drug resistance (Pavlova and Thompson, 2016; Wang et al., 2008).

Several recent studies have shown that an antioxidant environment likely benefits cancer progression. In one study, the Kras, Braf, and Myc oncogenes were found to stabilize the cytoprotective transcription factor Nrf2 (see Section 1.1.2.1), which upregulates antioxidant enzymes such as glutamate-cysteine ligase (GCL) (DeNicola et al., 2011). GCL catalyzes the rate-limiting step in the synthesis of glutathione, the most abundant antioxidant in the cell (Franklin et al., 2009). This finding demonstrated a previously unknown mechanism for oncogenesis, namely the establishment of
antioxidant conditions. One subunit of GCL was later found to be required for
tumourigenesis in a mouse model that spontaneously develops mammary tumours (Harris
et al., 2015). Another study found that feeding the antioxidants N-acetyl-L-cysteine
(NAC) or vitamin E caused increased tumour progression and decreased survival in mice
with K-Ras- and B-Raf-induced lung cancer (Sayin et al., 2014). This finding is
especially intriguing considering that antioxidant supplementation increased lung cancer
incidence in high-risk populations, as discussed previously (Omenn et al., 1996; The
Alpha-tocopherol Beta Carotene Cancer Prevention Study Group, 1994). Furthermore,
human melanomas xenografted into mice must overcome oxidative stress in the blood
and visceral organs in order to effectively metastasize to distant sites (Piskounova et al.,
2015). Several common drugs used in diabetes treatment, including dipeptidyl peptidase-
4 inhibitors saxagliptin and sitagliptin and the anti-neuropathic agent α-lipoic acid, were
also found to increase metastasis of liver cancer cells in mice by increasing Nrf2 activity,
thus promoting an antioxidant environment (Wang et al., 2016).

To summarize, although ROS likely contribute to cancer formation by causing DNA
damage, they also appear to have protective roles in cancer by preventing tumour
progression, metastasis, and disease resistance (Table 1.1). Intervention studies with
antioxidants have mostly been unsuccessful to date, and their interpretation is clouded,
either because the population being studied is unsuitable for such a trial (e.g. high-risk
participants may already be in the sub-clinical stages of cancer and thus should probably
not be treated with antioxidants) or potentially due to a lack of understanding of correct
doing and appropriate length of intervention. While there is vast potential for drugs that
modulate ROS levels, a deeper understanding of the roles of oxidative stress in cancer will be required to fully leverage such therapeutics in cancer.

1.1.1.1.2 Metabolic syndrome

Metabolic syndrome refers to a cluster of factors that increase risk for a number of diseases, including cardiovascular disease and diabetes. Risk factors that contribute to metabolic syndrome include abdominal obesity, high low-density lipoprotein and overall cholesterol levels, dyslipidemia, high blood pressure, and increased fasting blood sugar (Beltrán-Sánchez et al., 2013). In North America, nearly a quarter of the adult population is considered to have metabolic syndrome (Beltrán-Sánchez et al., 2013). Oxidative stress is a key player in the metabolic syndrome and many of its associated diseases. Here, I will discuss roles for oxidative stress in obesity and diabetes, and summarize antioxidant interventions that have been tested to date.

Obesity occurs due to the accumulation of fat, and is roughly defined as having a body mass index (BMI) equal to or greater than 30 in adults (Arroyo-Johnson and Mincey, 2016). Obese patients show increased levels of oxidative stress biomarkers such as lipid peroxides (Furukawa et al., 2004). Obese mice have increased ROS production in adipose tissue, concurrent with a decreased expression of antioxidant enzymes (Furukawa et al., 2004). Treating these mice with an NADPH oxidase inhibitor, which lowers ROS levels, leads to decreased plasma glucose and insulin levels, decreased dyslipidemia, and decreased hepatic steatosis (Furukawa et al., 2004). Activation of the Nrf2 transcription factor prevents high fat diet-induced obesity in mice (Yu et al., 2011).
However, one study also showed that mice overexpressing the H$_2$O$_2$-reducing enzyme glutathione peroxidase 1 (GPX1) had higher rates of obesity and insulin resistance at 24 weeks of age, due to interference in normal insulin signaling caused by GPX1 overexpression (McClung et al., 2004), demonstrating that the relationship between ROS and obesity is not straightforward.

One reason for increased oxidative stress in obesity is increased lipid levels, as fatty acids are broken down by mitochondrial β-oxidation. This process can create lipid peroxides, as well as increase electron flux into the electron transport chain (ETC) by synthesis of electron donors such as NADH and FADH$_2$, which can lead to a backup of electrons in the ETC (Giordano, 2005). Blocking electron flow through the ETC can lead to donation of single electrons to molecular oxygen by coenzyme Q to form superoxide (Turrens, 1997). In addition, adipose tissue is a source of proinflammatory cytokines, which stimulate the production of reactive species by immune cells such as macrophages and monocytes, thus contributing to chronic inflammation and oxidative stress (Fernández-Sánchez et al., 2011).

Obesity is frequently associated with Type 2 diabetes. Diabetes is a metabolic disease that is characterized by hyperglycemia, either due to defects in insulin production from autoimmune attack on the insulin-producing pancreatic β-cells (Type 1) or peripheral resistance to the effects of insulin (Type 2). However, the exact causes of diabetes are still unclear. Some agents used to induce diabetes in experimental mouse models, such as streptozotocin and alloxan, work at least partially by increasing oxidative stress in β-cells, but there is little evidence that this is relevant in human patients (Maritim et al., 2003). However, diabetes does correlate with oxidative stress, as biomarkers of oxidative
stress, such as lipid peroxides and 8-oxo dG are increased in the plasma and urine of diabetics (Halliwell and Gutteridge, 2015; Stephens et al., 2009). Increased oxidative stress can impair insulin-induced glucose uptake by muscle and adipose tissue (Maddux et al., 2001; Rudich et al., 1998), impair insulin synthesis (Matsuoka et al., 1997), and is hypothesized to lead to other diabetic complications such as peripheral neuropathy and retinopathy (Ceriello and Motz, 2004; Maritim et al., 2003).

At least some diabetic complications are likely caused by hyperglycemia-induced oxidative stress (Brownlee, 2005; Rolo and Palmeira, 2006). The cell types damaged by hyperglycemia, such as endothelial cells, cannot efficiently regulate glucose uptake in response to hyperglycemia (Kaiser et al., 1993). Increased cellular glucose uptake and subsequent metabolism causes elevated NADH generation, which acts as an electron donor for Complex I of the electron transport chain, thus promoting electron flow. This can cause increased leakage of electrons from the chain to form superoxide, similar to what happens in fatty acid β-oxidation, as discussed previously (Brownlee, 2005). Hyperglycemia can also lead to non-enzymatic glycation of proteins or lipids, which are oxidized by ROS to form advanced glycation end-products (Goh and Cooper, 2008; Goldin et al., 2006). Advanced glycation end-product formation can interfere with endogenous protein function, and their accumulation can damage connective tissue by promoting intermolecular collagen cross-linking (Goh and Cooper, 2008).

Unsurprisingly, a large number of studies have tested whether antioxidants might be a viable therapeutic in experimental models of metabolic disease, and clinical trials have been conducted with several antioxidant compounds. For example, in mouse models of diabetes, treatment with antioxidants such as vitamins C and E reduces markers of
oxidative stress in these animals (Johansen et al., 2005). In contrast to this study, meta-analyses showed that antioxidant vitamins have no effect on cardiovascular outcomes in diabetic patients in fifteen trials (Ye et al., 2013), or blood glucose and plasma insulin levels (Akbar et al., 2011) in 14 trials. However, vitamin E supplementation does decrease levels of glycated haemoglobin A₁c, a marker used for glycemia monitoring in type 2 diabetes (Akbar et al., 2011). Another antioxidant, α-lipoic acid, is an example of a compound with antioxidant activity that is in clinical use in the treatment of diabetic neuropathy (Mijnhout et al., 2012). It is currently approved for this purpose in Germany. However, it should be noted that only a small number of randomized, controlled, double-blind trials have been conducted for α-lipoic acid in diabetic neuropathy, and further studies are thus needed to demonstrate conclusively that it has beneficial effects in patients (Mijnhout et al., 2012).

While clinical data on the effects of antioxidants in metabolic diseases such as diabetes have been disappointing, there is still insufficient evidence to completely rule out a therapeutic role for antioxidants. In part, this is due to a lack of understanding of their mechanisms of action, how different antioxidants act at the molecular level, and the complexities involved in the metabolism of different antioxidants (Steinberg and Witztum, 2002). For example, most trials to date have tested vitamin E/α-tocopherol; however, even at very high dosages vitamin E does not decrease lipid peroxidation markers in healthy humans (Meagher et al., 2001). Furthermore, in the absence of a suitable co-antioxidant such as vitamin C, vitamin E can even act as a prooxidant (Stocker, 1999). As with the cancer trials there is also a need for agreement on markers and endpoints, better understanding of populations likely to benefit from antioxidant
treatment, and more data concerning appropriate length and time of intervention (Steinberg and Witztum, 2002).

1.1.1.1.3 Neurodegenerative disease

Oxidative stress has been implicated in a number of neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease (Barnham et al., 2004; Halliwell, 2006). Oxidative stress is thought to contribute to these diseases, as lipid peroxides, 8-oxo dG, and protein carbonylation occur at increased levels in the brains of patients with neurodegenerative disease (Halliwell, 2001). Moreover, levels of antioxidant enzymes such as catalase and SOD are increased in Alzheimer’s disease brains (Barnham et al., 2004; Halliwell, 2006). Treatment with the antioxidant NAC also restores cerebrovascular function in transgenic mice expressing the amyloid precursor protein, a model of Alzheimer’s disease (Nicolakakis et al., 2008). In addition, NAC prevents the loss of dopaminergic neurons in a mouse model of Parkinson’s disease (Park et al., 2004). Whether oxidative stress is a cause or consequence of neurodegenerative disease is unclear (Andersen, 2004).

Several clinical trials have been carried out to determine if antioxidants are a viable therapeutic for neurodegenerative disease. α-tocopherol slows disease progression in patients with moderate Alzheimer’s disease (Sano et al., 1997), although a similar dose does not prevent the progression of patients with mild cognitive impairment towards early Alzheimer’s disease (Petersen et al., 2005). In Parkinson’s disease, several antioxidants, including α-tocopherol, coenzyme Q₁₀, and glutathione, did not achieve significant changes in overall disease progression, although small improvements in
symptoms were observed in individual trials (Weber and Ernst, 2006). Collectively, these trials suffer from similar shortcomings as cancer and diabetes trials. Additionally, the administered compounds may not cross the blood-brain barrier efficiently (Halliwell, 2006). Further work, and perhaps new antioxidants, will be needed to conclusively determine whether ameliorating oxidative stress in neurodegenerative disease has any beneficial effects.

1.1.1.4 Aging

As previously described, the first suggestion that ROS may be important in vivo was the hypothesis that they may cause aging through a process of cumulative damage (Harman, 1956). Aging has been defined as “a persistent decline in the age-specific fitness components of an organism due to internal physiological deterioration” (Rose, 1994). A number of studies have shown that levels of oxidative damage increase with age across multiple species (Barja, 2002). Furthermore, many long-lived animal models, for instance Caenorhabditis elegans worms carrying insulin signaling pathway mutations and Drosophila melanogaster flies with a mutation in the methuselah gene, also show increased resistance to oxidative stress (Finkel and Holbrook, 2000). However, such studies only show correlation between oxidative stress and aging, not a causal link.

Several studies have taken genetic approaches to more definitively answer whether or not oxidative stress causes aging. If oxidative damage indeed caused aging, genetic ablations of antioxidant enzymes or regulators of such factors should show decreased lifespan; conversely, overexpression of such genes should cause increased lifespan. Many such studies focus on SOD. Superoxide is typically viewed as the major oxidative-
damage causing ROS, because it is the first ROS to be formed when electron leakage from the mitochondrial electron transport chain leads to donation of electrons to oxygen (Gems and Doonan, 2009). In *C. elegans*, SODs are not required for normal wild-type lifespan, as a quintuple mutant lacking all five *C. elegans* SODs does not show decreased lifespan (Van Raamsdonk and Hekimi, 2012). Furthermore, while overexpressing the two major SODs in *C. elegans*, sod-1 and sod-2, does increase lifespan, this was not due to decreased oxidative damage; in fact, worms overexpressing sod-1 showed increased protein carbonylation levels (Cabreiro et al., 2011). In mice, loss of CuZnSOD causes decreased lifespan (Elchuri et al., 2005), whereas overexpression is not sufficient to increase lifespan (Huang et al., 2000; Pérez et al., 2009). Complete loss of MnSOD in mice causes developmental defects, leading to lethality soon after birth (Lebovitz et al., 1996; Li et al., 1995), but heterozygous mice develop normally; these mice also show increased cancer incidence later in life, but have otherwise normal lifespan compared to wild-type mice (Van Remmen et al., 2003). Overexpression of MnSOD does not lead to increased lifespan in mice, either (Jang et al., 2009; Pérez et al., 2009). Curiously, in *D. melanogaster*, overexpression of both CuZnSOD and MnSOD results in longevity (Orr and Sohal, 1994; Sun and Tower, 1999; Sun et al., 2002), although the lifespan extensions observed by Orr and Sohal may have been overestimated due to the use of a control strain with a relatively short lifespan (Orr et al., 2003). Conversely, an MnSOD null mutant fly displays a shortened lifespan, as well as sensitivity to the oxidative stressor paraquat (Phillips et al., 1989). Similar experiments have been conducted with other antioxidant enzymes, for example catalase (Orr and Sohal, 1994; Petriv and Rachubinski, 2004; Pérez et al., 2009; Schriner et al., 2005) and glutathione peroxidase.
(Zhang et al., 2009b), with mixed results. On the whole, however, most of the genetic evidence to date does not support the classical free radical theory of aging (Blagosklonny, 2008; de Magalhães and Church, 2006; Gems and Doonan, 2009; Hekimi et al., 2011), although oxidative stress may contribute to age-related diseases such as those discussed above.

Interestingly, there is evidence that low levels of oxidative stress may be beneficial to longevity. In *C. elegans*, restricting glucose uptake causes an increase in mitochondrial respiration and a concomitant increase in oxidative stress, resulting in lifespan extension (Schulz et al., 2007). Treatment with antioxidants abolishes the lifespan extension seen in these worms, indicating that oxidative stress is required for increased lifespan under glucose-restricted conditions (Schulz et al., 2007). Long-lived insulin signaling mutant worms also generate a transient ROS signal that is required for their increased lifespan (Zarse et al., 2012). Long-lived germline-less *C. elegans* mutants show a similar increase in ROS, which is required for their extended lifespan (Wei and Kenyon, 2016). Other lifespan-extending interventions may also work at least partially through increasing ROS levels. Calorie restriction (CR), probably the most widely conserved experimental intervention known to increase lifespan, causes increased mitochondrial respiration in yeast and worms (Lin et al., 2002; Schulz et al., 2007; Sharma et al., 2011). However, it should be noted that some earlier studies observed lower rates of respiration in CR models (Barja, 2002), and several other hypotheses exist for the mechanism of CR action (Sinclair, 2005). Physical exercise is another intervention that is correlated with increased lifespan in humans (Lindsted et al., 1991; Manini et al., 2006) and causes an acute increase in oxidative stress (Radak et al., 2008). One study found that antioxidants
abolish this increase in oxidative stress and also prevent several beneficial outcomes of exercise (Ristow et al., 2009). While this on its own does not prove that exercise-induced oxidative stress causes increased longevity, it is a question that merits further study.
Table 1.1 Summary of animal model and clinical trial findings for and against roles of ROS in disease.

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<tr>
<th>Disease</th>
<th>Evidence from animal models</th>
<th>Evidence from selected clinical trials</th>
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<td>Positive evidence for ROS</td>
<td>Negative evidence for ROS</td>
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<td>Negative evidence for ROS</td>
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<td>Negative evidence for ROS</td>
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<td>Cancer</td>
<td>CuZnSOD&lt;sup&gt;−/−&lt;/sup&gt; mice: Increased liver cancer (Elchuri et al., 2005)</td>
<td>GCL required for tumourigenesis in mice (Harris et al., 2015)</td>
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<td></td>
<td>MnSOD&lt;sup&gt;−/+&lt;/sup&gt; mice: Increased cancer incidence (Van Remmen et al., 2003)</td>
<td>Antioxidants increase tumour progression in lung cancer (Sayin et al., 2014)</td>
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<td>GPx1,2&lt;sup&gt;−/−&lt;/sup&gt; mice: Increased intestinal cancer in <em>Helicobacter</em> infection (Chu et al., 2004)</td>
<td>Oxidative stress prevents metastasis (Piskounova et al., 2015)</td>
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<td>Antioxidants increase liver cancer metastasis (Wang et al., 2016)</td>
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<td>Linxian trial:</td>
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<td>Linxian trial: Combination of β-carotene, α-tocopherol and selenium associated with lower stomach cancer incidence (Blot et al., 1993)</td>
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<td>Obesity</td>
<td>NADPH oxidase inhibitor improved diabetes, dyslipidemia and hepatic steatosis in obese mice (Furukawa et al., 2004)</td>
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<td>Nrf2 activation prevents high fat diet-induced obesity in mice (Yu et al., 2011)</td>
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<td>Metabolic disease</td>
<td>GPX1 overexpression leads to increased obesity in mice (McClung et al., 2004)</td>
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<td>CARET trial: β-carotene and retinol associated with higher rates of lung cancer in smokers and asbestos-exposed workers (Omenn et al., 1996)</td>
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<td>ATBC trial: β-carotene associated with higher rates of lung, prostate and stomach cancer in male smokers (Albanes et al., 1995; The Alpha-tocopherol Beta Carotene Cancer Prevention Study Group, 1994)</td>
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<td>Disease</td>
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<td>Positive evidence for ROS</td>
<td>Negative evidence for ROS</td>
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<td>Secretion of pro-inflammatory cytokines by adipose tissue (reviewed in (Fernández-Sánchez et al., 2011))</td>
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<td>Diabetes</td>
<td>Induced models of diabetes in mice (streptozotocin, alloxan) (reviewed in (Maritim et al., 2003))</td>
<td>GPX1 overexpression leads to increased insulin resistance in mice (McClung et al., 2004)</td>
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<td>Vitamins C and E reduce oxidative stress markers in mouse models of diabetes (reviewed in (Johansen et al., 2005))</td>
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<td>Neuro-degenerative disease</td>
<td>Cerebrovascular function rescued in APP transgenic mice by NAC (Nicolakakis et al., 2008)</td>
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<td>Alzheimer's disease</td>
<td>NAC prevents loss of dopaminergic neurons in a mouse model of Parkinson's (Park et al., 2004)</td>
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<td>Disease</td>
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<td>Positive evidence for ROS</td>
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<tr>
<td>Aging</td>
<td><em>C. elegans</em>: Long-lived mutants have increased oxidative stress resistance (reviewed in (Finkel and Holbrook, 2000))</td>
<td><em>C. elegans</em>: SOD dispensable for lifespan (Van Raamsdonk and Hekimi, 2012); <em>sod-1/sod-2</em> overexpression does not increase lifespan by decreasing oxidative damage (Cabreiro et al., 2011); glucose restriction leads to lifespan extension (Schulz et al., 2007); <em>daf-2</em> worms require ROS for long lifespan (Zarse et al., 2012)</td>
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<td><em>D. melanogaster</em>: <em>methuselah</em> mutant has increased oxidative stress resistance (Lin et al., 1998); MnSOD null mutant decreases lifespan (Phillips et al., 1989); SOD overexpression increases lifespan (Sun and Tower, 1999; Sun et al., 2002)</td>
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<td><em>Mus musculus</em>: CuZnSOD knockout causes decreased lifespan (Elchuri et al., 2005)</td>
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<td><em>D. melanogaster</em>: Overexpression of SOD does not increase lifespan (Orr et al., 2003)</td>
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<td><em>M. musculus</em>: MnSOD&lt;sup&gt;+&lt;/sup&gt; mice have normal lifespan (Van Remmen et al., 2003); MnSOD overexpression does not increase lifespan (Jang et al., 2009; Pérez et al., 2009)</td>
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1.1.1.2 ROS have vital roles in health and homeostasis

While ROS have traditionally been thought of as damaging agents, a growing body of work demonstrates that they are in fact essential for maintaining a normal physiological state. For example, H$_2$O$_2$ is used as a signaling molecule in various biological pathways (Veal et al., 2007). Redox-sensitive enzymes are key to the regulation of these pathways (de Magalhães and Church, 2006; Soberman, 2003). Cells thus appear to have co-opted ROS as messengers in signal transduction pathways, while evolving sophisticated defense mechanisms to control ROS levels (D'Autréaux and Toledano, 2007; de Magalhães and Church, 2006; Holmström and Finkel, 2014; Pani et al., 2001; Soberman, 2003). Here, I summarize some physiological roles for ROS in vivo.

1.1.1.2.1 ROS in development

ROS play important roles in cell growth and proliferation, serving as signaling molecules in pertinent signaling pathways. For example, stimulation of cells with EGF leads to a transient increase in H$_2$O$_2$, which is required for phosphorylation of the EGF receptor and other downstream targets (Bae et al., 1997). ROS are thought to affect these signaling pathways via two mechanisms: by altering the intracellular redox state, and by modifying proteins within these pathways (Thannickal and Fanburg, 2000).

While roles for ROS in animal development are still unclear and, may be difficult to study, ROS are likely to be important regulators of development in vivo. Several studies show that in addition to regulating growth and proliferation, ROS are determinants of stem cell differentiation. Decreasing ROS levels by overexpressing antioxidant genes leads to
decreased differentiation in *Drosophila* multipotent haematopoietic stem cell progenitors, whereas increasing ROS levels by disrupting mitochondrial Complex I or deleting SOD2 increases differentiation (Owusu-Ansah and Banerjee, 2009). The differentiation of human mesenchymal stem cells into adipocytes also depends on mitochondrial ROS formation (Tormos et al., 2011). In the mouse D3 embryonic stem cell line, differentiation into cardiomyocytes is associated with a transient increase in endogenous ROS levels, and treatment with H$_2$O$_2$ enhanced differentiation while antioxidants suppressed differentiation (Sauer et al., 2000). Finally, in chick embryos, ganglion cell death can be prevented with antioxidants, but excess levels of antioxidants are also detrimental, suggesting that there is an optimum redox level for embryonic development (Castagné et al., 1999; Dennery, 2010).

### 1.1.1.2.2 ROS in immunity

ROS are important for both adaptive and innate immunity and are used by organisms to effectively clear pathogens. Perhaps their best-known role is the killing of engulfed pathogens by phagocytes, through the induction of a respiratory burst that generates large amounts of O$_2^-$ and H$_2$O$_2$ (Babior, 1984). In innate immunity, ROS mediate signaling downstream of Toll-like receptors to facilitate clearance of pathogens such as *Salmonella typhimurium* in macrophages (West et al., 2011). They are also required for signaling by inflammatory cytokines, such as tumour necrosis factor-$\alpha$ and interferon $\gamma$ (Sena and Chandel, 2012). In adaptive immunity, there is evidence that ROS are required for T cell activation. In support of this idea, treatment with antioxidants reduces antigen-specific T cell expansion upon viral infection in mice (Laniewski and Grayson, 2004).
1.1.1.2.3 ROS in autophagy

In autophagy, cellular components are degraded and recycled by double-membraned vesicles called autophagosomes (Glick et al., 2010; Mizushima, 2005). This pathway is conserved throughout eukaryotes and is activated under various stressful conditions, e.g. starvation and infection (Mizushima, 2005; Scherz-Shouval and Elazar, 2011). Autophagy is also essential for the removal of damaged cellular macromolecules, including those that have undergone oxidative damage. Defects in autophagy are implicated in a wide range of diseases, e.g. neurodegenerative disease, cancer, and Crohn’s disease (Jiang and Mizushima, 2014; Levine and Kroemer, 2008).

ROS induce autophagy, particularly superoxide (Chen et al., 2009), although H$_2$O$_2$ may also be important (Scherz-Shouval et al., 2007; Zhang et al., 2009a). The main source of ROS for stress-induced autophagy is the mitochondria, although NADPH oxidase can also contribute, particularly in antibacterial autophagy, which occurs in phagocytes (Scherz-Shouval and Elazar, 2011). An oxidative environment in the mitochondria leads to inhibition of a redox sensor, ATG4, that eventually allows the stabilization of a molecule that is crucial to autophagosome formation (Scherz-Shouval and Elazar, 2011). Autophagy is thus a redox-sensitive process that is activated in response to cellular stress.

1.1.2 Responses to oxidative stress

ROS can arise from a number of endogenous and exogenous sources. Under oxidative stress, cells respond with protective measures to ensure survival. As levels of oxidative stress rise, cells induce adaptation mechanisms in order to cope with injury, which may either be
reversible or permanent (Davies, 2000). Under high levels of oxidative stress, cells can undergo senescence, i.e. losing the ability to divide, or cell death, either by necrosis (unprogrammed cell death) or apoptosis (programmed cell death) (Ott et al., 2007; Tan et al., 1998) (Figure 1.1). Large bodies of work are devoted to each of these potential responses; here, I will focus on adaptive responses, particularly the induction of antioxidant and cellular repair responses.

All cells, including both prokaryotic and eukaryotic cells, encode enzymes that can modify or conjugate ROS to prevent them from reacting with cellular macromolecules. I previously discussed the SODs, which convert superoxide to less-reactive H$_2$O$_2$. In turn, catalase converts H$_2$O$_2$ can into water and oxygen. Reduced glutathione, the most abundant antioxidant in the cell, can donate electrons directly to ROS or to reduced disulphide bonds, thus acting as a ‘sacrificial’ molecule (Lushchak, 2012). Decreased glutathione levels are found many disease states, including Parkinson’s disease, chronic hepatitis C infection, and cystic fibrosis (Townsend et al., 2003). Some enzymes are also used to remove cellular components that have been damaged by ROS. For example, glutathione peroxidase can reduce lipid peroxides to alcohols (Halliwell and Gutteridge, 2015).

Under unstressed conditions, many protective or restorative enzymes are not expressed at high levels. However, under conditions of oxidative stress, levels of these enzymes increase. This can occur at several levels of regulation, including at the level of transcription (Finkel and Holbrook, 2000). Antioxidant gene expression is regulated by DNA-binding transcription factors, which are often redox-sensitive proteins or are under the control of redox-sensitive factors (Kobayashi et al., 2006; Sun and Oberley, 1996; Wakabayashi et al., 2004).
Both prokaryotes and eukaryotes contain transcription factors that regulate oxidative stress responses. In eukaryotes, transcription factors that regulate antioxidant transcriptional responses include Nrf2, Forkhead box O (FOXO), p53, NF-κB, and several others. Many of these transcription factors are normally repressed through degradation or by being kept in an inactive form. When oxidative stress arises, post-translational modifications allow them to escape degradation, and they accumulate in amounts large enough to activate cytoprotective target genes (Carter and Brunet, 2007; Itoh et al., 1999; Schreck et al., 2009; Vogelstein et al., 2000).

Many studies have elucidated the mechanisms of action for the transcription factors listed above. In the next section, I will focus on the transcription factor Nrf2, as it is a key regulator of the oxidative stress response. The roles and regulation of FOXO, p53, NF-κB and other transcription factors have been reviewed extensively elsewhere and do not fall within the scope of this thesis (Carter and Brunet, 2007; Schreck et al., 2009; Vogelstein et al., 2000).
Figure 1.1 Sources of ROS and their potential effects on cells.

ROS can arise from various endogenous and exogenous sources and can act as signaling molecules in critical pathways (blue), but can also have negative consequences on cells (red).

1.1.2.1 The transcription factor Nrf2 is a critical regulator of the oxidative stress response

Nrf2 is part of the Cap ‘n’ collar (CNC)-basic leucine zipper (bZIP) family of transcription factors, which are conserved throughout metazoans. In vertebrates, there are four family members: the p45 NFE2 (nuclear factor erythroid-derived 2) transcription factor, which functions in development; and three NFE2-related factors: Nrf1, Nrf2, and Nrf3 (Sykiotis and Bohmann, 2010). The three Nrf3s are broadly expressed, and although they regulate distinct gene programs, all three are involved in stress response regulation (Sykiotis
and Bohmann, 2010). Nrf2 is the most prominent of these factors, as it regulates a critical antioxidant response in oxidative stress (Itoh et al., 1997).

Nrf2 has several functional domains: a Neh2 domain near the N-terminus that binds the negative regulator Kelch-like ECH-associated protein 1 (Keap1), a transcriptional activation domain (TAD) just C-terminal to the Neh2 domain, a Neh1 domain that contains the CNC-bZIP DNA binding domain, a C-terminal Neh3 domain that is also required for transcriptional activation, and a Neh6 domain, which is crucial for Nrf2 turnover (Figure 1.2A) (Itoh et al., 1999; Loboda et al., 2016; McMahon et al., 2004; Nioi et al., 2005). Nrf2 binds the antioxidant response element (ARE), which has a core sequence of 5'-TGAG/CnnnGC-3', as a heterodimer with small Maf proteins (Itoh et al., 1997). AREs are found upstream of many antioxidant genes, including GSTs, NADPH:quinone oxidoreductase (NQO1), and heme oxygenase 1 (HO-1) (Loboda et al., 2016; Nguyen et al., 2009). Known Nrf2 targets include antioxidant and Phase II detoxification genes, lipid metabolism genes, and proteasome subunits (Kittingham et al., 2010; Kwak et al., 2003; Loboda et al., 2016). Under normal conditions, these genes are lowly expressed as Nrf2 is sequestered in the cytoplasm and targeted for degradation by Keap1, which acts as a substrate adaptor for a Cul3-based E3 ubiquitin ligase (Cullinan et al., 2004; Itoh et al., 1999; Kobayashi et al., 2004; Zhang et al., 2004). Keap1 contains redox-sensitive cysteine residues that can be reduced by electrophiles (Dinkova-Kostova et al., 2002). Some of these cysteine residues are critical for targeting Nrf2 for degradation, thus making Keap1 a redox-sensing regulator of Nrf2 that allows stabilization of Nrf2 under oxidative stress, allowing Nrf2 to translocate to the nucleus and activate target genes (Kobayashi et al., 2006; Wakabayashi et al., 2004) (Figure 1.2B).
Animal models and disease association studies support a role for Nrf2 as a critical regulator of the antioxidant response in vivo. Nrf2−/− mice are viable and are able to develop and reproduce normally (Chan et al., 1996). However, these mice are highly sensitive to oxidative stress, showing increased levels of oxidative stress markers and higher incidences of cancer and other diseases linked to oxidative stress (reviewed in (Sykiotis and Bohmann, 2010)). In humans, several single nucleotide polymorphisms in the Nrf2 gene NFE2L2 exist that are predicted to regulate its abundance; notably, some of these polymorphisms are associated with diseases such as asthma, systemic lupus erythematosus, vitiligo (loss of skin color in patches), and others (Cho et al., 2015). Moreover, somatic mutations in Nrf2 as well as in Keap1 have also been described in several cancers, including lung, head and neck, and gall bladder cancers (Hayes and McMahon, 2009; Sporn and Liby, 2012; Zhang, 2010). Many of these mutations lead to dissociation of Nrf2 from Keap1 and thus promote constitutive Nrf2 activation (Zhang, 2010). Such an effect may be beneficial in premalignant cells by preventing further oxidative damage, thus acting as a defense against carcinogens; however, in the later stages of tumourigenesis, Nrf2 protects against high ROS levels, aiding tumour progression and conferring resistance to radio- and chemotherapy (Sporn and Liby, 2012).
Figure 1.2 Nrf2 and SKN-1 regulate the antioxidant response in humans and *C. elegans*, respectively.

(A) Schematic diagram of Nrf2 and SKN-1 with functional domains highlighted. The Cap ‘n’ Collar (CNC), basic region (BR) and DIDLID domains are conserved between Nrf2 and SKN-1. (B) Schematic of SKN-1 isoforms showing location of its DNA binding domain and the *skn-1* RNAi clone. Adapted from (Blackwell et al., 2015). (C) Model of Nrf2 and SKN-1 activation. Under basal conditions, Keap1 and WDR-23 target Nrf2 and SKN-1 for ubiquitin-mediated proteasomal degradation, respectively. Oxidative stress caused by increased ROS levels suppresses the degradation of Nrf2/SKN-1, allowing them to bind antioxidant response elements upstream of target genes (via dimerization with small Maf proteins in the case of Nrf2) and activate target gene expression.
1.1.2.2  *C. elegans* as a model organism to study oxidative stress responses

Due to the limitations of studying oxidative stress *in vitro* and in cell culture, simple animal models are widely used to study oxidative stress responses *in vivo*. One of these is the nematode worm *C. elegans*, a small, genetically tractable model organism. The use of *C. elegans* as a model organism was first proposed by Sydney Brenner in the 1970s (Brenner, 1974). It has several qualities that make it an excellent model: worms can be cultured as hermaphrodites, making it possible to maintain clonal, isogenic populations. Additionally, males also exist, allowing for genetic crosses (Brenner, 1974). A plethora of genetic resources are available, including a sequenced genome, genome-wide RNA interference (RNAi) libraries collectively targeting >90% of the genome (Kamath et al., 2003; Rual et al., 2004), large-scale collections of promoter reporters, and a central repository of thousands of annotated mutant strains including a set of 2000 completely sequenced *C. elegans* strains collectively harbouring nearly a million mutations (C. elegans Deletion Mutant Consortium, 2012; Thompson et al., 2013). *C. elegans* has extremely stereotyped development – hermaphrodites have 959 cells, while males have 1031. The invariant lineage of all these cells has been mapped, allowing for intricate developmental studies (Sulston et al., 1983). Furthermore, these worms are transparent, allowing for examination of tissue morphology and enabling the convenient use of fluorescent reporters and myriad dyes in live animals. *C. elegans* mutants exhibit many phenotypes, both behavioural and morphological. Increasingly, *C. elegans* is becoming a popular model to study the genetics of physiology, for example in stress responses or aging (Gems and Doonan, 2009; Grants et al., 2015; Rodriguez et al., 2013). Of particular relevance to the study of stress responses and aging, however, is the short lifespan of these worms. Wild-type, isogenic *C. elegans* populations
live for up to a month, making them ideal for aging studies (Johnson and Wood, 1982). The ease with which large numbers of *C. elegans* can be cultured also allows for studies of population responses to stress agents. Additionally, several models of diseases in which oxidative stress is thought to play a crucial role, such as neurodegenerative diseases, have been created in *C. elegans* (Link, 2006). Thus, investigators can perform genetic and pharmacologic intervention studies in a whole organism in a rapid and efficient manner.

1.1.2.2.1 Caveats to using *C. elegans* to study oxidative stress

Despite the advantages listed above, there are some caveats to using *C. elegans* as a model to study oxidative stress responses. Firstly, unlike most mammals, *C. elegans* can tolerate a wide range of oxygen concentrations. They can survive 24 hours of anoxia (*i.e.* no oxygen) (Van Voorhies and Ward, 2000). Even more remarkably, they can reproduce for at least 50 generations in 100% oxygen, which causes fatal oxidative stress in most organisms in a short amount of time (Van Voorhies and Ward, 2000). The small size of *C. elegans* likely contributes to this wide range of oxygen tolerance. However, one study showed that *C. elegans* prefer 5-12% oxygen, adjusting their behavior to avoid hyperoxic conditions (Gray et al., 2004). This may reflect their natural habitat in microbe-rich settings such as decaying plant matter (Félix and Braendle, 2010). In the lab, *C. elegans* are normally cultured on *E. coli* bacterial lawns on the surfaces of agar petri dishes, where the oxygen concentration is presumably close to 21%. Very little work has been done to assess what effect these conditions have on lab-grown worms, compared to their counterparts in the wild. Based on Gray *et al.*’s work, it is possible that under lab conditions, *C. elegans* are actually experiencing hyperoxia, similar to cultured cells (Gray et al., 2004; Halliwell, 2003).
Although some disease models have been established in *C. elegans*, other diseases cannot be modeled in these worms. Cancer, for example, is difficult to model in *C. elegans*, as all somatic cells in adult worms are post-mitotic. Some exceptions to this are the use of the developing *C. elegans* vulva as a model of Ras-dependent cancers (Kaletta and Hengartner, 2006) and the formation of germline tumours in Notch mutants (Berry et al., 1997).

Nematodes also lack a circulatory system and an adaptive immune response (Kim and Ausubel, 2005; Van Voorhies and Ward, 2000). Finally, the small size of *C. elegans* means that tissue-restricted effects of oxidative stress can be difficult to study in this model, *e.g.* roles in neurons, which are affected in neurodegenerative diseases. Some genetic tools exist that can counter this problem, such as tissue-specific RNAi strains (Qadota et al., 2007) and tissue-specific promoters that allow expression of a gene of interest in tissue- or cell-type restricted fashion (Dupuy et al., 2007). It is also possible to isolate specific cells from *C. elegans*, which allows for gene expression and other analyses even in single cells (Kaletsky et al., 2016; Pauli et al., 2006; Spencer et al., 2014).

### 1.1.2.3 Oxidative stress response pathways in *C. elegans*

Oxygen sensing and responses to changes in oxygen levels are evolutionarily ancient biological processes. Thus, one would expect regulators of these responses to be evolutionarily conserved. In support of this, of the transcriptional regulators of oxidative stress responses described in *C. elegans* to date, all have human homologs. Most intensely studied among these are the Nrf2 homolog SKNhead-1 (SKN-1) and the FOXO homolog abnormal DAuer Formation-16 (DAF-16). The roles of these transcription factors in *C. elegans* are highlighted below.
1.1.2.3.1 SKN-1/Nrf2 is a key oxidative stress response regulator in *C. elegans*

*skn-1* was first described as a gene required for the formation of pharyngeal and intestinal cells by the EMS cell in the 4-cell stage *C. elegans* embryo (Bowerman et al., 1992). It subsequently emerged as a critical regulator of oxidative stress response genes in *C. elegans*, after investigators noted its similarity to other CNC transcription factors (An and Blackwell, 2003). Indeed, *skn-1* is required for *C. elegans* survival in many conditions that generate oxidative stress, including exposure to ROS-generating xenobiotics such as paraquat, sodium meta-arsenite, and juglone (An and Blackwell, 2003; Inoue et al., 2005; Park et al., 2009). It is also required for longevity, as worms lacking *skn-1* have a shorter lifespan than wild-type worms (An and Blackwell, 2003).

SKN-1 contains a number of structural similarities to Nrf2, including the presence of a CNC domain and basic region, as well as an N-terminal DIDLID motif. However, there are also some differences. Notably, SKN-1 lacks the bZIP domain found in mammalian Nrf, which is required for heterodimerization with the small Maf proteins (Blackwell et al., 1994; 2015). Instead, it contains a small, novel domain N-terminal to the CNC domain that binds the minor groove of DNA, allowing stable binding of SKN-1 to a single half-site motif (Blackwell et al., 1994) (Figure 1.2A). Four predicted isoforms of SKN-1 exist, SKN-1a-d (Figure 1.2B). SKN-1a is the longest SKN-1 isoform and contains a transmembrane region near its N-terminal end. It resides in endoplasmic reticulum (ER) and mitochondrial membranes but enters the nucleus after processing by proteases upon certain stimuli (Glover-Cutter et al., 2013; Hourihan et al., 2016; Paek et al., 2012). This activity is reminiscent of Nrf1, which also contains a transmembrane domain and localizes to the ER membrane.
(Radhakrishnan et al., 2014; Zhang et al., 2006). Additionally, Nrf2 can also localize to mitochondrial membranes as a ternary complex with Keap1 and the phosphoglycerate mutase family member PGAM5 (Lo and Hannink, 2008). SKN-1b is expressed constitutively in a pair of head neurons, the ASI neurons, and is required for dietary restriction-induced longevity but not oxidative stress responses (Bishop and Guarente, 2007). SKN-1c is expressed in the intestine and accumulates in intestinal nuclei under oxidative stress, and is thought to be the isoform analogous to Nrf2, i.e., it appears to be the dominant SKN-1 isoform in oxidative stress responses (An and Blackwell, 2003; Blackwell et al., 2015). SKN-1d is predicted to be expressed, but this has not yet been confirmed (Blackwell et al., 2015). Because my thesis work focuses primarily on the roles of SKN-1 in oxidative stress responses, ‘SKN-1’ refers to the SKN-1c isoform hereafter.

Like Nrf2, SKN-1 is normally targeted for degradation and only stabilizes in oxidative stress in wild-type worms (Choe et al., 2009). Surprisingly, *C. elegans* does not encode a Keap1 homolog. Instead, the WD40 repeat protein WDR-23 acts as a substrate adaptor for an E3 ubiquitin ligase, by binding both SKN-1 and the cullin4 ortholog damaged DNA binding protein 1 (Choe et al., 2009). In this manner, WDR-23 targets SKN-1 for ubiquitin-mediated degradation (Figure 1.2B). As expected, *wdr-23* knockdown or mutation causes increased levels of SKN-1 protein as well as activation of SKN-1 target genes (Choe et al., 2009). Like Keap1, WDR-23 contains a number of cysteine residues (Choe et al., 2009), but whether they are similarly modified by ROS to affect SKN-1 regulation is unknown.

The ease of genetic epistasis studies in *C. elegans* has helped elucidate several critical regulators upstream of SKN-1. These include the p38 mitogen-activated protein kinase (MAPK) pathway, the extracellular signal-related kinase (ERK) MAPK pathway, the
insulin/insulin growth factor-like signaling (IIS) pathway, the target-of-rapamycin (TOR) pathway, glycogen synthase kinase-3 (GSK-3), the dual oxidase BLI-3, the glp-1 Notch receptor, the ER stress sensor IRE-1, and the Skp1 homologs SKR-1/2 (An et al., 2005; Glover-Cutter et al., 2013; Hourihan et al., 2016; Inoue et al., 2005; Okuyama et al., 2010; Robida-Stubbs et al., 2012; Steinbaugh et al., 2015; Tullet et al., 2008; van der Hoeven et al., 2011; Wu et al., 2016). Notably, mutations in some of these pathways, such as the IIS and TOR pathways, as well as in glp-1, cause increased stress resistance and longevity (Arantes-Oliveira et al., 2002; Jia et al., 2004; Kenyon et al., 1993; Robida-Stubbs et al., 2012; Steinbaugh et al., 2015; Tullet et al., 2008; Vellai et al., 2003). Antioxidant target genes of SKN-1 are upregulated in these mutants, which presumably contributes to their enhanced stress resistance (Robida-Stubbs et al., 2012; Steinbaugh et al., 2015; Tullet et al., 2008). Whether upregulation of these genes is as critical to longevity is still debated, as SKN-1 also regulates genes involved in lipid metabolism and proteasomal degradation, both processes important for longevity (Li et al., 2011; Pang et al., 2014; Steinbaugh et al., 2015).

1.1.2.3.2 DAF-16/FOXO and the insulin/IGF-1-like signaling pathway

Mutations in the daf-2 gene cause a temperature-sensitive phenotype of constitutive dauer larva formation (Gems et al., 1998; Riddle, 1988). The dauer stage is a larval diapause in the C. elegans life cycle that occurs under conditions of low food availability, crowding, and high temperature. Dauer larvae are highly stress resistant and can survive without food for three to six months, traits that are presumably advantageous in the wild, when food sources are uncertain (Riddle, 1988). Once food becomes available again, dauers resume normal development and become reproductive adults (Riddle, 1988). In a key discovery,
Kenyon et al. showed that *daf*-2 mutants are long-lived as adults, with some mutant alleles causing lifespans twice as long as those of wild-type worms (Kenyon et al., 1993). Like many long-lived mutants, *daf*-2 adults are broadly stress-resistant, including resistance to oxidative stress (Kenyon, 2005). Genetic epistasis analysis revealed that *daf*-2 mutants require another gene called *daf*-16 for dauer formation, longevity, and stress resistance (Honda and Honda, 1999; Kenyon et al., 1993; Riddle et al., 1981; Vowels and Thomas, 1992). Notably, *daf*-2 is the *C. elegans* ortholog of mammalian insulin and IGF-1 receptors and *daf*-16 encodes the *C. elegans* ortholog of the FOXO transcription factor (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Subsequent work in *C. elegans* identified other members of the IIS pathway, most of whom also regulate dauer and lifespan phenotypes (Murphy and Hu, 2013).

DAF-16 localizes to the cytoplasm when DAF-2 is activated. However, when DAF-2 activity decreases, DAF-16 shuttles to the nucleus to activate its target genes (Henderson and Johnson, 2001; Lee et al., 2001). DAF-16-dependent genes fall into two classes: Class 1, which consists of genes that are upregulated in *daf*-2 mutants in a *daf*-16-dependent manner, and Class 2, genes that are downregulated in *daf*-2 mutants (Murphy et al., 2003). Class 1 genes largely consist of stress response genes, including catalases, the MnSOD *sod*-3, heat shock proteins that act as chaperones, cytochrome P450s, and other genes. Class 2 genes, on the other hand, consist of genes involved in growth and development (Murphy et al., 2003; Tepper et al., 2013). Loss of *daf*-16 leads to faster growth rate and causes decreased resistance to UV and heat stress, pointing to DAF-16 as a critical regulator of stress responses (Henderson and Johnson, 2001). This role of DAF-16 is conserved, as mammalian
FOXO similarly acts downstream of the insulin receptor and is also a key regulator of stress responses (Carter and Brunet, 2007; Kenyon, 2005).

DAF-16 is not the only transcription factor that acts downstream of the IIS pathway. Of particular relevance to this discussion is that SKN-1 is also a downstream target of the IIS pathway, acting parallel to DAF-16 (Ewald et al., 2015; Tullet et al., 2008). skn-1 is also required for the long lifespan and oxidative stress resistance of daf-2 mutants (Tullet et al., 2008), but is dispensable for dauer formation (Ewald et al., 2015; Tullet et al., 2008). Thus, while DAF-16 is a key determinant of the IIS pathway output, it is not the sole regulator of stress response downstream of this pathway.

1.2 The Mediator complex is a transcriptional coregulator

In eukaryotes, gene transcription is regulated by transcription factors that recognize and bind specific DNA sequences in promoters, enhancers, or silencers (Fuda et al., 2009). Through these physical interactions, transcription factors activate or repress nearby or distant genes. Although the ability to interact functionally with specific DNA elements is a key determinant in the selective regulation of gene expression, transcription factors do not regulate genes in isolation. Instead, they form regulatory complexes with transcriptional coregulators (coactivators and corepressors). Coregulators are essential accessory proteins that link transcription factors to the core transcriptional machinery such as RNA polymerase, or that modulate the structure of chromatin (Malovannaya et al., 2011; O’Malley et al., 2008; Spiegelman and Heinrich, 2004). The combinations of individual coregulators that act at a certain promoter ultimately determine whether a corresponding gene is induced or repressed in a particular cell type or physiological condition.
Among the many coregulators that are potentially available to transcription factors, the multiprotein Mediator complex (henceforth ‘Mediator’) plays a particularly interesting and central role (Allen and Taatjes, 2015; Malik and Roeder, 2010; Poss et al., 2013). Originally discovered and purified in yeast as a factor that promotes activator-dependent gene transcription (Flanagan et al., 1991; Kim et al., 1994; Koleske and Young, 1994), Mediator mechanistically influences transcription, RNA polymerase II activity, and chromatin structure and function in numerous ways. Crucially, it acts as a hub that integrates signals from multiple pathways to regulate transcriptional output.

1.2.1 Modules of the Mediator complex

Mediator is typically composed of 25-30 subunits. While composition varies by species, the key overall features of Mediator structure and function are evolutionarily conserved (Bourbon, 2008; Tsai et al., 2014). Mediator from yeast and human cells exhibits a similar overall architecture comprising four modules that perform somewhat separable functions: the head and middle modules contact Pol II, the tail module serves as a docking site for Mediator-binding transcription factors, and the dissociable kinase module regulates the activity of Mediator and of Mediator-binding transcription factors (Guglielmi et al., 2004; Tsai et al., 2014). Intriguingly, although head or middle module subunits are often broadly required for Mediator function and transcription, some tail and kinase module subunits are apparently not required for overall Mediator activity and instead are essential for specialized roles in developmental and physiological gene programs (Malik and Roeder, 2010; Poss et al., 2013). It is important to note that although emerging evidence hints at potential functions for some Mediator subunits independently of the complex and outside the nucleus (Cooper et
al., 2014; Huang et al., 2012), the specialized roles reviewed here are thought to originate not from subunit dissociation but due to individual activities while part of the complex.

1.2.2 Mediator mechanism of action in transcriptional regulation

In transcriptional activation, sequence-specific DNA-binding transcription factors bind their target sequences on DNA. Mediator forms physical interactions with transcription factors and links them to the transcriptional pre-initiation complex (PIC) (Figure 1.3A) (Conaway and Conaway, 2011a; Malik and Roeder, 2010). Such binding events may produce a specific output by inducing selective conformational changes, which are known to represent key effects of transcription factor binding to Mediator (Poss et al., 2013); indeed, Mediator is extraordinarily flexible, providing a malleable interface for transcription factors and RNA polymerases (Davis et al., 2002; Meyer et al., 2010; Näär et al., 2002; Taatjes et al., 2004; Tsai et al., 2014). Through its interactions with multiple transcription factors, Mediator thus integrates inputs from multiple signaling cascades to fine-tune downstream gene transcription.

1.2.2.1 Interactions with RNA polymerase II and general transcription factors

Upon binding of transcription factors to DNA and recruitment of the Mediator complex, RNA polymerase II (Pol II) and general transcription factors are assembled at promoters (Fuda et al., 2009). There are extensive contact interfaces between head and middle Mediator modules and the general transcription machinery (Davis et al., 2002; Tsai et al., 2014), with some subunits playing particularly crucial roles. For example, in yeast, the Mediator head module subunit MED17 binds the Rpb3 subunit of Pol II, and is required for recruitment of
Pol II in vivo (Soutourina et al., 2011). Mediator also binds Pol II at the C-terminal domain (CTD) of the Rpb1 subunit (Myers et al., 1998; Näär et al., 2002). The CTD is a multicopy heptapeptide repeat that is required for transcriptional activation by Mediator (Myers et al., 1998). It is a substrate for cyclin dependent kinase 8 (CDK8), the only Mediator subunit with known enzymatic activity. In yeast, CDK8 phosphorylates Ser5 of the CTD, resulting in inhibition of transcription by preventing Pol II interaction with DNA (Hengartner et al., 1998). On the other hand, human CDK8 phosphorylates the CTD at both Ser2 and Ser5 in vitro to promote thyroid hormone receptor (TR)-dependent transcription (Belakavadi and Fondell, 2010). Thus, the effects of CTD phosphorylation by CDK8 are complex and perhaps context-specific. The kinase module likely also regulates transcriptional activation through other interactions with the PIC, as some evidence suggests that while the entire Mediator complex is required to bridge sequence-specific transcription factors and the PIC, subsequent dissociation of the kinase module is required for release of Pol II and transcriptional activation (Jeronimo et al., 2016; Petrenko et al., 2016).

The PIC consists of Pol II and a number of general transcription factors (GTFs), which are required for transcriptional activation. Some Mediator subunits regulate the recruitment or activity of GTFs. At the level of recruitment, the head module subunit MED11 recruits the GTFs TFIIH and TFIIE, which are required for Pol II to begin transcription elongation (Esnault et al., 2008). Recruitment of TFIID and TFIIB are also dependent on Mediator (Baek et al., 2006; 2002). Mediator can also regulate the activity of TFIIH – phosphorylation of CDK7 subunit of TFIIH by CDK8 represses transcription (Akoulitchev et al., 2000). In summary, the interactions between Mediator and the PIC occur at multiple levels and are critical determinants of transcriptional output.
1.2.2.2 Interactions with sequence-specific transcription factors

Several Mediator subunits interact with DNA-binding sequence-specific transcription factors (Borggrefe and Yue, 2011; Malik and Roeder, 2010; Poss et al., 2013). Below I discuss some of the interactions pertinent to the topic of my thesis. Comprehensive reviews of Mediator-transcription factor interactions can be found in (Borggrefe and Yue, 2011) and (Poss et al., 2013).

Mammalian MED1, a Mediator subunit that locates to the tail-middle module interface (Tsai et al., 2014), is a critical signaling hub for a number of nuclear hormone receptors (NHRs) (Chen and Roeder, 2011); through these interactions, MED1 plays important roles in the regulation of metabolism. NHRs are a class of zinc-finger transcription factors that contain a ligand binding domain, which binds small molecules such as hormones and xenobiotics to control transcriptional activation. NHRs that bind MED1 include the thyroid hormone receptor, vitamin D receptor, hepatocyte nuclear factor 4α (HNF4α), peroxisome proliferator activated receptor-γ (PPARγ), estrogen receptor and glucocorticoid receptor (Fondell et al., 1996; Ge et al., 2008; 2002; Hittelman et al., 1999; Kang et al., 2002; Malik et al., 2002; Rachez et al., 1999). Thus, MED1 is a regulatory hub for many aspects of physiology. Binding of these NHRs is dependent on two LXXLL motifs in MED1, which interact with the AF2 domains of NHRs (Chen and Roeder, 2011). LXXLL motifs are also found in other transcriptional coregulators that interact with NHRs (Heery et al., 1997), as well as in other Mediator subunits, including MED14, MED25 and CDK8, all of which bind NHRs (Lee et al., 2007; Xie et al., 2015).
MED15 also interacts with many transcription factors, particularly those involved in metabolism. Yeast Med15 interacts with Gcn4, which regulates amino acid metabolism; with Oaf1, which regulates fatty acid oxidation; with the stress-responsive transcription factor Msn2; and with Pdr1, which regulates multidrug metabolism and resistance (Lallet et al., 2006; Park et al., 2000; Thakur et al., 2009; 2008). In mammals and C. elegans, MED15/MDT-15 binds the sterol regulatory element binding protein (SREBP), which regulates fatty acid desaturation, cholesterol synthesis (in mammals) and one-carbon cycle metabolism (in worms) (Walker et al., 2011; Yang et al., 2006). Interestingly, in C. elegans, most Mediator-binding NHRs bind at the MED15/MDT-15 tail module subunit instead of MED1/MDT-1.1/MDT-1.2 (Arda et al., 2010; Taubert et al., 2006). Many of these NHRs are involved in nutrient metabolism, including NHR-49, NHR-64 and NHR-114 (Arda et al., 2010; Gracida and Eckmann, 2013; Liang et al., 2010; Taubert et al., 2006). This suggests that MED15 is an evolutionarily ancient regulator of metabolism. While yeast do not have NHRs, they have NHR-like transcription factors, which also bind MED15 rather than MED1 (Thakur et al., 2008; 2009). As MED15 does not contain an LXXLL motif, the requirement of this motif for NHR binding most likely arose later in evolution. MED15 also binds the Smad2/3-Smad4 complex that effects transforming growth factor β (TGFβ) signaling (Kato et al., 2002), as well as p73, a p53 family member that activates cell cycle arrest and apoptosis in response to genotoxic stress (Satija and Das, 2016). Intriguingly, MED15 is overexpressed in a number of cancers, including castration-resistant prostate cancer, metastatic breast cancer and head and neck squamous cell carcinoma, and may contribute to cancer progression through modulation of TGFβ signaling (Shaikhibrahim et al., 2013; 2015; Zhao et al., 2013).
Although the sequence of MED15 is poorly conserved, it contains a conserved N-terminal KIX domain that is required for transcription factor binding (Novatchkova and Eisenhaber, 2004). However, multiple regions of MED15 contribute to such interactions. For instance, yeast Med15 binds Gcn4 using several protein-protein interfaces in multiple orientations, creating a ‘fuzzy complex’ consisting of weak hydrophobic interactions (Brzovic et al., 2011; Jedidi et al., 2010). Whether this applies to other MED15-transcription factor interactions is unknown.

1.2.2.3 Other roles for Mediator in transcription

Transcription is not just regulated at the initiation stage, but also at the elongation and termination stages, and Mediator plays roles in these processes as well. Initiated Pol II can pause 30-50 nucleotides downstream of the transcriptional start site and then be released by the cyclin dependent kinase positive transcription elongation factor b (P-TEFb), which is part of a large complex known as the super elongation complex (SEC) (Conaway and Conaway, 2013). Mediator is important for transcriptional elongation as well as initiation. In *Drosophila*, recruitment of Mediator to promoters of heat shock genes coincides with the release of paused Pol II, suggesting that Mediator recruitment promotes Pol II release (Park et al., 2001). One study showed that the MED23 subunit was required to release paused Pol II at the *Egr1* gene promoter in mouse embryonic stem cells (Wang et al., 2005). Additionally, both the MED26 and CDK8 subunits are required to recruit P-TEFb and the SEC to a subset of genes, providing a mechanistic explanation for Mediator’s role in elongation (Donner et al., 2010; Takahashi et al., 2011).
Mediator is also required for efficient transcription termination. In yeast lacking the MED18 subunit, Pol II accumulates at the 3’ end of MED18-dependent genes (Mukundan and Ansari, 2011). MED18 recruits cleavage factor 1 to the 3’ end of genes and is also required for gene looping by cross-linking the 5’ and 3’ ends of genes (Mukundan and Ansari, 2011; 2013), both of which are required for proper transcriptional termination.

Finally, Mediator also interacts with chromatin modifiers to regulate transcriptional activation or silencing. The MED12 kinase module subunit binds the RE1 silencing transcription factor and the methyltransferase G9a, which is required for methylation of H3K9 to repress neuronal genes in extraneuronal cells (Ding et al., 2008). MED23 is required for histone H2B mono-ubiquitination via its association with an E3 ubiquitin ligase complex (Yao et al., 2015), and MED25 facilitates the acetylation of histone H3K27 at the promoter of a HNF4α-dependent cytochrome P450 gene, leading to target gene activation (Englert et al., 2015).

1.2.3 Evolutionary conservation of the Mediator complex

Mediator is evolutionarily conserved and found in all eukaryotes; however, as noted, the exact subunit composition varies by species. For example, a number of subunits are not present in yeast, but are found in metazoans and some other unicellular eukaryotes: these are MED23, MED25, MED26, MED28, and MED30 (Bourbon, 2008). Moreover, some subunits have species-specific paralogs. For example, CDK19, MED12L, and MED13L are mammalian-specific paralogs of CDK8, MED12, and MED13, respectively (Conaway and Conaway, 2011b). Additionally, many Mediator subunits display limited sequence similarity between species; thus, some homology assignments (e.g. MED2/MED29, MED3/MED27,
and MED5/MED24) are tenuous and await experimental validation (Bourbon, 2008). For nomenclature conventions of Mediator subunits, see (Bourbon et al., 2004).

Despite the low sequence conservation at the individual Mediator subunit level and the variability of Mediator subunit collections in different species, the complex’s overall architecture is apparently also conserved. Using cryo-electron microscopy to observe individual Mediator subunits, Tsai et al. found that module composition and interactions are similar between yeast and human Mediator, and that conserved Mediator subunits show similar intra-Mediator interactions in both species (Guglielmi et al., 2004; Imasaki et al., 2011; Larivière et al., 2013; Tsai et al., 2014). However, human-specific Mediator subunits enable intra-Mediator interactions not seen in yeast Mediator, leading to structural differences. Most notably, the human Mediator tail module forms more extensive contacts with the head and middle modules, compared with yeast (Tsai et al., 2014).

1.2.3.1.1 The Mediator complex of C. elegans

While many biochemical studies of the Mediator complex have been conducted, mostly in yeast and mammalian cell culture, the functions of individual subunits in vivo are understudied. Understanding these functions is important, as mutations in Mediator subunits have been identified in multiple human diseases, including cancer and neurodevelopmental disorders (Napoli et al., 2012; Spaeth et al., 2011); importantly, individual Mediator subunit mutations evoke highly various phenotypes, attesting to the individual roles that each subunit plays. Studies to delineate these functions are hampered by the fact that most Mediator subunit knockout mice are not viable (Wang et al., 2009; Westerling et al., 2007). Conditional knockouts have been generated to study tissue or developmental stage-specific
roles of some Mediator subunits, but are comparatively costlier and more laborious to generate. Other model organisms such as *C. elegans* have thus been critical to our understanding of the functions of Mediator subunits, especially in development and physiology (Grants et al., 2015).

Although it has not yet been purified biochemically, sequence comparisons predict that *C. elegans* Mediator consists of 29 subunits (Table 1). Approximately one third of the 29 *C. elegans* Mediator subunits have been examined using *in vivo* promoter::GFP fusions, and most of these reporters appear to be expressed in a relatively broad manner (*e.g.* (McKay et al., 2003; Steimel et al., 2013; Taubert et al., 2006)). These findings align well with the view that Mediator is likely required for the bulk of Pol II transcription, and suggest that tissue-restricted expression is unlikely to be a key driver of Mediator subunit selectivity.

Functional studies using genome-wide RNAi libraries and/or strains carrying mutations in Mediator subunit genes suggest that many Mediator components are essential for viability or fertility in *C. elegans* (Kamath et al., 2003). These requirements are underscored by the fact that three Mediator subunit genes were initially discovered as essential (lethal, *let*) genes: *mdt-13* as *let-19*, *mdt-6* as *let-425*, and *mdt-7* as *let-49* (Kwon et al., 2001; Wang et al., 2004; Yoda et al., 2005) (Table 1.2). Although these studies likely underestimate the number of essential Mediator subunits (due to RNAi causing a reduction rather than a loss of gene function and because certain tissues are refractory to RNAi), it is also worth noting that some Mediator subunit null mutants do not display lethality or sterility (*e.g.* *cdk-8*) (Grants et al., 2016; Steimel et al., 2013). Therefore, these Mediator subunits appear to participate in specific signaling events rather than being generally required for transcription. A summary of
Mediator subunits in *C. elegans* and their hypothesized locations within the complex is given in Table 1.2 and Figure 1.3B.

![Mediator mode of action and hypothetical architecture in C. elegans.](image_url)

**Figure 1.3 Mediator mode of action and hypothetical architecture in C. elegans.**

(A) Diagram of Mediator mode of action. Mediator bridges DNA-binding sequence-specific transcription factors and the PIC to regulate transcription. (B) Model showing the hypothetical structure of *C. elegans* Mediator, with putative locations of individual subunits based on (Tsai et al., 2014). Subunits with a dashed outline lack apparent *C. elegans* orthologs. Where homology between yeast and *C. elegans* subunits is tenuous, both yeast and *C. elegans* names are given (MDT-29/MED2, MDT-27/MED3, and MDT-24/MED5). Adapted from (Grants et al., 2015).
List of *C. elegans* Mediator subunits and their mammalian orthologs, alternative names, and module locations.

List of *C. elegans* Mediator subunits and their mammalian orthologs (based on (Bourbon, 2008)), alternative *C. elegans* names, sequence number, and locations within Mediator complex modules (based on (Tsai et al., 2014)). Table adapted from (Grants et al., 2015).

<table>
<thead>
<tr>
<th>Subunits</th>
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<th>Alternative <em>C. elegans</em> names</th>
<th>Sequence number</th>
<th>Module</th>
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<td>H14E04.5</td>
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### 1.2.4 Mediator regulates physiological functions in *C. elegans*

A growing number of studies have identified roles for Mediator in regulating physiology in *C. elegans*. Below, I highlight Mediator-dependent regulation of lipid metabolism and stress responses in *C. elegans*. In particular, several studies have shown that the MDT-15/MED15 subunit is a critical regulator of both processes, which I will discuss in detail.

#### 1.2.4.1 Mediator in lipid metabolism

The tail module subunit gene *mdt-15* is essential for the regulation of lipid metabolism in *C. elegans*. MDT-15 physically interacts with nuclear hormone receptors (NHRs) homologous to mammalian HNF4α, such as NHR-49 and NHR-64, and also binds SBP-1, the worm homolog of sterol regulatory element binding protein (SREBP) (Figure 1.4) (Arda et al., 2010; Taubert et al., 2006; Yang et al., 2006). *sbp-1*, *nhr-49*, and *mdt-15* are all required for the expression of the fatty acid (FA) desaturase genes *fat*-5, -6, and -7, which generate mono- and polyunsaturated FAs (MUFAs and PUFAs). Both types of FAs are required for many aspects of *C. elegans* physiology; accordingly, worms depleted of *mdt-15* exhibit pleiotropic phenotypes that can be partially rescued with dietary PUFAs (Hou et al.,
2014; Taubert et al., 2006; Yang et al., 2006). The partial nature of this rescue suggests that, although a substantial fraction of its role in animal physiology entails maintaining normal FA desaturation, *mdt-15* must also be essential for additional processes (see Section 1.2.4.2).

*mdt-15*-dependent FA production is particularly important for membrane lipid homeostasis. Worms depleted of *mdt-15* activate the ER stress response due to imbalances in ER membrane lipid composition (Hou et al., 2014). Moreover, an *mdt-15* gain-of-function mutation suppresses the cold-sensitive phenotype of a *C. elegans* adiponectin receptor mutant (Svensk et al., 2013). Cold sensitivity is intrinsically linked to membrane fluidity, which in turn is affected by membrane lipid composition, again implicating *mdt-15* in membrane lipid homeostasis (Guschina and Harwood, 2006). Additional mutations that increase unsaturated FA production and suppressed this cold-sensitive phenotype were also identified in *nhr-49* (Svensk et al., 2013). Intriguingly, the mutations in *mdt-15* and *nhr-49* lie within or near domains that mediate the physical interaction between the two proteins; in fact, one of the *nhr-49* mutations increased binding to MDT-15, whereas another one abolished the interaction but still upregulated NHR-49 and MDT-15 target genes, possibly due to a conformational change that causes constitutive activation of NHR-49 (Lee et al., 2016). The activation of FA desaturases by MDT-15 and SBP-1, which leads to *de novo* FA synthesis, is also required to counter toxicity caused by excessive dietary glucose (Lee et al., 2015).

Lipid molecules, and *mdt-15*-dependent production thereof, have been proposed to play a role in the regulation of longevity. *mdt-15* is required for the long lifespan of *daf-2* mutants (Zhang et al., 2013); however, the specificity of this requirement is difficult to interpret, as *mdt-15* is similarly essential for the normal lifespan of wild-type worms and several other
long-lived mutants (Rogers et al., 2011; Taubert et al., 2006; Zhang et al., 2013).

Nevertheless, in the wild type background, PUFA supplementation partially rescues the short lifespan of \textit{mdt-15}–depleted worms (Taubert et al., 2006), suggesting that \textit{mdt-15} indeed assures normal life span through its requirement for the synthesis of certain lipid molecules.

In addition to its role in FA desaturation, MDT-15 also regulates FA \(\beta\)-oxidation genes (Taubert et al., 2006). \textit{nhr-49} and \textit{mdt-15} are particularly important to induce a subset of these genes in response to fasting (Taubert et al., 2006; Van Gilst et al., 2005a). Additional MDT-15–binding transcription factors, especially NHRs (Arda et al., 2010), are likely involved in various aspects of \textit{mdt-15}–dependent regulation of lipid metabolism, possibly performing redundant roles or alternatively adapting more specialized functions (Taubert et al., 2011). For instance, NHR-64 also regulates some \(\beta\)-oxidation genes and suppresses the synthesis of monomethyl branched chain FAs (Liang et al., 2010).

\textit{mdt-15} is required to express genes involved in lipid breakdown (\textit{e.g.} \(\beta\)-oxidation genes) and in lipid synthesis (\textit{e.g.} FA desaturases). What, then, is the overall effect of \textit{mdt-15} loss on fat storage? Worms subjected to \textit{mdt-15} depletion exhibit the Clear phenotype often associated with reduced fat storage (as seen in worms with reduced \textit{sbp-1/SREBP} levels) (Yang et al., 2006). In agreement with this finding, \textit{mdt-15} depletion results in reduced staining with the lipid-labeling dye Oil Red O (Arda et al., 2010). In contrast, recent quantification of overall extractable fats in \textit{mdt-15}–depleted worms and \textit{mdt-15} hypomorph mutants revealed that stored triglyceride abundance resembled that of wild-type worms (Hou et al., 2014). One way to reconcile these findings is that potentially the overall neutral lipid levels are similar in wild-type worms and in animals lacking \textit{mdt-15}, yet the assembly of
subcellular structures such as lipid droplets is impaired; this would explain the discrepancy between the gross morphological phenotypes and the extract-based lipid analysis.

*mdt-15*’s role in the regulation of lipid metabolism is evolutionarily conserved. In yeast, the MDT-15 ortholog MED15 also regulates genes involved in β-oxidation (Thakur et al., 2009). The interaction between MDT-15 and NHRs may also be conserved, as Gal11 interacts with Oaf1, a member of the Zinc-cluster type transcription factor family that is related to NHRs (Näär and Thakur, 2009; Phelps et al., 2006). In mammals, the MDT-15 ortholog MED15 regulates lipid metabolism by interacting physically and functionally with SREBP, the ortholog of SBP-1 (Yang et al., 2006). However, MED15 is not known to interact with NHRs, which primarily bind Mediator through MED1 (Borggreve and Yue, 2011), as discussed. Thus, whereas *C. elegans* MDT-15 interacts physically and functionally with transcription factors that regulate lipid balance, mammalian MED15 may have evolved into a more specialized regulator of certain aspects of lipid metabolism, distributing its broad ancestral regulatory roles to other subunits.

### 1.2.4.2 Mediator in detoxification and stress responses

Besides its role in lipid metabolism, *mdt-15* is also required for various stress responses. Gene expression studies revealed a large number of putative xenobiotic detoxification genes that depend on *mdt-15* for expression, both in the absence of toxins and when worms are exposed to xenobiotic compounds (Figure 1.4) (Taubert et al., 2008). Accordingly, *mdt-15* is required for resistance to the xenobiotics fluoranthene and RPW-24 (Pukkila-Worley et al., 2014; Taubert et al., 2008). *mdt-15* also engages in the innate immune response against the opportunistic bacterial pathogen *Pseudomonas aeruginosa* (Figure 1.4). Specifically, *mdt-15*
induces immune effectors downstream of the p38 MAPK PMK-1 upon \textit{P. aeruginosa} infection, thus mediating pathogen resistance (Pukkila-Worley et al., 2014). In contrast, \textit{mdt-15} is not required for thermotolerance or for resistance to the glycosylation inhibitor tunicamycin; similarly, the induction of detoxification genes in response to some pesticides is not blocked by loss of \textit{mdt-15} (Hou et al., 2014; Jones et al., 2013; Taubert et al., 2008). This demonstrates that MDT-15 is required for specific adaptive responses rather than being a universal stress resistance factor, and argues against the possibility that knockdown of \textit{mdt-15} simply renders worms too sick to mount a response against all stresses. Instead, the specificity of \textit{mdt-15} action likely originates from the ability of the MDT-15 protein to selectively bind transcription factors that implement particular stress responses.

Mediator subunits other than \textit{mdt-15} may also be required for stress responses. Two Mediator subunits were identified in a screen for genes conferring stress responses and/or longevity (Shore et al., 2012). \textit{mdt-26} is required to induce chaperones of the ER and mitochondrial UPR, as well as a detoxification gene responsive to sodium azide. \textit{mdt-26} knockdown causes decreased resistance to sodium azide, cadmium, and paraquat (Shore et al., 2012). Additionally, \textit{mdt-26} is required for the normal lifespan of wildtype worms and for the longevity of \textit{daf-2} and \textit{eat-2} (a genetic mimic of dietary restriction) mutants (Samuelson et al., 2007; Shore et al., 2012). Therefore, \textit{mdt-26} may have broad cytoprotective activities and deserves further attention, as relevant mechanisms remain undefined at this time.

The same screen also identified putative roles for the kinase module subunit gene \textit{mdt-12}, which is weakly required for detoxification gene induction, resistance to paraquat and sodium azide, and for the extended lifespan of some mitochondrial mutants, but not \textit{daf-2} or
eat-2 mutants (Shore et al., 2012). Another study showed that *mdt-12* is essential to induce the oxidative stress response gene *gcs-1* upon arsenite exposure, although it is not required for arsenite resistance *per se* (Crook-McMahon et al., 2014). In summary, Mediator subunits are required to regulate various stress responses; however, they are not simply generally required for stress responses, but rather are able to respond to specific stresses with tailored responses, likely through binding to stress-specific transcription factors.

**Figure 1.4 Known transcription factor interactions and functions of *C. elegans* MDT-15.**

MDT-15 interacts with several transcription factors to regulate lipid metabolism and detoxification programs in *C. elegans*. The dashed line between MDT-15 and NHR-64 indicates that MDT-15’s role in NHR-64 function has not yet been validated. MDT-15-interacting transcription factors in xenobiotic detoxification and innate immunity are still unknown.
1.3 Concluding remarks, hypothesis and objectives

*C. elegans* is a powerful model organism for the study of Mediator subunit functions *in vivo*. In particular, the MDT-15/MED15 subunit has been shown to be a critical regulator of lipid metabolism and stress responses in this model. However, while MDT-15 is known to partner with several DNA-binding transcription factors, including NHR-49, SBP-1, to regulate lipid metabolism (Arda et al., 2010; Taubert et al., 2006; Yang et al., 2006), no such partnerships have been described for MDT-15 dependent stress responses.

Microarray analysis of worms depleted of *mdt-15* by RNAi show that *mdt-15* is required for the expression of numerous stress response genes (see Chapter 2). These include oxidative stress-responsive genes, many of which are dependent on the transcription factor SKN-1/Nrf2 (Oliveira et al., 2009; Taubert et al., 2008). However, physical interactions between MDT-15 and SKN-1 have not been described. Additionally, SKN-1-independent oxidative stress response genes have been described, and some of these are also dependent on MDT-15 (Oliveira et al., 2009; Taubert et al., 2008). Therefore, the overall hypothesis of my thesis is that MDT-15 is a novel regulator of oxidative stress responses in *C. elegans*. The individual objectives are as follows:

1. To determine if MDT-15 is a coactivator of SKN-1 in the regulation of SKN-1-dependent oxidative stress response genes, and;

2. To determine if MDT-15 regulates SKN-1-independent oxidative stress response genes by interacting with a known MDT-15 transcription factor partner.
Chapter 2: The Mediator subunit *mdt-15* is required for both SKN-1-dependent and -independent oxidative stress responses in *C. elegans*

2.1 Synopsis

Reactive oxygen species (ROS) are vital for signaling and various physiological processes, but can also damage cellular macromolecules; thus, organisms tightly control ROS levels. One defense mechanism is to increase expression of detoxification genes, which can metabolize and remove excess ROS. Expression of these genes is tightly regulated and is specific to the type of oxidative stress present. Here, I show that in *C. elegans*, a subunit of the Mediator transcription coregulator complex, *mdt-15*, is required for the responses to two distinct types of oxidative stress. Loss of *mdt-15* phenocopied loss of the cytoprotective transcription factor *skn-1*, a key regulator of oxidative stress responses, as both genes were required to induce SKN-1 target genes in response to oxidative stress. MDT-15 physically interacted with SKN-1 in a yeast two-hybrid assay, indicating that it may be a *bona fide* SKN-1 coregulator. This interaction requires a region of MDT-15 that has not previously been described to bind MDT-15 transcription factor partners. Interestingly, I also found that MDT-15 is required for a SKN-1-independent oxidative stress response. This requirement can be separated from MDT-15’s known roles in lipid metabolism. A known MDT-15-binding transcription factor, NHR-64, was required for resistance but not the transcriptional response to oxidative stress. The MDT-15 transcription factor partner(s) that regulates the SKN-1-independent oxidative stress response therefore remains unknown. In summary, MDT-15 acts as a novel regulator of the oxidative stress response in *C. elegans*, by
interacting with and regulating the activity of multiple sequence-specific transcription factors.

2.2 Background

The Mediator is a large, multi-subunit protein complex conserved throughout eukaryotes that acts as a transcriptional coregulator, chiefly through its interactions with sequence-specific transcription factors and the pre-initiation complex. In *C. elegans*, the Mediator subunit MDT-15 was initially described as a regulator of lipid metabolism, specifically fatty acid desaturation and β-oxidation genes. To regulate these genes, MDT-15 interacts with the transcription factors SBP-1 and NHR-49 (Taubert et al., 2006; Yang et al., 2006). Worms depleted of *mdt-15* by RNAi show a decrease in polyunsaturated fatty acid (PUFA) levels. Many of the pleiotropic phenotypes of *mdt-15*(RNAi) worms, for instance larval development, brood size, and intestinal fat storage, can be rescued by feeding either oleic acid, the direct product of the Δ9 fatty acid desaturases *fat-6* and *fat-7*, or downstream PUFAs (Taubert et al., 2006; Yang et al., 2006). Notably, however, some phenotypes cannot be rescued in this way: for example, *mdt-15*(RNAi) causes extremely shortened lifespan (approximately one-third that of control worms), which only shows slight improvement with PUFA feeding (Taubert et al., 2006). Inadequate PUFA rescue is unlikely to be a major factor in these experiments as fatty acid profiles of PUFA-rescued *mdt-15*(RNAi) worms resemble that of wild-type worms (Hou et al., 2014). Instead, *mdt-15* is most likely also required to express non-lipid metabolism genes.

Microarray analysis of *mdt-15*(RNAi) worms showed that in addition to lipid metabolic genes, *mdt-15* is also required to express many genes with putative roles in detoxification, as
determined by gene ontology (GO) analysis. These include genes within the cytochrome P450 (CYP450), glutathione S-transferase (GST), UDP-glucuronosyl/UDP-glucosyltransferase (UGT) and short-chain dehydrogenase/reductase classes, which are all involved in xenobiotic detoxification (Taubert et al., 2008). Further studies showed that mdt-15 is required for resistance against a number of xenobiotics, such as fluoranthene, β-naphthoflavone and the heavy metal cadmium (Taubert et al., 2008). The role of MDT-15 in xenobiotic detoxification is likely conserved, as the yeast orthologue of MDT-15, Gal11, is also required for resistance to antifungal drugs via its interaction with a nuclear receptor-like transcription factor, Pdr1 (Thakur et al., 2008). However, the transcription factor(s) that MDT-15 partners with to regulate these responses are still unknown: SBP-1 and NHR-49 are not required for the expression of detoxification genes, and another MDT-15-binding transcription factor, NHR-8, was not required either, despite being a known regulator of other detoxification programs (Taubert et al., 2008).

Xenobiotic toxicity often occurs through mechanisms related to oxidative stress. For instance, a xenobiotic may itself be a reactive molecule, it may indirectly cause oxidative stress by interfering with intracellular antioxidant defenses e.g. by depleting glutathione, or its metabolism by detoxification enzymes such as CYP450s may generate reactive species (Halliwell and Gutteridge, 2015). Thus, the transcriptional responses to xenobiotics and oxidative stress are similar. In C. elegans, a key regulator of oxidative stress responses is the transcription factor SKN-1, the orthologue of mammalian Nrf2 (An and Blackwell, 2003). Interestingly, many SKN-1-regulated genes fall within the same gene classes as those regulated by MDT-15 (Oliveira et al., 2009). In fact, genes downregulated on mdt-15(RNAi) and those upregulated by SKN-1 show a small but significant overlap (Table 2.1).
Furthermore, MDT-15-dependent genes also show significant overlaps with gene sets induced by exposure to other oxidative stresses, for example sodium arsenite (henceforth referred to as ‘arsenite’), hyperoxia and juglone (Table 2.1) (Oliveira et al., 2009; Park et al., 2009; Przybysz et al., 2009). The responses to these stresses are known to require SKN-1, further suggesting a functional link between SKN-1 and MDT-15.

Transcriptional responses to distinct oxidative stresses vary depending on the type of stress. For instance, genes induced by arsenite are largely distinct from genes induced by another stressor, the organic peroxide tert-butyl hydroperoxide (tBOOH) (Oliveira et al., 2009). Arsenite attacks the thiol groups of glutathione and other peptides and promotes ROS production (Shi et al., 2004), whereas tBOOH acts at least partially by promoting lipid peroxidation (Masaki et al., 1989). The tBOOH response is largely SKN-1-independent; the transcription factor that regulates this response has yet to be identified (Oliveira et al., 2009). Intriguingly, MDT-15-dependent genes show a near-significant overlap with tBOOH-induced genes ($p=0.057$). Since many stress response genes are expressed lowly under non-stressed conditions, and the MDT-15 microarray experiment was not performed on worms undergoing oxidative stress, it is possible that the overlap is an underestimate and that MDT-15 may, in fact, also be required for the tBOOH response, in coordination with at least one unknown transcription factor.
Table 2.1 Overlaps between MDT-15-dependent genes and oxidative stress response or SKN-1-dependent genes.

Number of genes expected to overlap between lists of relevant sizes, the number of genes that actually overlap, and p-values (Fisher’s exact test) indicating significance of said overlap. The expected overlap is the fraction of MDT-15 regulated genes multiplied with the fraction of stress responsive genes.

<table>
<thead>
<tr>
<th></th>
<th>Down in mdt-15 RNAi (187 genes; (Taubert et al., 2008))</th>
</tr>
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<tbody>
<tr>
<td><strong>SKN-1 induced</strong></td>
<td><strong>233 genes</strong> (Oliveira et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Expected overlap: 4.0</td>
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<tr>
<td></td>
<td>Actual overlap: 13 (10 have predicted SKN-1 binding sites)</td>
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<tr>
<td></td>
<td>P-value 1.85E-04</td>
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<tr>
<td><strong>Arsenite-induced</strong></td>
<td><strong>118 genes</strong> (Oliveira et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Expected overlap: 2.0</td>
</tr>
<tr>
<td></td>
<td>Actual overlap: 11 (10 have predicted SKN-1 binding sites)</td>
</tr>
<tr>
<td></td>
<td>P-value 5.44 e-06</td>
</tr>
<tr>
<td><strong>tBOOH-induced</strong></td>
<td><strong>285 genes</strong> (Oliveira et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Expected overlap: 4.9</td>
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<td></td>
<td>Actual overlap: 9</td>
</tr>
<tr>
<td></td>
<td>P-value 0.057</td>
</tr>
<tr>
<td><strong>Hyperoxia (100% O2)</strong> induced</td>
<td><strong>948 genes</strong> (Park et al., 2009)</td>
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<td></td>
<td>Expected overlap: 16.3</td>
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<tr>
<td></td>
<td>Actual overlap: 37</td>
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<td></td>
<td>P-value 2.17E-06</td>
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<tr>
<td><strong>Juglone-induced in L4</strong></td>
<td><strong>103 genes</strong> (Przybysz et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Expected overlap: 1.8</td>
</tr>
<tr>
<td></td>
<td>Actual overlap: 10</td>
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<tr>
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<td>P-value 1.02E-05</td>
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2.3 Results

2.3.1 MDT-15 is required for survival in oxidative stress

Depletion of mdt-15 by feeding RNA interference (RNAi) causes decreases in gene expression levels of many oxidative stress response genes. To test whether these changes in gene expression caused oxidative stress sensitivity, I quantified population survival of wild-type worms and mdt-15(tm2182) hypomorphic mutants (Taubert et al., 2008) on 5 mM arsenite (As) and on 6 mM tBOOH. I found that mdt-15(tm2182) mutants were hypersensitive to both stressors (Figure 2.1A, Table 2.2). Additionally, depleting mdt-15 by
RNAi also caused tBOOH sensitivity (Figure 2.7, Table 2.4). Therefore, mdt-15 is required for normal oxidative stress resistance.

Figure 2.1 mdt-15 reduction-of-function mutants are hypersensitive to oxidative stress.

Survival plots of wild-type N2 and mdt-15(tm2182) worms on (A) 5 mM arsenite (As) and (B) 6 mM tBOOH. One representative experiment out of three independent repeats is shown.

Table 2.2 Statistics for individual mdt-15(tm2182) oxidative stress survival experiments.

Number of subjects is denoted as follows: Dx=Number of deaths that occurred during the assay; Nx=Total number of animals used in assay; Cx=Number of censored events (i.e. worms that ruptured at the vulva, underwent internal hatching of the progeny, or crawled off the plate). All p-values are derived using the log-rank (Mantel-Cox) test. ***p<0.001.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Strain</th>
<th>Experiment no.</th>
<th>Median survival (hours)</th>
<th>Number of subjects (Dx/Nx (Cx))</th>
<th>p-value vs. N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM arsenite</td>
<td>N2</td>
<td>1</td>
<td>&gt;100</td>
<td>3/107 (0)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>100</td>
<td>90/105 (5)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>&gt;100</td>
<td>45/107 (1)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>mdt-15 (tm2182)</td>
<td>1</td>
<td>24</td>
<td>104/105 (1)</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>24</td>
<td>98/100 (2)</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>24</td>
<td>105/105 (0)</td>
<td>&lt;0.0001***</td>
</tr>
<tr>
<td>Condition</td>
<td>Strain</td>
<td>Experiment no.</td>
<td>Median survival (hours)</td>
<td>Number of subjects (Dx/Nx (Cx))</td>
<td>p-value vs. N2</td>
</tr>
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</tr>
<tr>
<td>6 mM tBOOH N2</td>
<td>1</td>
<td>48</td>
<td>84/114 (28)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>35</td>
<td>83/111 (28)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>48</td>
<td>84/106 (19)</td>
<td>NA</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>49</td>
<td>67/105 (38)</td>
<td>NA</td>
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<tr>
<td>mdt-15 (tm2182)</td>
<td>1</td>
<td>24</td>
<td>104/105 (1)</td>
<td>&lt;0.0001***</td>
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<tr>
<td></td>
<td>2</td>
<td>10</td>
<td>17/17 (0)</td>
<td>&lt;0.0001***</td>
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<td></td>
<td>3</td>
<td>24</td>
<td>102/105 (3)</td>
<td>&lt;0.0001***</td>
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<tr>
<td></td>
<td>4</td>
<td>24</td>
<td>76/76 (0)</td>
<td>&lt;0.0001***</td>
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2.3.2 Levels and localization of oxidative stress-responsive transcription factors are unchanged in mdt-15 loss- or reduction-of-function backgrounds

To test that the oxidative stress sensitivity of mdt-15(tm2182) mutants was not due to reduced expression of known oxidative stress-responsive transcription factors such as SKN-1 or DAF-16 (An and Blackwell, 2003; Murphy et al., 2003), mRNA and protein levels of both transcription factors in mdt-15(RNAi) and mdt-15(tm2182) worms were measured. Real-time PCR (qPCR) was used to quantify mRNA levels of skn-1 and daf-16 in vivo. mdt-15 depletion or mutation did not significantly alter skn-1 levels, and actually increased daf-16 levels (Figure 2.2A-B). I also used translational GFP reporters to assess the levels and the nuclear localization of DAF-16 and SKN-1, as they are known to alter upon exposure to stress (An and Blackwell, 2003; Henderson and Johnson, 2001), and found that they were similar in control(RNAi) and mdt-15(RNAi) worms (Figure 2.2C-D). Thus, the phenotypes of mdt-15(RNAi) and mdt-15(tm2182) worms are unlikely to originate from compromised DAF-16 or SKN-1 expression or localization.
Figure 2.2 Levels and localization of DAF-16 and SKN-1 are unchanged in mdt-15 loss- or reduction-of-function backgrounds.

(A) mRNA fold change of skn-1 and daf-16 in mdt-15(RNAi) worms relative to control(RNAi) (n=4). mRNA levels were normalized to act-1, ama-1, cdc-42, and tba-1; error bars represent SEM. (B) Same as (A) but with N2 and mdt-15(tm218) worms. *p<0.05. (C) Fluorescence micrographs showing DAF-16::GFP expression in control(RNAi) and mdt-15(RNAi) worms, with or without sodium arsenite treatment. (D) Fluorescence micrographs showing SKN-1B/C::GFP expression in control(RNAi) and mdt-15(RNAi) worms. Because SKN-1 was not readily detectable, worms were treated with wdr-23(RNAi) to increase SKN-1 levels (WDR-23 is a negative SKN-1 regulator (Choe et al., 2009)). Autofluorescence is shown in the TxRed channel (magenta).

2.3.3  mdt-15 is required to induce SKN-1 target genes in response to arsenite

MDT-15 target genes show a significant overlap with SKN-1 target genes in the arsenite response. To test whether mdt-15 is required to induce arsenite response genes, synchronized wild-type worms were grown to the L4 stage on control and mdt-15 RNAi, then exposed
them to 5 mM arsenite for four hours and used qPCR to quantify oxidative stress gene expression. Arsenite reproducibly induced the six tested genes more than two-fold in control(RNAi) worms, and inductions of four genes were significantly reduced in mdt-15(RNAi) worms (Figure 2.3A-B). Thus, mdt-15 is required to induce arsenite response genes, and mdt-15(RNAi) phenocopies skn-1(RNAi). I note that while the skn-1 RNAi clone may target all skn-1 isoforms (Figure 1.2B), skn-1c is the major isoform that regulates oxidative stress responses. To support the RNAi studies, I similarly exposed synchronized wild-type and mdt-15(tm2182) L4 larvae to arsenite. mdt-15(tm2182) mutants showed significantly impaired arsenite inductions for five genes and reduced basal levels of three genes, resembling mdt-15(RNAi) worms (Figure 2.3C-D). Importantly, similar results were obtained with a one-hour arsenite exposure (Figure 2.3E-F), suggesting that compromised gene induction, not indirect effects, causes the oxidative stress sensitivity of mdt-15(tm2182) worms.
Figure 2.3 *mdt-15* is required for the SKN-1-dependent arsenite response.

(A) Fold changes of mRNA levels (relative to untreated control(RNAi) worms) in L4 wild-type worms grown on control, *mdt-15*, or *skn-1* RNAi and treated with 5 mM sodium arsenite for four hours (n=4). mRNA levels
were normalized to act-1, ama-1, cdc-42, and tba-1. (B) Fold inductions for (A). (C) mRNA fold changes
(relative to untreated N2 worms) in N2 and mdt-15(tm218) worms exposed to 5 mM As for 4 hours (n=5).
mRNA levels were normalized to act-1, ama-1, cdc-42, and tba-1. (D) Fold inductions for (C). (E) mRNA fold
changes (relative to untreated N2 worms) in N2 and mdt-15(tm218) worms exposed to 5 mM As for one hour
(n=5). mRNA levels were normalized to act-1, tba-1, and ubc-2. (F) Fold inductions for (E). Error bars
represent SEM. For (A), (C), and (E), * **Gene expression levels differ significantly from non-treated
control(RNAi) or N2 worms (p<0.05 and p<0.01 respectively). † † † Gene expression levels differ significantly
from As-treated control(RNAi) or wild-type worms (p<0.05 and p<0.01 respectively).

To corroborate the qPCR data, I studied worms expressing transcriptional gcs-1p::gfp or
gst-4p::gfp reporters (An and Blackwell, 2003; Leierson et al., 2003). In L4 stage gcs-1p::gfp
worms, the induction of intestinal GFP by a four-hour arsenite exposure was severely
compromised when worms were grown on mdt-15 RNAi (Figure 2.4A). To ensure that this
phenotype was not caused by impaired development due to mdt-15 depletion, I treated late
L4 gcs-1p::gfp worms with RNAi for 48 hours and then exposed them to arsenite for four
hours, i.e. allowing worms to complete development before exposing them to RNAi.

Induction of gcs-1p::gfp by arsenite remained mdt-15 dependent in this adult-only RNAi
regimen (Figure 2.4B). I observed a similar mdt-15 requirement in worms expressing a gst-
4p::gfp reporter (Figure 2.4C). Notably, mdt-15 depletion reduced intestinal gst-4p–driven
fluorescence but evoked hypodermal fluorescence not seen in control(RNAi) animals,
perhaps reflecting compensatory, mdt-15–independent gst-4 induction (Figure 2.4C).

Together, these data suggest that MDT-15 coregulates the transcriptional stress response to
arsenite by affecting a subset of arsenite responsive genes.
Figure 2.4 *mdt-15* is required to induce SKN-1-dependent transcriptional GFP reporters on arsenite.  

(A) DIC and fluorescence micrographs show *gcs-1p::gfp* worms grown on control or *mdt-15* RNAi to the L4 stage and then treated with 5 mM sodium arsenite for four hours. One of three repeats is shown. (B) *gcs-1p::gfp* worms grown to the L4 stage and then treated with control or *mdt-15* RNAi for 2 days were subsequently exposed to 5 mM arsenite for four hours. (C) Same as (A), except with *gst-4p::gfp* worms.
2.3.4 MDT-15 is required to induce SKN-1 targets in worms with elevated SKN-1 levels

As Mediator subunits are tethered to genomic regulatory elements by transcription factors, we hypothesized that MDT-15 may be a coregulator for SKN-1. First, wdr-23(tm1817) loss-of-function (LOF) mutants (Choe et al., 2009) were used to test whether MDT-15 and SKN-1 cooperate. WDR-23 is part of a ubiquitin ligase complex that promotes SKN-1 degradation; thus, wdr-23(tm1817) worms exhibit increased levels of SKN-1 and SKN-1 target genes. If mdt-15 were required to express SKN-1 targets, mdt-15 depletion should suppress SKN-1–dependent gene inductions in wdr-23(tm1817) mutants. To test this hypothesis, mRNA levels in developmentally synchronized wild-type and wdr-23(tm1817) worms grown on control, mdt-15, and skn-1 RNAi were quantified. The upregulation of several SKN-1 targets in wdr-23(tm1817) mutants was strongly and similarly reduced by mdt-15 and skn-1 depletion (Figure 2.5A). Depletion of wdr-23 in mdt-15(tm2182) mutants by RNAi also caused significantly impaired induction of SKN-1 targets (Figure 2.5B). Thus, the increased expression of SKN-1 targets in wdr-23 LOF worms requires mdt-15.

Some Mediator subunits act in gene-specific fashion, but others are broadly required for transcription (Holstege et al., 1998; Poss et al., 2013; van de Peppel et al., 2005). To address subunit specificity in SKN-1 target gene transcription, we tested whether MDT-6 was required for the SKN-1–dependent inductions in wdr-23 LOF mutants. MDT-6 is part of the head module of the Mediator complex, which interacts with RNA polymerase II (Davis et al., 2002; Malik and Roeder, 2010); loss of mdt-6 in the developing C. elegans embryo causes lethality, suggesting that it is broadly required for transcription (Kwon et al., 1999). However, unlike mdt-15 RNAi, mdt-6 RNAi did not prevent the induction of SKN-1 targets.
in \textit{wdr-23} mutants (Figure 2.5B), although both RNAi clones delay growth and development. These data provide evidence for Mediator subunit specificity and demonstrate that developmental arrest \textit{per se} is not sufficient to block SKN-1 target induction.

\textbf{Figure 2.5} \textit{mdt-15} is required for the upregulation of SKN-1 targets in \textit{wdr-23} LOF backgrounds. (A) Relative mRNA fold changes of SKN-1 targets in L4 stage wild-type N2 or \textit{wdr-23} \textit{(tm1817)} worms grown on either control, \textit{mdt-15}, \textit{skn-1}, or \textit{mdt-6} RNAi (\textit{n}=4). (B) Fold changes of SKN-1 targets in N2 and \textit{mdt-15} \textit{(tm2182)} worms grown on control and \textit{wdr-23} RNAi, relative to control(RNAi) (\textit{n}=4). mRNA levels were normalized to \textit{act-1}, \textit{ama-1}, \textit{cdc-42}, and \textit{tba-1}; error bars represent SEM. *\textit{p}<0.05.

2.3.5 The MDT-15 protein physically interacts with SKN-1 independently of its KIX domain

If MDT-15 acts as a SKN-1 coregulator, the two proteins should associate physically. To test whether SKN-1 binds MDT-15, I used the yeast two-hybrid system. MDT-15 contains an N-terminal KIX-domain that binds nuclear hormone receptors (NHRs) and the lipogenic transcription factor SBP-1 (Taubert et al., 2006; Yang et al., 2006). As this is the only recognized transcription factor binding domain in MDT-15, we hypothesized that the KIX-domain (aa1-124; Figure 2.6A) might associate physically with SKN-1. However, although
the MDT-15-KIX bait was expressed (Figure 2.6C), it did not interact with SKN-1 in yeast-two-hybrid assays (Figure 2.6B).

The yeast MDT-15 orthologue Gal11 uses multiple surfaces to bind the transcription factor Gcn4, with the KIX-domain playing a minor role (Herbig et al., 2010; Jedidi et al., 2010). Thus, I tested whether SKN-1 interacted with two longer baits, MDT-15-NT (aa 1-338) and near-full-length MDT-15ΔCT (aa 1-600; Figure 2.6B; full-length MDT-15 strongly autoactivates (data not shown), as expected from a protein that acts as a coactivator, and hence cannot be used as bait in the Y2H system). While MDT-15-NT failed to interact, MDT-15ΔCT strongly and specifically bound SKN-1c (Figure 2.6B; binding to SKN-1a and SKN-1b was undetectable; SKN-1d was not tested). In fact, MDT-15ΔCT bound SKN-1 more strongly than the known MDT-15 binding partner NHR-49 (Figure 2.6D) (Taubert et al., 2006).

The KIX-domain is not sufficient for SKN-1 binding, but may be required. To test this, I assayed binding of SKN-1c to an MDT-15ΔCT variant lacking the KIX-domain (MDT-15ΔKIXΔCT; aa 125-600). Binding of SKN-1c to MDT-15ΔKIXΔCT was as strong as binding to MDT-15ΔCT (Figure 2.6B), indicating that the KIX-domain is dispensable for SKN-1c binding.

The mdt-15(tm2182) allele contains an in-frame deletion that removes 161 amino acids from the MDT-15 protein (Goh et al., 2014). Because this mutant is sensitive to arsenite, it is possible that the region deleted in this strain is involved in SKN-1 binding. An MDT-15tm2182ΔCT bait (aa 1-393–553-600), which recapitulates the tm2182 deletion, showed weak SKN-1c binding compared to MDT-15ΔCT (Figure 2.6B). Thus, a likely molecular explanation for the inability of mdt-15(tm2182) worms to induce SKN-1 targets is that the
tm2182 mutation compromises MDT-15 binding to SKN-1c. However, I note that the expression of the MDT-15tm2182ΔCT (Figure 2.6C) bait is also very weak, which may account for its lack of binding to SKN-1, although it is only slightly weaker than the MDT-15ΔCT construct, which binds strongly. I also tested whether the deleted region (MDT-15-del; aa 393-552) was sufficient for SKN-1c binding, but it failed to interact with SKN-1c above background levels, suggesting that it is not (Figure 2.6B).

**Figure 2.6** MDT-15 physically interacts with SKN-1.

(A) Schematic of MDT-15 fusion proteins tested for interaction with SKN-1c. (B) Relative interaction strength between Gal4DBD-MDT-15 variants and Gal4AD-SKN-1c. Values indicate average interaction strength in percent, calculated from Miller units (n>4 per plasmid combination); error bars represent SEM. (C) Expression of Gal4DBD::MDT-15 variants used in (B). Asterisks indicate the expected size of individual fusion proteins. (D) Relative interaction strength of MDT-15ΔCT binding to SKN-1 vs. NHR-49.
2.3.6 MDT-15’s role in tBOOH resistance is independent from its role in fatty acid desaturation

Unlike *mdt-15, skn-1* is largely dispensable for the tBOOH response (Oliveira et al., 2009), implicating SKN-1–independent mechanisms for MDT-15 in this context. One possibility is that the altered fatty acid profiles of *mdt-15* worms (Taubert et al., 2006; Yang et al., 2006) underlie their tBOOH sensitivity. The fatty acid desaturases *fat-6* and *fat-7* are MDT-15 targets (Taubert et al., 2006; Yang et al., 2006), and RNAi against either enzyme causes sensitivity against the oxidative stressor paraquat (Horikawa and Sakamoto, 2009). To test whether fatty acid desaturases are required for tBOOH resistance, I exposed *fat-6(tm331); fat-7(wa36)* double mutants (Brock et al., 2007) to tBOOH. Surprisingly, these worms were not tBOOH sensitive (Figure 2.7A, Table 2.3), perhaps because their unusual C18 polyunsaturated fatty acids (PUFAs) substitute for normal C20 PUFAs (Brock et al., 2007). *fat-6* RNAi, which depletes both *fat-6* and *fat-7* due to their high sequence similarity (Brock et al., 2006), also failed to evoke tBOOH sensitivity, despite delaying development (Figure 2.7, Table 2.4). This suggests that reduced *fat-6* and *fat-7* expression does not cause the tBOOH sensitivity of *mdt-15* worms. To corroborate these data, I allowed wild-type and *mdt-15(tm2182)* worms to complete development in the presence of exogenous PUFAs, then exposed these rescued worms to tBOOH. Although fertility, mobility, and development were improved in PUFA-fed *mdt-15(tm2182)* mutants, they remained fully tBOOH sensitive (Figure 2.7C, Table 2.5). Thus, reduced PUFA levels are unlikely to cause the tBOOH sensitivity of *mdt-15(tm2182)* mutants. In fact, wild-type worms were slightly tBOOH hypersensitive in the presence of exogenous PUFAs (Figure 2.7C, Table 2.5). Furthermore, *fat-5, fat-6,* and *fat-7* mRNA levels decreased following tBOOH exposure (Figure 2.7D).
Taken together, these data argue that altered fatty acid profiles are unlikely to cause the tBOOH sensitivity of mdt-15 worms.

Figure 2.7 MDT-15 regulates oxidative stress responses independently of fatty acid desaturation.

(A) Survival plots of N2 and fat-6(tm331); fat-7(wa36) worms on 6 mM tBOOH. (B) Survival plots of control(RNAi), mdt-15(RNAi), and fat-6(RNAi) worms on 6 mM tBOOH. (C) Survival plots of wild-type N2 and mdt-15(tm2182) worms on 6 mM tBOOH following development on PUFAs. For all survival plots, one representative experiment out of three independent repeats is shown. (D) Relative mRNA fold changes of fat-5, -6, and -7 in wild-type worms in unstressed conditions and after four hours on 7.5 mM tBOOH (n=4). mRNA levels were normalized to act-1, tba-1, and ubc-2; error bars represent SEM. *p<0.05
Table 2.3 Statistics for individual fat-6(tm331);fat-7(wa36) tBOOH survival experiments.

Number of subjects denoted and statistics calculated as described in Table 2.2. *p<0.05, ***p<0.001.

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<td>4</td>
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<td>45/120 (75)</td>
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<tr>
<td>(wa36)</td>
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<td>97</td>
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Table 2.4 Statistics for individual RNAi-treated tBOOH survival experiments.

Number of subjects denoted and statistics calculated as described in Table 2.2. **p<0.01, ***p<0.001.

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Table 2.5 Statistics for individual PUFA-treated tBOOH survival experiments.

Number of subjects denoted and statistics calculated as described in Table 2.2. *p<0.05, ***p<0.001.

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<td>Experiment no.</td>
<td>Median survival (hours)</td>
<td>Number of subjects (Dx/Nx (Cx))</td>
<td>p-value vs. no treatment</td>
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<td>---------------</td>
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<td>24</td>
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<td>0.0345*</td>
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2.3.7 MDT-15 is required for the SKN-1-independent response to tBOOH

To test whether mdt-15 is required for the transcriptional tBOOH response, L4 stage control(RNAi), mdt-15(RNAi), and skn-1(RNAi) worms were exposed to 7.5 mM tBOOH for four hours. mdt-15, but not skn-1, was required to induce some, but not all genes in response to tBOOH, again affecting both basal levels and fold-inductions (Figure 2.8A). I obtained similar data with mdt-15(tm2182) mutants, with three of six genes displaying MDT-15–dependent regulation in tBOOH (Figure 2.8C-D; some genes show increased basal expression in mdt-15(tm2182) mutants). As with arsenite, a one-hour exposure to tBOOH showed early dependence of mdt-15 for at least two genes (Figure 2.8E-F; several genes were not well induced after one hour). Thus, mdt-15 is selectively required for the transcriptional response to two compounds evoking distinct oxidative stress signatures.
Figure 2.8 mdt-15 is required for the SKN-1-independent transcriptional response to tBOOH.

(A) Fold changes of mRNA levels in L4 wild-type N2 worms grown on control, mdt-15, or skn-1 RNAi and treated with 7.5 mM tBOOH for four hours, relative to untreated control (RNAi) (n=4). mRNA levels were normalized to act-1, ama-1, cdc-42, and tba-1. (B) Fold inductions for (A). (C) Fold changes in tBOOH responsive genes in N2 and mdt-15(tm2182) worms exposed for four hours to 7.5 mM tBOOH, relative to untreated control (RNAi) (n=4). mRNA levels were normalized to act-1, ama-1, cdc-42, and tba-1. (D) Fold inductions for (C).
untreated N2 worms (n=4). mRNA levels were normalized to act-1, tba-1 and ubc-2. (D) Fold inductions for (B). (E) mRNA fold changes (relative to untreated N2) in N2 and mdt-15(tm2182) worms exposed to 7.5 mM tBOOH for one hour (n=5). mRNA levels were normalized to act-1, tba-1, and ubc-2. (F) Fold inductions for (E). Error bars represent SEM. For (A), (C) and (E), * **Gene expression levels differ significantly from non-treated control(RNAi) or N2 worms (p<0.05 and p<0.01 respectively). † ††Gene expression levels differ significantly from As-treated control(RNAi) or wild-type worms (p<0.05 and p<0.01 respectively).

2.3.8 An MDT-15-interacting transcription factor is required for resistance but not the transcriptional response to tBOOH

SKN-1 is dispensable for tBOOH induced transcription, whereas MDT-15 is necessary (Figure 2.8A-C). MDT-15 binding transcription factors other than SKN-1 must therefore confer transcriptional tBOOH responses and tBOOH resistance. To test this hypothesis, I quantified the tBOOH sensitivity of previously characterized nhr-64(ok1957) and nhr-49(nr2041) null mutants (Liang et al., 2010; Van Gilst et al., 2005b); both genes encode MDT-15–binding transcription factors (Taubert et al., 2006). I found that nhr-64(ok1957) mutants were sensitive to tBOOH but not arsenite, whereas nhr-49(nr2041) worms were sensitive to both molecules (Figure 2.9A-D, Table 2.6, Table 2.7). However, while nhr-49 RNAi also causes paraquat sensitivity (Horikawa and Sakamoto, 2009), prior microarray studies of nhr-49(nr2041) mutants revealed no link to stress responses (Pathare et al., 2012), and nhr-49(RNAi) worms normally induce xenobiotic response genes upon toxin exposure (Taubert et al., 2008). Therefore, I subsequently decided to focus on whether NHR-64 is required for the tBOOH response.

NHR-64 regulates fat metabolism genes, but whether or not it also regulates oxidative stress response genes has not been studied (Liang et al., 2010). Thus, I quantified tBOOH
responsive genes in wild-type worms and nhr-64(ok1957) mutants by qPCR. Tested genes were only mildly affected in L4 stage nhr-64(ok1957) mutants, and induction by tBOOH was not significantly compromised (Figure 2.9E; note that basal levels were increased in nhr-64(ok1957) mutants). Further work is required to define the molecular cause of tBOOH sensitivity in these mutants.

Aside from the KIX-domain, the MDT-15 surfaces involved in NHR-64 binding have not been comprehensively tested. I found that NHR-64 interacted with MDT-15-KIX, -NT, and -ΔCT, as expected because they all contain the KIX-domain (Figure 2.9F). Unlike SKN-1, NHR-64 failed to bind MDT-15-ΔKIXΔCT, demonstrating that the KIX-domain is sufficient and necessary for NHR-64 binding (Figure 2.9F). Like SKN-1, NHR-64 bound only weakly to MDT-15tm2182-ΔCT, suggesting that the region deleted in mdt-15(tm2182) worms is involved in both NHR-64 and SKN-1 interactions (Figure 2.9F). Thus, two separable regions in MDT-15 are required for NHR-64 binding, although the KIX-domain is sufficient for partial binding. Reduced binding of MDT-15 to NHR-64 (or other factors required for the tBOOH response) might be responsible for the tBOOH sensitivity of mdt-15(tm2182) mutants.
Figure 2.9 MDT-15-binding transcription factors are required for resistance to tBOOH.

(A-B) Survival plots of wild-type N2 and *nhr-64(ok1957)* worms on 5 mM arsenite and 6 mM tBOOH. (C-D) Survival plots of wild-type N2 and *nhr-49(nr2041)* worms on 5 mM arsenite and 6 mM tBOOH. For all survival
plots, one representative experiment out of three independent repeats is shown. (E) mRNA fold changes in L4 stage N2 and nhr-64(ok1957) worms treated with 7.5 mM tBOOH for four hours, relative to untreated N2 worms (n=4). mRNA levels were normalized to act-1, tba-1, and ubc-2; error bars represent SEM. *Gene expression levels differ significantly from untreated N2 worms (p<0.05). (G) Relative interaction strength between Gal4DBD-MDT-15 variants and Gal4AD-NHR-64. Values indicate average interaction strength in percent, calculated from Miller units (n>4 per plasmid combination); error bars represent SEM.

Table 2.6 Statistics for individual mutant As survival experiments.

Number of subjects denoted and statistics calculated as described in Table 2.2. *p<0.05, ***p<0.001

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Table 2.7 Statistics for individual mutant tBOOH survival experiments.

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2.4 Discussion

2.4.1 A novel role for MDT-15 in the oxidative stress response

ROS possess both beneficial and detrimental properties, making tight control of their levels necessary. Here, I report a novel role for the *C. elegans* Mediator subunit MDT-15 in the oxidative stress response, involving a distinct functional region required for interactions with at least two transcription factors, including the well-characterized stress regulator SKN-1.

2.4.2 *C. elegans* MDT-15 is required for at least two distinct oxidative stress responses

My data show that *mdt-15* is required for two oxidative stress responses. Specifically, its mutation or depletion prevents normal gene inductions by, and renders worms sensitive to arsenite and tBOOH. Worms mount distinct defenses against each molecule: SKN-1 is required to induce arsenite response genes, but only regulates a small proportion of tBOOH response genes, despite being required for tBOOH resistance (An et al., 2005; Oliveira et al., 2009). That MDT-15 is essential for both responses suggests that it has a broad role in cytoprotective pathways (Figure 2.10).

The actions of MDT-15 are specific and not a consequence of sickness or impaired development because: (i) *mdt-15* RNAi in fully developed adults causes a defective arsenite response (Figure 2.4B); (ii) PUFA complementation rescues the development, fertility, and
mobility of *mdt-15* worms (Taubert et al., 2006; Yang et al., 2006) but does not rescue tBOOH sensitivity (Figure 2.7C); (iii) *fat-6(RNAi)* worms and *fat-6; fat-7* double mutants are not susceptible to tBOOH, despite pleiotropic phenotypes resembling *mdt-15* worms (Figure 2.7A-B); (iv) *mdt-15* depletion or mutation specifically impairs the transcriptional response and sensitivity to oxidative stress, but does not block the transcriptional heat shock response, affect thermotolerance, or cause sensitivity to tunicamycin-induced protein misfolding (Hou et al., 2014; Taubert et al., 2008); and (v) *mdt-6* RNAi causes larval arrest yet fails to block SKN-1–dependent gene inductions in *wdr-23(-)* worms, unlike *mdt-15* RNAi (Figure 2.5A). Moreover, MDT-15’s yeast orthologue Gal11 was identified in a screen for genes involved in oxidative stress sensitivity (Thorpe et al., 2004), suggesting that this is an evolutionarily conserved role for MED15 proteins.
Figure 2.10 Model of MDT-15’s role in oxidative stress responses.

MDT-15 is required for transcriptional responses to oxidative stress induced by both arsenite and tBOOH. MDT-15 regulates arsenite-responsive genes, likely by acting as a coactivator for SKN-1. In the tBOOH response, a small proportion of genes require SKN-1 for induction, but the majority of tBOOH response genes are regulated by MDT-15 and a separate, unidentified transcription factor. Additionally, the MDT-15-binding transcription factor NHR-64 is specifically required for resistance to tBOOH but not arsenite, but its role in the transcriptional response to tBOOH is still unclear.

2.4.3 MDT-15 is a putative coactivator of SKN-1

Previously, the gene host cell factor-1 (HCF-1) had been identified as a negative coregulator of SKN-1 (Rizki et al., 2012). Here I show that MDT-15 physically binds SKN-1 in a yeast two-hybrid system and is required for activation of SKN-1-dependent genes. This
is the first report of a positive coregulator for SKN-1. The interaction is KIX-domain independent, as MDT-15 instead associates with SKN-1c via a region partially deleted in
\textit{mdt-15}\textit{(tm2182)} mutants (Figure 2.6B). The tm2182 region is also required for NHR-64 binding, although NHR-64 also requires the KIX-domain (Figure 2.9F). It is more likely that the tm2182 region is directly involved in protein binding rather than causing MDT-15 misfolding, because MDT-15tm2182 is transcriptionally active in yeast suggesting that it adopts functional conformation, is expressed at wild-type levels \textit{in vivo}, and \textit{tm2182} causes hypomorph phenotypes that are much less severe than \textit{mdt-15} depletion by RNAi (Goh et al., 2014). Such a multi-surface interaction between MDT-15 and NHR-64 resembles the interaction between Gcn4 and Gal11 in yeast (Herbig et al., 2010; Jedidi et al., 2010).

One potential caveat of this finding is the known high false positive rate of yeast two-hybrid systems; the putative interaction between MDT-15 and SKN-1 could be further tested using an alternative method to detect protein-protein interactions, such as GST pull-down assays, or by using chromatin immunoprecipitation (ChIP) to test for MDT-15 at SKN-1-dependent promoters. However, supporting my data that MDT-15 acts as a SKN-1 coactivator, a recent paper described a gain-of-function (GOF) mutation in \textit{skn-1} that caused altered lipid metabolism in \textit{C. elegans}; \textit{mdt-15} was required for the phenotypes of this GOF mutant (Pang et al., 2014). While indirect, this is an independent piece of genetic evidence for MDT-15’s role as a coactivator of SKN-1, in a distinct biological context \textit{i.e.} lipid metabolism (see further discussion of these findings in 2.4.4). Intriguingly, a recent study found that the Mediator subunit MED16 interacts with the SKN-1 orthologue Nrf2 to regulate antioxidant genes in both mice and human cell lines (Sekine et al., 2016). Within the human Mediator complex, MED16 is located adjacent to MED15 in the tail module (Tsai et
al., 2014); however, there is no known orthologue of MED16 in *C. elegans*, even though a MED16 orthologue exists in *S. cerevisiae* (Grants et al., 2015; Tsai et al., 2014). It is possible that the ancestral SKN-1 protein in *C. elegans* co-opted MDT-15 as a binding surface to Mediator. In future it would be interesting to test whether MED15 is also able to physically interact with Nrf2 in mammalian cells.

2.4.4 \( \Delta 9 \) fatty acid desaturases are not required for oxidative stress resistance

As MDT-15 is a known coregulator of lipid metabolism genes, it was important to test whether its roles in oxidative stress responses were separable from its roles in lipid metabolism. A previous paper found that depleting the fatty acid desaturases *fat-6* or *fat-7* causes paraquat sensitivity (Horikawa and Sakamoto, 2009). I found that *mdt-15(tm2182)* and *nhr-49(nr2041)* mutants, which strongly downregulate these genes and show altered fatty acid profiles (Taubert et al., 2006; Van Gilst et al., 2005b; Yang et al., 2006), are also sensitive to oxidative stress. This initially suggested that fatty acid imbalance might cause oxidative stress susceptibility. However, *fat-6(RNAi)* and *fat-6(tm331); fat-7(wa36)* worms were insensitive to tBOOH despite pleiotropic phenotypes, and PUFA complementation failed to protect *mdt-15(tm2182)* mutants from tBOOH (Figure 2.7A-C). In fact, I found that fatty acid desaturases were downregulated by tBOOH (Figure 2.7D). Synthesizing PUFAs in an oxidizing environment may be undesirable as they are peroxidation targets. Accordingly, I observed that exogenous PUFAs caused slight tBOOH sensitivity in wild-type animals (Figure 2.7C). Thus, the altered fatty acid composition of *mdt-15* worms is unlikely to cause its oxidative stress sensitivity, although it impacts growth, fertility, and mobility (Taubert et al., 2006; Yang et al., 2006). The discrepancy between my findings and those of Horikawa
and Sakamoto’s could be due to the difference in the oxidative stressor used: Horikawa and Sakamoto used paraquat, a herbicide that acts through the formation of superoxide ($O_2^{•-}$) (Bus et al., 1974). tBOOH, on the other hand, is an organic peroxide containing a ROOH group. The O–O bond within this group is easily broken to form an alkoxy radical (RO•), which accelerates lipid peroxidation (Halliwell and Gutteridge, 2015; Masaki et al., 1989). Perhaps oleic acid and/or PUFA’s are protective against superoxide (Richard et al., 2008), but are unnecessary and perhaps even detrimental on tBOOH.

Recent data from other groups also indicate that lipid metabolism and oxidative stress responses are separable in *C. elegans*. Steinbaugh et al. found that long-lived germline-less *glp-1(bn18ts)* mutants show increased SKN-1 nuclear localization and concomitant upregulation of *gst-4* due to changes in lipid balance caused by loss of the germline. This activation of SKN-1 and its targets are dependent on *sbp-1* (Steinbaugh et al., 2015). SBP-1 is a known transcription factor partner of MDT-15 that activates *fat-5, -6, and -7* (Walker et al., 2011; Yang et al., 2006). However, in *glp-1* mutants depleted of *sbp-1*, an increase in SKN-1 nuclear localization is still observed upon the addition of arsenite (Steinbaugh et al., 2015). This indicates that loss of *sbp-1* and downregulation of PUFA synthesis, which similarly occurs in *mdt-15* loss/reduction-of-function, does not affect these worms’ ability to respond to oxidative stress. Furthermore, Pang et al. recently described the enhanced breakdown of fat stores during fasting in *alh-6* mutant worms, which occurs in a SKN-1 and MDT-15-dependent manner (Pang et al., 2014). They also found that the direct SKN-1 target *gst-4* is upregulated in fasted *alh-6* worms, but this upregulation could not be abolished by adding the antioxidant N-acetylcysteine (NAC). In contrast, while adding arsenite also increases *gst-4* expression in *alh-6* mutants, NAC prevents *gst-4* upregulation in this context.
(Pang et al., 2014). This shows that SKN-1 regulates $gst-4$ in both fasting and oxidative stress, but that the signals regulating SKN-1 activity are distinct in these two processes (Figure 2.11). Taken together, my data and others’ support the hypothesis that lipid metabolism and oxidative stress responses are biologically separable, and that even severe changes in lipid composition do not necessarily disrupt stress responses.

![Figure 2.11](image)

**Figure 2.11** SKN-1 is independently activated by oxidative stress and disruptions to lipid balance.

Under conditions of either oxidative stress or altered lipid metabolism, SKN-1 activity increases and SKN-1 target genes such as $gst-4$ are upregulated (Pang et al., 2014; Steinbaugh et al., 2015). The signals regulating SKN-1 activity in these two conditions are distinct. SKN-1 is also activated in an $sbp-1$-dependent manner in some conditions of altered lipid balance e.g. in $glp-1$ mutants (Steinbaugh et al., 2015).
2.4.5 MDT-15 likely coregulates the tBOOH response with an unidentified transcription factor

Many tBOOH response genes require MDT-15, but not SKN-1, for wild-type expression levels. Since MDT-15 is not known to interact directly with DNA, it likely binds at least one other transcription factor to coregulate these genes. While NHR-64, a known MDT-15 partner, is required for tBOOH but not arsenite resistance, it was not required for the expression of the tBOOH response genes tested here. One caveat is that I only tested a small number of these genes; it is possible that NHR-64 regulates other tBOOH response genes. However, I note that these are some of the most highly and consistently upregulated genes in the tBOOH response: \textit{fmo-2}, for example, is one of the most highly induced genes in tBOOH exposure, both in this study and in that of Oliveira et al (Oliveira et al., 2009). As with SKN-1, the transcriptional response to oxidative stress thus does not always correlate with resistance to that particular stressor. In chapter 3, I will discuss a functional RNAi screen using a promoter::GFP reporter fusion to identify genes required to induce the tBOOH response.
Chapter 3: Nuclear hormone receptor NHR-49 is required for an oxidative stress response in *C. elegans*

3.1 Synopsis

Oxidative stress is caused by a buildup of excess reactive oxygen species (ROS) that can be damaging to cells. Organisms have evolved highly complex and specific systems to detoxify different types of ROS. A key regulator of the oxidative stress response is the transcription factor Nrf2 (the ortholog of SKN-1 in *Caenorhabditis elegans*), which induces many antioxidant genes in oxidative stress. However, exposure to the organic peroxide tert-butyl hydroperoxide (tBOOH) induces a largely SKN-1-independent response in *C. elegans*, implying the utilization of an independent transcription factor in the regulation of this response. By conducting a candidate reverse genetic screen, I show that the nuclear hormone receptor *nhr-49* is required for the tBOOH response. I find that *nhr-49* is both necessary and sufficient for normal resistance to tBOOH. Furthermore, the NHR-49-dependent response is also induced in fasting and in long-lived germline-less *glp-1* mutants, and *nhr-49* is required for resistance to starvation and the resistance of *glp-1* mutants to tBOOH. Previous work showed that the induction of *fmo-2*, a strongly NHR-49-dependent target gene, in fasting is regulated by a separate transcription factor, HLH-30. I show that *hlh-30* is only partially required for the *nhr-49*-dependent tBOOH and fasting response. Together with other findings, these data suggest that two distinct upstream signaling pathways, one dependent on NHR-49 and one on HLH-30, can converge on similar downstream targets. In summary, this work describes a novel role for NHR-49 in an oxidative stress response, and shows that
NHR-49 and HLH-30 likely receive distinct upstream signals when worms are exposed to different stresses in order to coordinate stress responses that determine survival.

3.2 Background

Cells induce highly specific responses to distinct stresses, which present different challenges to cells and organisms. For example, cells will respond differently to heat, cold, or osmotic stress. The specificity of stress responses is enabled by the activation of stress response enzymes, such as detoxification enzymes, chaperones and membrane transporters, which act on their respective substrates to allow cells to return to homeostatic conditions (Halliwell and Gutteridge, 2015). Activation of these enzymes is primarily regulated by stress-responsive transcription factors, which induce their expression on exposure to the relevant stressors (Carter and Brunet, 2007; Loboda et al., 2016; Schreck et al., 2009; Sykiotis and Bohmann, 2010; Vogelstein et al., 2000).

Oxidative stress occurs when the amount of reactive oxygen species (ROS) in a cell exceeds the capabilities of antioxidant defenses to remove them (Sies, 1991). ROS can arise from normal metabolism, e.g. due to electron leakage from the mitochondria, or from exogenous sources such as heavy metals or xenobiotic molecules (Finkel and Holbrook, 2000; Halliwell and Gutteridge, 2015; Lenaz, 2001; Turrens, 1997). Many different types of ROS and ROS-derived molecules exist; these molecules are removed or detoxified by specific enzymes. For example, superoxide is modified by superoxide dismutase to produce the less reactive hydrogen peroxide, which is then converted by catalase into water and oxygen (McCord and Fridovich, 1969). On the other hand, glutathione can act as an electron donor to detoxify a number of ROS including hydroxyl and alkoxyl radicals, and it can also
reduce oxidized disulphide bonds (Lushchak, 2012). Oxidative stress responses can thus be tailored to the type of ROS causing the stress, presumably in response to upstream signals. For instance, Zhuang et al. showed that the stress-activated p38 mitogen-activated protein kinase (MAPK) pathway regulates apoptosis in response to singlet oxygen, but not hydrogen peroxide (Zhuang et al., 2000).

The nematode worm *Caenorhabditis elegans* is commonly used to study oxidative stress responses, as it is a simple, genetically tractable model organism and many of the pathways that regulate these responses are conserved between worms and humans. For instance, in response to the heavy metal arsenite (As), it induces a response that is almost entirely dependent on the cytoprotective transcription factor SKN-1, the *C. elegans* homolog of Nrf2 (Oliveira et al., 2009). However, the response to the organic peroxide tert-butyl hydroperoxide (tBOOH) is distinct from the arsenite response, and is largely SKN-1-independent (Oliveira et al., 2009). This implies the presence of a separate, unidentified transcription factor that regulates this SKN-1-independent response. Previously, I determined that the transcriptional coregulator *mdt-15* is required for both the As and tBOOH responses (see Chapter 2). MDT-15 likely activates the As response by acting as a SKN-1 coactivator (see Section 2.4). Since the sequence-specific transcription factor that regulates the SKN-1-independent tBOOH response is unknown, I hypothesized that MDT-15 interacts with an as yet unidentified transcription factor to regulate in this context.

Many transcription factors have been described to bind MDT-15 in large-scale protein-protein interaction screens (Arda et al., 2010; Reece-Hoyes et al., 2013; Taubert et al., 2006; Yang et al., 2006). I thus hypothesized that one of these transcription factors is required for the SKN-1-independent tBOOH response. Here, I conducted a candidate reverse genetic
screen and identified the *C. elegans* nuclear hormone receptor NHR-49 as a novel regulator of the tBOOH response. NHR-49 is a well-characterized MDT-15-binding partner that regulates genes involved in various aspects of lipid metabolism. I further show that *nhr-49*-dependent stress response genes are also upregulated in fasting, and that *nhr-49* is required for resistance to both tBOOH and fasting. Finally, previous work showed that *nhr-49* is required for the long lifespan and altered lipid metabolism in *glp-1* mutants (Ratnappan et al., 2014). I found that *nhr-49*-dependent stress response genes are also upregulated in long-lived germline-less *glp-1* mutants, which are also highly resistant to tBOOH in an *nhr-49*-dependent manner; while not conclusive, these results suggest that *nhr-49*-dependent stress response genes may contribute to *glp-1* longevity, as there is a correlation between long-lived mutant strains and increased stress resistance (Finkel and Holbrook, 2000). In summary, NHR-49 is a novel regulator of a SKN-1-independent oxidative stress response, which is also induced in several other physiological conditions *i.e.* fasting and germline loss. This establishes a new role for a transcription factor that was previously only described to function in the regulation of fatty acid β-oxidation and desaturation, and has implications for other *nhr-49*-dependent functions, for example in lifespan regulation.

### 3.3 Results

#### 3.3.1 A candidate reverse genetic screen identifies NHR-49 as a regulator of a SKN-1-independent tBOOH response gene

First, I used a candidate reverse genetic approach to identify transcription factors required for the tBOOH response. To do this, I sought to identify regulators of a highly tBOOH-responsive, SKN-1-independent gene, *fmo-2* (Oliveira et al., 2009). I used a
transcriptional GFP reporter containing a 1536 bp region upstream of the *fmo-2* transcriptional start site (Figure 3.1A). This reporter was only weakly visible in basal, unstressed conditions, but was strongly induced upon exposure to tBOOH. The induction of the reporter was *mdt-15*-dependent and *skn-1*-independent, as expected from previous gene expression data (Figure 3.1C). Knockdown of *nhr-64* by RNAi also did not impair *fmo-2* induction, agreeing with qPCR analysis of the *nhr-64* null mutant (Table 3.1, also see Figure 2.9). These results recapitulated the expression pattern of endogenous *fmo-2* mRNA on tBOOH, validating the *fmo-2p*::GFP reporter as a tool to identify novel regulators of the tBOOH response.

I then conducted a candidate RNAi screen to search for transcription factors required to upregulate the *fmo-2p*::GFP reporter in response to tBOOH exposure. I focused on transcription factors known to physically interact with MDT-15 (Table 3.1). I depleted each transcription factor in *fmo-2p*::GFP bearing transgenic worms, initiating RNAi in synchronized L1 worms. Once worms had reached the young adult stage, they were placed on 10 mM tBOOH for three hours to identify RNAi clones that prevented *fmo-2p*::GFP reporter induction by tBOOH (Figure 3.1B).

Of the 18 transcription factors tested, I identified one hit: only *nhr-49* was required to induce the *fmo-2p*::GFP reporter on tBOOH (Figure 3.1C, Table 3.1). NHR-49 is a known transcription factor partner of MDT-15 that regulates fatty acid β-oxidation, desaturation, and sphingolipid metabolism. Importantly, *fmo-2p*::GFP worms depleted of another MDT-15 partner transcription factor, *sbp-1*, were still able to induce *fmo-2* (Figure 3.1C). This is notable because SBP-1 and NHR-49 both regulate Δ9 fatty acid desaturases, and loss of *sbp-1, nhr-49*, or *mdt-15* causes dramatically altered fatty acid profiles in worms, with reduced
unsaturated fatty acids and increased saturated fatty acids. Such changes can affect gene expression profiles, and others have suggested that they may influence oxidative stress responses (Horikawa and Sakamoto, 2009). However, my data suggest that unsaturated fatty acid deficiency caused by sbp-1 RNAi does not prevent fmo-2 induction, and it is therefore unlikely to contribute to the defects in nhr-49(RNAi) worms.
Figure 3.1 Depletion of \textit{nhr-49} by RNAi leads to loss of \textit{fmo-2} induction on tBOOH.

(A) Diagram of the \textit{fmo-2p::GFP_{NLS}} reporter used in the RNAi screen. (B) Outline of the RNAi screen performed to identify MDT-15-binding transcription factors that regulate \textit{fmo-2} in response to oxidative stress.
(C) *fmo-2p::GFP* is induced on tBOOH in an *mdt-15*-dependent, *skn-1*-independent manner. Depletion of *nhr-49* by RNAi also prevents *fmo-2* induction on tBOOH, whereas depletion of another regulator of Δ9 fatty acid desaturases, *sbp-1*, does not affect *fmo-2* induction.

**Table 3.1 List of transcription factors tested in the RNAi screen.**

List of transcription factors tested in Figure 3.1, with references to publications where their interaction with MDT-15 was described. A transcription factor was only considered to be required for *fmo-2* induction if the requirement was shown in three independent repeats. For detailed results of individual repeats, please refer to Appendix A.1.

<table>
<thead>
<tr>
<th>RNAi condition</th>
<th>Required for <em>fmo-2</em> induction?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td><em>mdt-15</em></td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td><em>skn-1</em></td>
<td>-</td>
<td>(Goh et al., 2014)</td>
</tr>
<tr>
<td><em>nhr-4</em></td>
<td>-</td>
<td>(Reece-Hoyes et al., 2013)</td>
</tr>
<tr>
<td><em>nhr-8</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-10</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-12</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-28</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-49</em></td>
<td>+</td>
<td>(Taubert et al., 2006)</td>
</tr>
<tr>
<td><em>nhr-64</em></td>
<td>-</td>
<td>(Taubert et al., 2006)</td>
</tr>
<tr>
<td><em>nhr-69</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-86</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-97</em></td>
<td>-</td>
<td>Taubert et al., unpublished</td>
</tr>
<tr>
<td><em>nhr-112</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-114</em></td>
<td>- (mild suppressor)</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>nhr-138</em></td>
<td>-</td>
<td>(Reece-Hoyes et al., 2013)</td>
</tr>
<tr>
<td><em>nhr-273</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>npax-2</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>hlh-8</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>ztf-2</em></td>
<td>-</td>
<td>(Arda et al., 2010)</td>
</tr>
<tr>
<td><em>sbp-1</em></td>
<td>-</td>
<td>(Yang et al., 2006)</td>
</tr>
</tbody>
</table>
3.3.2 *nhr-49* is required for the tBOOH response

Since *fmo-2* is only one of over 200 SKN-1-independent tBOOH response genes, I next tested whether *nhr-49* was required to induce other such genes. I harvested N2 and *nhr-49(nr2041)* null mutants that were either unstressed or exposed to 7.5 mM tBOOH for four hours and measured expression of SKN-1-independent tBOOH response genes (as determined by (Oliveira et al., 2009)) by qPCR. Of 20 genes tested, I found 14 that required *nhr-49* either for basal expression or for induction on tBOOH (Figure 3.2A-B, also see Appendix A.1). The differences in fold-induction of tBOOH response genes are modest between N2 and *nhr-49(nr2041)* worms (Figure 3.2B), likely reflecting lower basal expression of these genes in unstressed *nhr-49(nr2041)* mutants. Taken together with my previous finding that *nhr-49(nr2041)* mutants are more sensitive to tBOOH than wild-type worms (Figure 2.9), I concluded that *nhr-49* is required for both the transcriptional response and the organismal resistance to tBOOH.

While *skn-1* is largely dispensable for the transcriptional response to tBOOH, it is still required for a small number of genes in this response (Oliveira et al., 2009). I therefore tested whether *nhr-49* may also be required for arsenite response genes, in addition to its role in the tBOOH response. Of the five arsenite response genes tested (which I previously showed required *mdt-15*, see Figure 2.3), only *gst-4* required *nhr-49* for normal basal expression levels, and none showed significant impairment in induction on exposure to 5 mM arsenite for 4 hours (Figure 3.2C-D). Therefore, *nhr-49* is not required to induce at least some arsenite response genes, although it is possible that it is required for other genes in this response. Unbiased gene expression analysis e.g. microarray or RNA-seq is required to further determine if *nhr-49* is required for any part of the transcriptional response to arsenite.
Figure 3.2 *nhr-49* is required for the response to tBOOH but not arsenite.

(A) Fold changes of mRNA levels (relative to untreated N2 worms) in L4-stage N2 and *nhr-49(nr2041)* worms treated with 7.5 mM tBOOH for four hours (n=5). (B) Fold inductions of tBOOH response genes in N2 vs. *nhr-49(nr2041)* worms on tBOOH. (C) mRNA fold changes (relative to untreated N2 worms) in N2 and *nhr-49(nr2041)* worms exposed to 5 mM arsenite for 4 hours (n=4). (D) Fold inductions of arsenite response genes in N2 vs. *nhr-49(nr2041)* worms on As. Error bars represent SEM. For (A) and (C), **,**,**,** Gene expression levels differ significantly from non-treated N2 worms (p<0.01 and p<0.001 respectively). ††, ††† Gene expression levels differ significantly from tBOOH-treated N2 worms (p<0.01 and p<0.001 respectively). For (B) and (D), *p<0.05, **p<0.01.
3.3.3 An *nhr-49* GOF mutation is sufficient for the tBOOH response

*nhr-49* is required for the tBOOH response. Thus, I next tested whether three putative GOF *nhr-49* alleles, *nhr-49(et7)*, *nhr-49(et8)* and *nhr-49(et13)*, were sufficient for this response. These alleles were isolated in a forward genetic screen for regulators of fatty acid desaturation (Svensk et al., 2013). *fmo-2* was upregulated in all three mutants under unstressed conditions (Figure 3.3A). Therefore, *nhr-49* GOF is sufficient for *fmo-2* induction.

To further confirm the sufficiency of *nhr-49* GOF alleles, I crossed the *fmo-2p::GFP* reporter into the *nhr-49(et13)* background. I chose the *et13* allele because it showed the strongest upregulation of *fmo-2* (Figure 3.3A). Compared to worms carrying the reporter in a wild-type background, *nhr-49(et13); fmo-2p::GFP* worms were fluorescent under control conditions (Figure 3.3B). Next, I hypothesized that if MDT-15 is a bona fide coregulator of NHR-49 in the regulation of the tBOOH response, the upregulation of *fmo-2p::GFP* seen in the *nhr-49(et13)* background should be dependent on *mdt-15*. I confirmed that this was true, as depletion of *mdt-15* by RNAi abrogated *fmo-2p::GFP* expression in the *nhr-49(et13)* background (Figure 3.3B). *nhr-49(et13); fmo-2p::GFP* worms showed fluorescence in the pharynx, which was not seen in the wild-type background (Figure 3.3B). The pharyngeal *fmo-2* expression in the *nhr-49(et13)* background was not abrogated by *mdt-15(RNAi)*; in *C. elegans*, the pharynx is naturally more refractory to RNAi (Kumsta and Hansen, 2012). Additionally, I found that *mdt-15* is also required for the upregulation of two other tBOOH response genes, *K05B2.4* and *icl-1/gei-7*, in *nhr-49(et13)* worms (Figure 3.3C).

Finally, I investigated whether *nhr-49* GOF is sufficient to confer resistance to tBOOH. I measured population survival of *nhr-49(et13)* worms compared to N2 on 6 mM tBOOH and
found that they showed slight resistance to tBOOH (Figure 3.3D, Table 3.2). Therefore, nhr-49 is not only required, but nhr-49 GOF is also sufficient for the response and resistance to tBOOH.

Figure 3.3 nhr-49 GOF is sufficient for the response and resistance to tBOOH.

(A) mRNA fold changes (relative to N2 worms) of three putative nhr-49 GOF mutants (n=3). Synchronized L4 stage worms were used for qPCR. Error bars represent SEM. *Gene expression levels differ significantly from N2 worms (p<0.05). (B) nhr-49(et13); fmo-2p::GFP show intestinal and pharyngeal fluorescence in unstressed conditions, unlike WT worms carrying the reporter (Figure 3.1C). Intestinal fmo-2p::GFP expression is abrogated in worms depleted of both mdt-15 and nhr-49 by RNAi. (C) mRNA fold changes (relative to N2 (Control RNAi) worms) of tBOOH response genes in N2 and nhr-49(et13) worms treated with control and mdt-15 RNAi (n=5). Error bars represent SEM. *p<0.01, ***p<0.001 (D) Survival plots of N2 and nhr-49(et13) worms on 6 mM tBOOH (p<0.05). One representative experiment out of four independent repeats is shown.
Table 3.2 Statistics for individual nhr-49(et13) tBOOH survival experiments.

Number of subjects is denoted as follows: Dx=Number of deaths that occurred during the assay; Nx=Total number of animals used in assay; Cx=Number of censored events (*i.e.* worms that ruptured at the vulva, underwent internal hatching of the progeny, or crawled off the plate). All *p*-values are derived using the log-rank (Mantel-Cox) test. *p*<0.05, **p*<0.001

<table>
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<th>Strain</th>
<th>Experiment no.</th>
<th>Median survival (hours)</th>
<th>Number of subjects (Dx/Nx (Cx))</th>
<th><em>p</em> value vs. N2</th>
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<td>45</td>
<td>81/118(37)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>3</td>
<td>47</td>
<td>66/116(50)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30</td>
<td>76/129 (53)</td>
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</tr>
<tr>
<td>nhr-49(et13)</td>
<td>1</td>
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<td>54/119(65)</td>
<td>0.0492*</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>39/136(97)</td>
<td>0.1352</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>67</td>
<td>29/121(92)</td>
<td>0.0002***</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30</td>
<td>69/125 (56)</td>
<td>0.0007***</td>
</tr>
</tbody>
</table>

3.3.4 Three known NHR-49 partner transcription factors are not required for the tBOOH response

In mammals, many NHRs bind target genes as heterodimers. While little work has been done to define *C. elegans* NHR partnerships, protein-protein interaction screens have found that NHR-49 may bind multiple potential partner transcription factors (Pathare et al., 2012; Reece-Hoyes et al., 2013). The interactions of three of these partners with NHR-49 have been further validated: these are NHR-13, -66, and -80 (Pathare et al., 2012). These transcription factors partner with NHR-49 to regulate distinct transcriptional programs: NHR-13 and NHR-80 appear to activate fatty acid desaturation genes, while NHR-66 suppresses sphingolipid catabolism genes, a function of NHR-49 that has not been studied in
great detail (Goudeau et al., 2011; Pathare et al., 2012). Further roles of these three NHR-49 partner transcription factors have not yet been defined.

To investigate whether any of these three NHR-49 partners might also be involved in the oxidative stress response, I tested whether null mutants of nhr-13, -66, and -80 were required for the tBOOH response. I found that all mutants showed normal expression levels of three tBOOH-responsive genes on exposure to tBOOH, including fmo-2 (Figure 3.4). Therefore, I concluded that nhr-13, -66, and -80 are individually not required for the tBOOH response.

Figure 3.4 Three NHR-49 transcription factor partners are not required for the tBOOH response.

Fold changes of mRNA levels (relative to N2 untreated worms) in L4 N2, nhr-13(gk796), nhr-66(ok940), and nhr-80(tm1011) worms treated with 7.5 mM tBOOH for 4 hours (n≥3). Error bars represent SEM. ***, *** Gene expression levels differ significantly from N2 untreated worms (p<0.05, p<0.01 and p<0.001 respectively).
3.3.5 NHR-49 regulates a common transcriptional response in oxidative stress and fasting

As previously described, NHR-49 regulates fatty acid β-oxidation genes, which are upregulated upon fasting to utilize lipid stores for energy (Van Gilst et al., 2005a). Interestingly, in a published microarray analysis of worms undergoing acute fasting, I noted that several genes induced in fasting are also tBOOH response genes, e.g. fmo-2, K05B2.4, and icl-1 (Table 3.3) (Uno et al., 2013). To determine if there is a common transcriptional signature in fasting and oxidative stress, I compared tBOOH response genes with genes induced after nine hours of fasting to determine if there was a significant overlap between these datasets (Oliveira et al., 2009; Uno et al., 2013). I found a statistically significant overlap between SKN-1-independent tBOOH response genes and genes induced after nine hours of fasting ($p<2.2\times10^{-16}$ as determined by Fisher’s exact test) (Figure 3.5A, Table 3.3). To test whether nhr-49 is also required to induce these genes in fasting, I fasted synchronized WT and nhr-49(nr2041) null mutant L4 worms for eight hours and measured the induction of nhr-49-dependent tBOOH response genes in these worms. I found that most of these genes are also induced upon fasting in an nhr-49-dependent manner (Figure 3.5B). Therefore, nhr-49 is required for a common transcriptional response to two stresses, tBOOH-induced oxidative stress and fasting.

Finally, I investigated whether nhr-49 was also required for resistance to chronic starvation. When C. elegans hatch in conditions where food is lacking, worms are capable of arresting at the young L1 larval stage for several weeks; when food becomes available, the surviving worms resume normal larval development (Lee and Ashrafi, 2008). I hatched WT and nhr-49(nr2041) null worms in liquid media without food and determined how long
arrested L1s were able to resume development when transferred to food. *nhr-49(nr2041)* mutants ceased being able to resume development earlier than WT larvae, indicating that *nhr-49* is required for resistance to starvation (Figure 3.5C). Surprisingly, the *nhr-49(et13)* GOF mutation was not sufficient to confer resistance to starvation (Figure 3.5D). While the reason for this is unknown, the *nhr-49(et13)* mutation is a missense mutation (V411E) that is hypothesized to disrupt the ability of NHR-49 to undergo conformational changes upon activation or repression (Lee et al., 2016). It is possible that this mutation affects NHR-49 activity in the presence of tBOOH, but not under conditions of starvation, thereby explaining the lack of resistance of this strain to starvation.

![Venn diagram showing the overlap between genes upregulated on tBOOH in a SKN-1-independent manner and after 9 hours of fasting.](A)

![Relative mRNA levels (relative to fed)](B)

![Percentage recovered over days of starvation](C)

![Percentage recovered over days of starvation](D)

**Figure 3.5** *nhr-49* regulates a common transcriptional response in oxidative stress and fasting. (A) Venn diagram showing the overlap between genes upregulated on tBOOH in a SKN-1-independent manner and after 9 hours of fasting. *p*<2.2x10^{-16}, Fisher’s exact test. (B) Fold changes of mRNA levels (relative to fed
N2 worms) in L4 WT or nhr-49(nr2041) worms that were either fed or fasted for 8 hours (n=3). Error bars represent SEM. ****Gene expression levels differ significantly from fed N2 worms (p<0.05, p<0.01 and p<0.001 respectively). †††††Gene expression levels differ significantly from fasted N2 worms (p<0.05 and p<0.01 respectively). (C) Percentages of N2 and nhr-49(nr2041) worms that were able to resume development after L1 starvation over time (p<0.05, determined by calculating area under the curve). (D) Percentages of N2 and nhr-49(et13) worms that were able to resume development after L1 starvation over time (p=0.857).

**Table 3.3 List of genes upregulated in fasting and tBOOH-induced oxidative stress.**

The table shows genes that are upregulated by tBOOH in a SKN-1-independent manner (Oliveira et al., 2009) and after 9 hours of fasting (Uno et al., 2013).

<table>
<thead>
<tr>
<th>WormBase Sequence ID</th>
<th>Gene Name</th>
<th>Notes</th>
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</thead>
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<tr>
<td>B0213.15</td>
<td>cyp-34A9</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>B0222.9</td>
<td>gad-3</td>
<td>Xanthine dehydrogenase</td>
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<tr>
<td>B0272.4</td>
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<td>C03B1.13</td>
<td></td>
<td>Orthologue of human solute carrier family 2</td>
</tr>
<tr>
<td>C05E4.9</td>
<td>gei-7/lcl-1</td>
<td>Isocitrate lyase/malate synthase</td>
</tr>
<tr>
<td>C06B3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C08F11.13</td>
<td>srr-6</td>
<td>Serpentine receptor, class R</td>
</tr>
<tr>
<td>C13D9.1</td>
<td>nnt-1</td>
<td>Nicotinamide nucleotide transhydrogenase</td>
</tr>
<tr>
<td>C15H9.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C25F6.2</td>
<td>dlg-1</td>
<td>Discs large homologue</td>
</tr>
<tr>
<td>C46F4.2</td>
<td>acs-17</td>
<td>Acyl-CoA synthetase</td>
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<td>oac-14</td>
<td>O-acyltransferase homologue</td>
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<tr>
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<td></td>
</tr>
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</tr>
<tr>
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<td>dct-7</td>
<td>DAF-16/FOXO-controlled, germline tumour affecting</td>
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<td>F45D3.4</td>
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</tr>
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<td>SCP-like extracellular protein</td>
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<td>Orthologue of human enoyl CoA hydratase 1,</td>
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<td><strong>Gene Name</strong></td>
<td><strong>Notes</strong></td>
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<td>far-7</td>
<td>Fatty acid/retinol binding protein</td>
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<td>Caenorhabditis epoxide hydrolase</td>
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<td>fmo-1</td>
<td>Flavin-containing monoxygenase</td>
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<tr>
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<td>fmo-2</td>
<td>Flavin-containing monoxygenase</td>
</tr>
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<td>Metallothionein</td>
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<td>R09D1.11</td>
<td>chil-23</td>
<td>Chitinase-like</td>
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<td>Catechol-O-methyltransferase family</td>
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<td>nlp-25</td>
<td>Neuropeptide-like protein</td>
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<td>cpt-3</td>
<td>Carnitine palmitoyl transferase</td>
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<td>Y5H2B.5</td>
<td>cyp-32b1</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>ZC443.3</td>
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<td>Ortholog of human filamin A interacting protein 1-like</td>
</tr>
<tr>
<td>ZK550.6</td>
<td></td>
<td>Ortholog of the human gene phytanoyl-CoA hydroxylase</td>
</tr>
</tbody>
</table>

3.3.6 The *fmo-2* regulator HLH-30/TFEB does not play a major role in the tBOOH response

*fmo-2* was recently identified as a regulator of age-associated autofluorescence in *C. elegans* (Leiser et al., 2015). *fmo-2* appears to play a role in aging and stress resistance in *C. elegans*, as overexpression of *fmo-2* extends lifespan and increases resistance to a number of stresses, including heat, ER stress, and reductive stress (Leiser et al., 2015).

Leiser *et al.* determined that *fmo-2* is transcriptionally regulated by the transcription factor HLH-30, the *C. elegans* orthologue of the basic helix-loop-helix transcription factor EB (TFEB), a regulator of autophagy genes (Lapierre et al., 2013). *hlh-30* is required to induce *fmo-2* in conditions of fasting and hypoxia (Leiser et al., 2015). Based on this data, I hypothesized that HLH-30 might also be required for the induction of *fmo-2* in the tBOOH response, as well as to induce other tBOOH response genes.
To test the requirement for *hlh-30* in the tBOOH response, I exposed *hlh-30(tm1978)* null mutants to tBOOH and measured tBOOH response gene expression. I found that, unlike in the *nhr-49* null mutants, *fmo-2* induction showed only a partial requirement for *hlh-30*; this requirement is not statistically significant, perhaps due to the variation in relative *fmo-2* mRNA levels on tBOOH (Figure 3.6). Furthermore, of all other genes tested, only *sodh-1* showed a partial requirement for *hlh-30*. I therefore conclude that *hlh-30* plays only a minor role in the tBOOH response.

While *hlh-30* is required to induce *fmo-2* in fasting, whether it regulates a broader transcriptional response in this context is unknown. I therefore also tested whether *hlh-30* was required to induce other fasting response genes. I found that, similarly to its role in the tBOOH response, *hlh-30* was partially required to induce *fmo-2* and *sodh-1*, but was not required for any of the other genes tested. Therefore, *hlh-30* is only partially required for the shared transcriptional response in tBOOH treatment and fasting.

![Figure 3.6](image)

**Figure 3.6** *hlh-30* is partially required for the shared response to tBOOH and fasting.

(A) Fold changes of mRNA levels (relative to N2 untreated worms) in L4 N2 and *hlh-30(tm1978)* worms treated with 7.5 mM tBOOH for 4 hours (n=5). (B) Same as (A), but with worms fasted for 8 hours (n=3). Error
bars represent SEM. * * * * * * Gene expression levels differ significantly from N2 untreated worms ($p<0.01$, $p<0.001$ and $p<0.0001$ respectively).

3.3.7 NHR-49 acts either downstream or in parallel to HLH-30

Since NHR-49 regulates the tBOOH response and *hlh-30* is also partially required for this response (particularly in the induction of *fmo-2*), I further investigated the relationship between these two transcription factors. To do this, I used LOF mutations in both genes, as well as the *nhr-49(et13)* GOF mutation and a strain that overexpresses HLH-30 (Lapierre et al., 2013; Svensk et al., 2013; Van Gilst et al., 2005b). I reasoned that basal levels of *fmo-2* and other tBOOH response genes should be increased in the GOF/overexpressors; however, if this upregulation was lost when these worms were crossed to LOFs, then the LOF should act downstream or in parallel to the former.

First, I crossed a *hlh-30p::hlh-30::GFP* strain, which overexpresses HLH-30, to the *nhr-49(nr2041)* null mutant. I found by qPCR that three tBOOH response genes are only slightly upregulated in the HLH-30-overexpressing strain, and that this upregulation was lost when the *nhr-49(nr2041)* mutation was present; in fact, expression of target genes was reduced to similar levels as in the *nhr-49(nr2041)* single mutant (Figure 3.7A). *Vice versa*, I tested the expression of these genes in the *nhr-49(et13)* GOF mutant crossed to the *hlh-30(tm1978)* null mutant. As before, I found that *nhr-49(et13)* alone caused a substantial upregulation of *fmo-2* and other tBOOH response genes. Interestingly, this effect was not lost in the *nhr-49(et13); hlh-30(tm1978)* double mutant, which showed a similar gene expression pattern to the *nhr-49(et13)* single mutants (Figure 3.7B). I also tested gene expression levels of both transcription factors in both null mutant backgrounds and found that expression of neither
transcription factor was substantially altered (Figure 3.7C-D). Therefore, the effects seen are likely not due to changes in transcription of either nhr-49 or hlh-30. In sum, these results suggest that NHR-49 either works downstream of HLH-30, since it is required for upregulation of tBOOH response genes in the HLH-30 overexpressing background, or that NHR-49 may work in parallel to HLH-30 to regulate fmo-2 and other tBOOH response genes. Since the fmo-2 promoter contains a HLH-30 binding site, it seems more likely that NHR-49 and HLH-30 act in parallel, at least in the regulation of fmo-2.

Figure 3.7 NHR-49 works either downstream or in parallel to HLH-30 in the tBOOH response.

(A) mRNA fold changes of tBOOH response genes in L4 hlh-30p::hlh-30::GFP worms carrying the nhr-49(nr2041) null mutation (n≥3). (B) mRNA fold changes of tBOOH response genes in L4 nhr-49(et13) GOF mutants carrying the hlh-30(tm1978) null mutation (n≥3). (C) mRNA fold changes of nhr-49 and hlh-30 in L4

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hlh-30p:hlh-30::GFP worms carrying the nhr-49(nr2041) null mutation. (D) mRNA fold changes of nhr-49 and hlh-30 in L4 nhr-49(et13); hlh-30(tm1978) double mutants. All fold changes are relative to N2 worms. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001.

3.3.8  *fmo-2* is required for resistance to starvation but not tBOOH

*fmo-2* is highly upregulated in both tBOOH and fasting; thus, I hypothesized that it is required for resistance to both stresses. To test this, I performed a starvation resistance assay and found that an *fmo-2(ok2147)* null mutant was sensitive to starvation, as expected (Figure 3.8A). Conversely, an FMO-2 overexpressing strain (Leiser et al., 2015) was resistant to starvation (Figure 3.8B). Surprisingly, *fmo-2(ok2147)* mutants were resistant to tBOOH, whereas overexpression of FMO-2 caused sensitivity to tBOOH (Figure 3.8C-D). While the reason for this is unclear, *C. elegans* is more likely to encounter and evolve resistance to periods of starvation than high levels of oxidants in the wild. FMO-2 function *in vivo* may therefore be more important in contributing to survival during starvation. Interestingly, human FMO1-3 has been found to release 30-50% of oxygen consumed as H$_2$O$_2$, raising the possibility that *fmo-2* activity may generate ROS that activate pro-survival pathways, but in the presence of tBOOH also contributes to additional toxicity (Siddens et al., 2014).
Figure 3.8 *fmo-2* is required for resistance to starvation but not tBOOH.

(A) Percentages of N2 and *fmo-2(ok2147)* worms that were able to resume development after L1 starvation over time (*p*<0.05). (B) Percentages of N2 and FMO-2-overexpressing worms that were able to resume development after L1 starvation over time (*p*<0.05). (C) Survival plots of N2 and *fmo-2(ok2147)* worms on 6 mM tBOOH (*p*<0.01). One representative experiment out of 4 independent repeats is shown. (D) Survival plots of N2 and FMO-2-overexpressing worms on 6 mM tBOOH (*p*<0.0001). One representative experiment out of 4 independent repeats is shown.

Table 3.4 Statistics for individual *fmo-2(ok2147)* and FMO-2 OEx tBOOH survival experiments.

Number of subjects denoted and statistics calculated as described in Table 3.2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Experiment no.</th>
<th>Median survival (hours)</th>
<th>Number of subjects (Dx/Nx (Cx))</th>
<th><em>p</em> value vs. N2</th>
</tr>
</thead>
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<td>67/112 (45)</td>
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<tr>
<td></td>
<td>2</td>
<td>41</td>
<td>41/118 (77)</td>
<td>NA</td>
</tr>
<tr>
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<td>52</td>
<td>48/107 (59)</td>
<td>NA</td>
</tr>
<tr>
<td>Strain</td>
<td>Experiment no.</td>
<td>Median survival (hours)</td>
<td>Number of subjects (Dx/Nx (Cx))</td>
<td>p value vs. N2</td>
</tr>
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<td>--------------</td>
<td>----------------</td>
<td>-------------------------</td>
<td>----------------------------------</td>
<td>----------------</td>
</tr>
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<td>fmo-2(ok2147)</td>
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<td>76/110 (34)</td>
<td>0.0015**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>51.5</td>
<td>32/66 (34)</td>
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</tr>
<tr>
<td></td>
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<td>69</td>
<td>36/113 (77)</td>
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<td>61/129 (68)</td>
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<td>84/115 (31)</td>
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<td>59/114(55)</td>
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</tr>
</tbody>
</table>

### 3.3.9 nhr-49 is required for increased tBOOH resistance in glp-1(e2141) mutants

Many *C. elegans* regulators of lipid metabolism are required for longevity, both in wild-type and long-lived backgrounds (Goudeau et al., 2011; Ratnappan et al., 2014; Taubert et al., 2006; Van Gilst et al., 2005b; Zhang et al., 2013). A previous study found that *nhr-49* was required for the long lifespan of *glp-1(e2141)* Notch receptor mutants (Ratnappan et al., 2014). At the restrictive temperature of 25°C, germline stem cells in these mutants cannot proliferate, thus causing sterility. Notably, these worms are also long-lived compared to WT worms (Arantes-Oliveira et al., 2002). Ratnappan *et al.* suggested that the requirement for *nhr-49* in the *glp-1* background was due to *nhr-49*’s role in lipid metabolism (Ratnappan et al., 2014). In particular, several *nhr-49*-dependent β-oxidation genes were upregulated in the *glp-1* background and were required for *glp-1* longevity (Ratnappan et al., 2014). Similar, *nhr-49*-dependent desaturases are also upregulated in this background and required for longevity of *glp-1* mutants (Goudeau et al., 2011; Ratnappan et al., 2014).
Given my findings that tBOOH response genes also require *nhr-49* for upregulation, I hypothesized that these genes may also be upregulated in *glp-1* mutants, and that these mutants have increased resistance to tBOOH. First, I crossed the *nhr-49(nr2041)* null mutant into the *glp-1(e2141)* background. I determined by qPCR that a number of NHR-49 dependent tBOOH response genes were upregulated in the *glp-1* background, and that this upregulation was no longer present in the double mutant (Figure 3.9). However, most of the changes in gene expression seen in *glp-1* worms were far milder compared to those seen in tBOOH or fasting.

Additionally, I also tested whether *glp-1* mutants are resistant to tBOOH in an *nhr-49*-dependent manner. I found that *glp-1* worms are highly resistant to tBOOH, whereas *glp-1;nhr-49* worms show near-wild type sensitivity. Therefore, *nhr-49* is required for the resistance of *glp-1* to tBOOH-induced oxidative stress.

![Graph](image)

**Figure 3.9** *nhr-49* is required for the upregulation of tBOOH/fasting response genes and tBOOH resistance of *glp-1(e2141)* mutants.

(A) mRNA fold changes of tBOOH/fasting response genes (relative to N2 worms) in L4 stage *glp-1(e2141)*, *nhr-49(nr2041)* and double mutants. Error bars represent SEM. **,*****Gene expression levels differ significantly from N2 worms (*p*<0.01 and *p*<0.001, respectively). ††††††††††Gene expression levels differ
significantly from glp-1(e2141) worms (p<0.05, p<0.01 and p<0.001, respectively). (B) Survival plots of N2, glp-1(e2141) and glp-1(e2141);nhr-49(nr2041) worms on 6 mM tBOOH (glp-1(e2141) vs N2: p<0.0001; glp-1(e2141);nhr-49(nr2041) vs N2: p<0.01). One representative experiment out of four independent repeats is shown.

Table 3.5 Statistics for individual glp-1(e2141) and glp-1(e2141);nhr-49(nr2041) tBOOH survival experiments.

Number of subjects denoted and statistics calculated as described in Table 3.2.

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<th>Strain</th>
<th>Experiment no.</th>
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<th>p value vs. glp-1(e2141)</th>
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<tr>
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<td>23</td>
<td>(36/50 (14))</td>
<td>0.0101*</td>
<td>&lt;0.0001****</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>23</td>
<td>(67/80 (13))</td>
<td>&lt;0.0001****</td>
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</tr>
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3.4 Discussion

3.4.1 A novel role for NHR-49 in an oxidative stress response

In the previous chapter, I showed that the transcriptional coregulator mdt-15 is required for the responses to two distinct oxidative stresses, arsenite and tBOOH. In the arsenite response, MDT-15 likely works by interacting with the transcription factor SKN-1 (see Chapter 2). However, most tBOOH response genes do not require skn-1 for their induction, although mdt-15 is required (Oliveira et al., 2009). Therefore, I reasoned that another MDT-
15 partner transcription factor is required for the tBOOH response. Here, I found that nhr-49 is required to induce a number of SKN-1-independent tBOOH response genes, some of which are also induced in other stresses such as fasting.

### 3.4.2 nhr-49 regulates tBOOH response genes in an mdt-15-dependent manner

By conducting a candidate reverse genetic screen, I identified nhr-49 as a regulator of the highly tBOOH-responsive, SKN-1-independent gene fmo-2 (Figure 3.10A). I further confirmed that nhr-49 is also required to induce a number of other known SKN-1-independent tBOOH response genes. In agreement with this, I previously found that nhr-49 is required for resistance to tBOOH (Figure 2.9D). nhr-49 is also sufficient for the tBOOH response, as putative nhr-49 GOF mutants show upregulation of tBOOH response genes in unstressed conditions compared to WT worms. As expected, this upregulation was dependent on mdt-15, as depletion of mdt-15 by RNAi abrogated the increased expression of fmo-2 and other tBOOH response genes in nhr-49(et13) GOF mutants.

A common criticism raised in studies of mutants with defective responses to exogenous stressors is that such mutants are already ‘sick’ and thus less able to respond to stress, rendering them more sensitive. This is unlikely to be the case with nhr-49 null mutants, as while they are short-lived, noticeable deleterious phenotypes (e.g. formation of vacuoles in the intestine and gonad and gonadal necrosis) do not manifest until around day 3 of adulthood (Van Gilst et al., 2005b). nhr-49 is also required to regulate Δ9 fatty acid desaturases and thus null mutants show altered lipid balance, which is thought to contribute to their shortened lifespan (Goudeau et al., 2011; Van Gilst et al., 2005b) and may also contribute to stress sensitivity, including potentially some types of oxidative stress.
(Horikawa and Sakamoto, 2009). However, I previously showed that these desaturases are not required for tBOOH resistance and are in fact downregulated on tBOOH exposure (Figure 2.7D). Additionally, another MDT-15-binding transcription factor, sbp-1, which also regulates Δ9 fatty acid desaturases and whose depletion causes strongly altered fatty acid compositions in *C. elegans* (Ashrafi et al., 2003; Yang et al., 2006), is not required for *fmo-2* induction. Therefore, I conclude that *nhr-49* is a *bona fide* transcriptional regulator of the tBOOH response in *C. elegans*. While a previous study found that *nhr-49* was not required for xenobiotic detoxification genes, the tBOOH response genes in this study are distinct from those genes, indicating that this is a stress-specific response (Taubert et al., 2008).

Combinatorial interactions with coregulators such as MDT-15 can fine-tune the transcriptional output of transcription factors in response to developmental and environmental signals (Malik and Roeder, 2010). Another layer of specificity can also be conferred through dimerization with other transcription factors. This is a mode of regulation used by many NHRs (Evans and Mangelsdorf, 2014). I found that three putative NHR-49 heterodimerization partners, NHR-13, NHR-66 and NHR-80, which are predicted to regulate lipid metabolism programs in partnership with NHR-49 (Pathare et al., 2012), were not required for the tBOOH response. However, I note that I did not test whether these NHRs may regulate the tBOOH response redundantly, for example with double or triple mutants, although Pathare *et al.* found that single mutations in these genes were sufficient to confer gene expression and phenotypic changes (Pathare et al., 2012). NHR-49 also binds a number of other NHRs *in vitro*, including itself, *i.e.* it may act as a homodimer at some loci (Pathare et al., 2012; Reece-Hoyes et al., 2013). It is therefore likely that NHR-49 works with another as yet unidentified NHR to regulate tBOOH response genes.
Importantly, not all tBOOH response genes require \textit{nhr-49} for their induction (see Appendix 1). Many \textit{nhr-49}-independent genes such as \textit{ttr-37} and \textit{fmo-1} are, however, dependent on \textit{mdt-15} (Figure 2.8). Therefore, another MDT-15-binding transcription factor may be required to induce these genes. Candidate RNAi screens using transcriptional reporters for \textit{nhr-49}-independent tBOOH response genes could be used to identify these factors.

3.4.3 The \textit{nhr-49}-dependent tBOOH response is also activated in fasting and downstream of \textit{glp-1}

\textit{NHR-49} is known to induce fatty acid β-oxidation genes in response to fasting. Chamoli \textit{et al.} noted that one potential benefit of shifting to a β-oxidation program is that more FADH$_2$ is generated relative to NADH compared to when glucose is used as an energy source (Chamoli \textit{et al.}, 2014). FADH$_2$ is oxidized at Complex II of the mitochondrial electron transport chain, which produces less ROS by electron leakage compared to Complex I, which oxidizes NADH (Lenaz, 2001; Turrens, 1997). Therefore, upregulation of β-oxidation generates less ROS compared to glucose metabolism and is hypothesized to be overall beneficial to an animal that is already undergoing a fasting stress.

Here, I show that the \textit{NHR-49}-dependent stress response is also upregulated upon fasting (Figure 3.10B), and that one of its targets genes, \textit{fmo-2}, confers resistance to starvation. The relationship between fasting and oxidative stress is complex. Some studies have shown that ROS levels are increased in fasting (Marczuk-Krynicka \textit{et al.}, 2003; Sorensen \textit{et al.}, 2006). While high levels of ROS are detrimental, ROS (in particular superoxide) are required to stimulate autophagy, a process by which cellular materials are degraded and recycled to
promote survival under conditions of starvation (Chen et al., 2009; Scherz-Shouval et al., 2007; Zhang et al., 2009a). Taken together, it is likely that under conditions of starvation, intracellular ROS levels must be regulated to ensure that they cause minimal damage, while still being present at sufficient levels to stimulate autophagy. Perhaps NHR-49 regulates ROS levels to ensure that this balance is maintained in starvation; this could be tested using ROS-sensing fluorescent dyes (Back et al., 2012a; Tauffenberger and Parker, 2014) to measure ROS levels in fed/fasted wild-type and nhr-49 mutant worms.

*nhr-49* is also required for the extended lifespan of *glp-1* mutants (Ratnappan et al., 2014). This was previously thought to be due solely to *nhr-49*’s role in lipid metabolism. However, I have shown that tBOOH/fasting response genes are also upregulated in *glp-1* worms, and that *glp-1* mutants are highly resistant to tBOOH in an *nhr-49*-dependent manner (Figure 3.10C). While the reason these genes are upregulated in *glp-1* worms is unknown, one could speculate that this mimics a fasting ‘signal’ which leads to a dietary restriction-like response, causing the increased lifespan of these worms. Future studies will be required to determine if the upregulation of tBOOH/fasting response genes are required for *glp-1* longevity.
Figure 3.10 Model for NHR-49 and HLH-30-dependent gene regulation.

(A) In tBOOH-induced oxidative stress, fmo-2 and other SKN-1-independent stress response genes are induced by NHR-49, with MDT-15 acting as a coactivator. HLH-30 is partially required for this response. (B) In fasting, NHR-49 and MDT-15 activate fmo-2 and other stress response genes as well as fatty acid β-oxidation genes, whereas HLH-30 activates fmo-2 and autophagy genes. (C) In germline-less glp-1 mutants, NHR-49 regulates fmo-2 and other stress response genes as well as fatty acid β-oxidation and desaturation genes, while HLH-30 activates autophagy genes; whether HLH-30 is also required to induce fmo-2 in the glp-1 background is unknown.

3.4.4 fmo-2 is activated by distinct upstream regulatory inputs

Previously, fmo-2 was known to be upregulated by two stresses, hypoxia and fasting (Leiser et al., 2015; Shen et al., 2005). Leiser et al. showed that this was dependent on the transcription factor HLH-30. In hypoxia, the hypoxia inducible factor HIF-1 acts in the neurons to activate HLH-30 in the intestine, which induces fmo-2 cell autonomously (Leiser et al., 2015). The exact mechanism by which HIF-1 activates HLH-30 is unknown. However, while hlh-30 is required for fmo-2 activation in fasting, hif-1 is not (Leiser et al., 2015). My findings show that NHR-49’s role in activating fmo-2 is likely independent of HLH-30.
Furthermore, a number of tBOOH/fasting-induced genes require nhr-49, but not hlh-30, for expression and/or induction. While I have not determined if HIF-1 also acts upstream of NHR-49, the fact that it is not required for fmo-2 induction by HLH-30 in fasting (Leiser et al., 2015) likely means that it does not, although further experiments would be required to test this.

Interestingly, upregulation of fmo-2 has also been reported in response to stresses other than the ones already mentioned here. For instance, it is the highest-upregulated gene in response to C. elegans infection with the pathogen Staphylococcus aureus (Visvikis et al., 2014). Here, the induction of fmo-2 is hlh-30-independent, even though hlh-30 regulates the majority (77%) of genes that are activated upon S. aureus exposure (Visvikis et al., 2014). fmo-2 is also induced by silver nanoparticles in a HIF-1-dependent manner, as well as in the clk-1 and isp-1 mitochondrial mutants, which are long-lived, in a HIF-1-independent manner (Eom et al., 2013; Lee et al., 2010). Therefore, there appear to be at least two regulatory axes that lead to the activation of fmo-2: one that is dependent on HLH-30 (sometimes downstream of HIF-1, as in the case of hypoxia), and one that requires NHR-49. Another transcription factor could be required in the case of S. aureus. This is summarized in (Figure 3.11). It would be interesting to known how such different stresses can converge on only two transcription factors and regulate a single gene in such a combinatorial fashion. One could speculate that perhaps some of these stresses give rise to similar signaling molecules that activate only one of these pathways, therefore leading to response specificity.

Despite being strongly induced in diverse conditions, fmo-2’s biological function remains elusive. fmo-2 belongs to the flavin-containing monooxygenase (FMO) enzyme family, which is predicted to function in xenobiotic detoxification. FMOs catalyze the oxygenation
of a number of xenobiotic and endogenous substrates (Phillips and Shephard, 2008; Ziegler, 2002). A number of endogenous physiological roles have been found for mammalian FMOs, for example in the metabolism of lipids, cholesterol, and glucose (Gonzalez Malagon et al., 2015; Schugar and Brown, 2015; Warrier et al., 2015). There are five members of the FMO family in C. elegans; precise roles for most of these have not been defined, save for a role in osmoregulation for fmo-4 (Hirani et al., 2016). The closest human homologue of fmo-2 is FMO5. Interestingly, Fmo5 knockout mice show several metabolic phenotypes, including an age-related lean phenotype, increased fatty acid oxidation in white adipose tissue but decreased oxidation in skeletal muscle, and decreased levels of several enzymes involved in glucose and lipid metabolism in the liver (Gonzalez Malagon et al., 2015). Future unbiased gene expression and metabolite analysis may provide further insight into the role of fmo-2 in C. elegans.
Figure 3.11 Model of pathways that regulate fmo-2 in C. elegans.

fmo-2 is activated in response to several stresses, including tBOOH-induced oxidative stress, fasting, hypoxia and S. aureus infection. In tBOOH and fasting, fmo-2 is induced by NHR-49 and HLH-30. The hypoxia response requires hif-1, which signals HLH-30 to activate fmo-2; whether NHR-49 is also required for fmo-2 activation in hypoxia is unknown. Finally, S. aureus infection causes fmo-2 activation in an HLH-30-independent manner; therefore, it is possible that NHR-49 activates fmo-2 in this context.

3.4.5 Conservation between NHR-49 and its mammalian orthologs

There are 284 NHRs in C. elegans, an unusually large number compared to 48 in humans, that has been hypothesized to reflect the constantly changing environment that this nematode encounters in the wild (Sluder et al., 1999). Of these, 269, including NHR-49, are derived from an ancestral homologue related to hepatocyte nuclear factor 4 (HNF4).
HNF4 is involved in the development of organs such as the pancreas, liver, and intestines in mammals, as well as in glucose and lipid metabolism (Duncan et al., 1994; Hayhurst et al., 2001; Odom, 2004; Stoffel and Duncan, 1997; Watt et al., 2003). However, due to nhr-49's role in fatty acid β-oxidation, several papers have hypothesized that it is in fact a functional homologue of another mammalian nuclear receptor, peroxisome proliferator-activated receptor α (PPARα) (Ratnappan et al., 2014; Van Gilst et al., 2005a; 2005b). In fact, modeling of the NHR-49 structure onto experimentally derived HNF4α and PPARα structures showed robust overlays in both cases, although there was a slightly better fit with HNF4α (Lee et al., 2016). As there is no sequence orthologue of PPARα in C. elegans, it is possible that NHR-49 has evolved similar roles to PPARα despite being derived from a HNF4α ancestor.

Several papers point to a potential role of PPARα in stress responses such as heat stress and acetaminophen toxicity in mammals (Anderson et al., 2004; Chen et al., 2000; Vallanat et al., 2010). Interestingly, one study described an increase in weight reduction and oxidative stress markers in livers of fasted PPARα null mice (Abdelmegeed et al., 2009). The livers of these mice also showed lower antioxidant enzyme activity, although the mRNA expression levels of these genes were not measured (Abdelmegeed et al., 2009). It is therefore possible that mammalian PPARα is also involved in stress responses. If so, this could present a biomedical application for modulating stress responses in human diseases, as PPARα can be targeted by small molecules: the fibrates are a class of drugs that agonize PPARα and are commonly used to treat dyslipidemia (Tenenbaum and Fisman, 2012). Intriguingly, fibrates can extend C. elegans lifespan in an nhr-49-dependent manner (Brandstädt et al., 2013). Whether or not fibrates have a similar effect in mammals remains to be seen.
Chapter 4: Discussion

Redox homeostasis is critical for health. In order to prevent injury caused by oxidative stress, cells can activate enzymes and other molecules that either remove ROS or repair damage to cellular macromolecules. This is often regulated at the level of transcription by sequence-specific transcription factors and their coregulators. In this thesis, I investigated the hypothesis that the Mediator subunit \textit{mdt-15} is required for oxidative stress. I described a mechanism by which MDT-15 regulates an oxidative stress response via the conserved cytoprotective transcription factor SKN-1. I also found that \textit{mdt-15} is required for a SKN-1-independent oxidative stress response through another transcription factor partner, the nuclear hormone receptor NHR-49. This work identifies novel roles for two transcriptional regulators in the oxidative stress response, and may have therapeutic implications for diseases in which oxidative stress plays a major role.

4.1 \textit{mdt-15} and two of its transcription factor partners are required for oxidative stress responses in \textit{C. elegans}

Worms carrying an \textit{mdt-15} reduction-of-function mutation exhibit pleiotropic phenotypes, many of which are caused by defects in fatty acid desaturation. However, some of these phenotypes cannot be rescued by feeding unsaturated fatty acids, leading to the hypothesis that MDT-15 regulates other gene programs (Taubert et al., 2008). \textit{mdt-15}-dependent genes, as determined by microarray analysis, showed significant overlap with a number of oxidative stress response gene sets. In this thesis, I used genetic, molecular, and biochemical analyses to determine that MDT-15 is a coactivator of SKN-1 in the SKN-1-dependent oxidative stress response. Furthermore, I found that MDT-15 and its known
partner NHR-49, a nuclear hormone receptor with previously described roles in lipid metabolism, regulates SKN-1-independent oxidative stress response genes. Interestingly, NHR-49 also upregulates these genes when worms are starved, as well as in the long-lived glp-1 background; therefore, this transcriptional program likely facilitates adaptation to various types of changes in the environment, such as the presence of toxic stresses or food levels. In total, these findings show that MDT-15 is a central regulator of oxidative stress responses in C. elegans, by partnering and acting as a coactivator for at least two different transcription factors (Figure 4.1 and Figure 4.2).

In mammals, several coactivators for Nrf2 have already been described, including the chromo-ATPase/helicase DNA-binding protein family member CHD6, CREB-binding protein (CBP)/p300, and, interestingly, the Mediator subunit MED16 (Katoh et al., 2001; Nioi et al., 2005; Sekine et al., 2016; Zhu and Fahl, 2001). Human p300 binds SKN-1 and drives SKN-1-dependent transcription, suggesting that CBP/p300 is a conserved coactivator of SKN-1 (Walker et al., 2000). Additionally, the human host cell factor 1-related (HCF-1) transcriptional coregulator was previously shown to be a negative coregulator of SKN-1 (Rizki et al., 2012). HCF-1 likely binds SKN-1 in the nucleus to prevent its accumulation in intestinal nuclei, although the exact mechanism for this is still unknown (Rizki et al., 2012). It is possible that other, as yet unidentified coregulators also work with SKN-1 to regulate its target genes.

SKN-1 is considered the main oxidative stress-responsive transcription factor in C. elegans. It is required for survival on various different oxidative stressors, including arsenite, tBOOH, paraquat, etc (An and Blackwell, 2003; An et al., 2005; Inoue et al., 2005). However, Oliveira et al. showed that the vast majority of tBOOH-induced genes are actually
SKN-1-independent, although the SKN-1-dependent genes are likely required for survival (Oliveira et al., 2009). The transcription factor(s) that regulates the SKN-1-independent response had not previously been identified. In Chapter 2, I showed that *mdt-15* is required to induce SKN-1-independent tBOOH response genes, raising the possibility that an MDT-15-binding transcription factor regulates these genes. In Chapter 3, I showed that *nhr-49* is required to induce at least some of these genes, including the highly tBOOH-responsive SKN-1-independent gene *fmo-2*. Therefore, I have not only identified a novel transcription factor that is required for an oxidative stress response, but also a previously unknown role for NHR-49.

Figure 4.1 Model depicting the roles of MDT-15 and its partner transcription factors in oxidative stress and other physiological conditions.

When worms are exposed to arsenite, a SKN-1-dependent transcriptional response is activated by SKN-1, with MDT-15 and the Mediator complex acting as a coactivator. The response to tBOOH is partially dependent on SKN-1, but also requires NHR-49 working together with MDT-15. NHR-49-dependent tBOOH response genes
are also induced in other conditions such as fasting and germline loss due to *glp-1* mutation. These conditions have also been reported to activate SKN-1 (Paek et al., 2012; Steinbaugh et al., 2015).

**Figure 4.2 Updated transcription factor interactions and functions of *C. elegans* MDT-15.**

In this thesis, I have shown that MDT-15 interacts with SKN-1 to regulate a SKN-1-dependent oxidative stress response, and that it cooperates with NHR-49 to regulate a SKN-1-independent oxidative stress response. Novel interactions and functions are highlighted in bold.

### 4.2 Limitations and caveats

In this thesis, I show that *mdt-15* and *nhr-49* are not only required for transcriptional responses to oxidative stress, but are also required for organismal resistance to this stress (*i.e.* improved population survival upon stress). Survival on oxidative stress-inducing compounds, such as I described in this thesis, is a widely used and generally accepted method of measuring sensitivity to oxidative stress in the *C. elegans* field (An and Blackwell, 2003; An
et al., 2005; Chen et al., 2013; Inoue et al., 2005; Li et al., 2008; Przybysz et al., 2009; Steinbaugh et al., 2015). The sensitivity of mdt-15 and nhr-49 loss/reduction-of-function mutants to oxidative stress is likely due to their inability to mount proper defensive responses to abnormally high levels of oxidative damage. However, I have not shown that these mutants in fact have increased oxidative damage or internal ROS levels compared to wild-type worms. This could be determined by measuring biomarkers of oxidative stress (e.g. lipid peroxidation, protein carbonylation) and using ROS-sensing fluorescent dyes or transgenic fluorescent reporters to measure internal levels of ROS in vivo. Such dyes and reporters have been developed for use in C. elegans, and can be used to determine ROS levels in different mutant backgrounds (Back et al., 2012a; 2012b; Tauffenberger and Parker, 2014).

One caveat to my studies is that direct targets of MDT-15 and NHR-49 have not yet been identified. Conducting chromatin immunoprecipitation (ChIP) on a tagged MDT-15 protein followed by qPCR or sequencing could identify direct targets of MDT-15. For NHR-49, a putative DNA binding site was previously described using in vitro methods (Weirauch et al., 2014). However, many of the nucleotides in this sequence are low confidence, making identification of potential binding sites in the genome challenging. Again, techniques such as ChIP could allow identification of direct NHR-49 targets as well as the elucidation of a more well-defined consensus NHR-49 binding site.

One question I have not addressed in this study is whether MDT-15 and its transcription factor partners regulate oxidative stress response genes cell autonomously or non-autonomously. Many mdt-15-dependent genes as well as stress-responsive genes such as gcs-1, fmo-2 are primarily expressed in the intestine, the main site of contact with ingested material in C. elegans (An and Blackwell, 2003; Leiser et al., 2015; Taubert et al., 2008).
*mdt-15* is expressed in the intestine and head neurons of worms (Taubert et al., 2006). SKN-1 has four isoforms, with only one being required for the oxidative stress response (Blackwell et al., 2015). This isoform, SKN-1c, is normally expressed in the intestine at low levels, but accumulates in intestinal nuclei upon oxidative stress (An and Blackwell, 2003). This selective induction of SKN-1c strongly supports the hypothesis that MDT-15 and SKN-1 act together in the intestine to regulate SKN-1-dependent oxidative stress response genes.

In the case of NHR-49, the evidence that it functions with MDT-15 in the intestine is less clear. NHR-49 is also widely expressed, including in the intestine, hypodermis, and many neurons (Ratnappan et al., 2014; Van Gilst et al., 2005b). While many NHR-49-dependent genes, both in stress responses and in lipid metabolism, are intestinally expressed (Mullaney and Ashrafi, 2009; Van Gilst et al., 2005a), NHR-49 can function in other tissues to regulate these genes in cell non-autonomous fashion. Burkewitz *et al.* showed that NHR-49 can act either in the neurons to regulate the β-oxidation gene *acs-2* in the intestine, or in the intestine itself (Burkewitz *et al.*, 2015). However, I note that I observed loss of *fmo-2* induction by knocking down *mdt-15* and *nhr-49* with RNAi; in *C. elegans*, the nervous system is refractory to feeding RNAi (Kennedy *et al.*, 2004). This suggests that at least in the tBOOH response, MDT-15 and NHR-49 do not operate in the neurons to regulate *fmo-2*.

### 4.3 MDT-15 is a key regulator of responses to environmental stimuli

In this work, I describe a novel role for *mdt-15* in both SKN-1-dependent and – independent oxidative stress responses. Collectively, this and other published data show that MDT-15/MED15 is an evolutionarily ancient coregulator and signaling hub for
transcriptional adaptation to external stimuli such as nutrition availability and presence of toxic stresses.

In *C. elegans*, *mdt-15/MED15* is required for fatty acid desaturation and β-oxidation, through its interactions with SBP-1/SREBP and NHR-49/PPARα (Lee et al., 2015; Taubert et al., 2006; Yang et al., 2006). A recent study showed that *mdt-15* is also required for the upregulation of a starvation response by a *skn-1* GOF mutation (Pang et al., 2014). It is also critical for xenobiotic detoxification: *mdt-15/MED15* is required for resistance to organic carcinogens and the response to heavy metals (Taubert et al., 2008). Additionally, *mdt-15/MED15* is required for the innate immune response (Pukkila-Worley et al., 2014).

In yeast, MED15 (also known as Gal11) regulates fatty acid β-oxidation through its interaction with the Oaf1 transcription factor; amino acid biosynthesis via Gcn4; and xenobiotic detoxification and other stress responses through the Pdr1 and Msn2/Msn4 transcription factors, respectively. Conversely, in human cell lines, MED15 regulates fatty acid desaturation and cholesterol metabolism through its interaction with the SREBP transcription factor (Yang et al., 2006). A recent paper also found that MED15 interacts with phosphorylated p73 to regulate genotoxic stress response genes in human cell lines (Satija and Das, 2016).

What factors might act upstream to regulate MDT-15 and its targets? Most studies to date suggest that MDT-15/MED15, and the rest of the Mediator complex, is recruited to target gene promoters by partner transcription factors that bind specific DNA sequences (Conaway and Conaway, 2011a; Malik and Roeder, 2010; Poss et al., 2013). It is likely that environmental changes activate upstream signaling pathways that lead to binding of these transcription factors at their target promoters, which then require MDT-15/MED15 and the
Mediator complex for transcriptional activation. However, MDT-15 may also be directly regulated, e.g. by modulation of its protein levels or by post-translational modifications. Evidence for this hypothesis is provided by a study on yeast Gal11/MED15, which is phosphorylated to suppress osmotic stress-induced transcription under normal conditions (Miller et al., 2012). Another study found that human MED15 was bound and targeted for degradation by the E3 ubiquitin ligase TRIM11 (Ishikawa et al., 2006). These results were later independently confirmed by a study that aimed to map the human coregulator ‘complexome’, which found that MED15 exists in two stable complexes, one with the larger Mediator complex and another with TRIM11 (Malovannaya et al., 2011). Together, these findings show that post-translational modification of MED15 is capable of regulating both activity and levels of MED15. Whether this also occurs in *C. elegans* has yet to be determined; however, a large-scale protein-protein interaction screen of *C. elegans* proteins identified a putative physical interaction of MDT-15 with MEL-26, an adaptor for the CUL-3-containing E3 ubiquitin ligase (Li, 2004). Therefore, MEL-26 could represent a novel regulator that may influence MDT-15 levels in context dependent fashion, a notion that warrants further investigation.

4.4 The relationship between lipid metabolism and oxidative stress response transcriptional programs

Lipid metabolism and oxidative stress responses are biological processes that are disrupted in many diseases, for instance in cancer and metabolic disease (Brownlee, 2005; Eckel et al., 2005; Pavlova and Thompson, 2016). Prior to my study, MDT-15 and NHR-49 were already known to be critical regulators of lipid metabolism (Taubert et al., 2006; Van
Gilst et al., 2005a; 2005b). SKN-1 can also regulate lipid metabolism genes, particularly in β-oxidation (Paek et al., 2012). This raises the possibility of a relationship between lipid metabolism and oxidative stress responses, since MDT-15, NHR-49, and SKN-1 all regulate both programs. Therefore, I investigated the overlap between lipid biology and oxidative stress resistance in several instances in this thesis.

In Chapter 2, I used a combination of approaches to determine whether *mdt-15*-dependent Δ9 fatty acid desaturases were required for oxidative stress resistance. Depleting the critical MDT-15 (and NHR-49) targets *fat-6* and *fat-7* did not cause sensitivity to tBOOH, and feeding *mdt-15* mutants unsaturated fatty acids did not rescue their oxidative stress sensitivity, either, even though many other phenotypes were rescued. Additionally, fatty acid desaturases decrease in expression levels upon tBOOH stress. This means that Δ9 fatty acid desaturases are not required for resistance to tBOOH, and that they may even be detrimental to resistance. Furthermore, in Chapter 3 I showed that the lipogenic transcription factor *sbp-1*, which also regulates Δ9 fatty acid desaturases, was not required for *fmo-2* induction on tBOOH. The β-oxidation gene *acs-2* is also not induced in tBOOH (unpublished data); additionally, other β-oxidation genes were not identified in microarray data of tBOOH-induced genes (Oliveira et al., 2009), making it unlikely that NHR-49-dependent β-oxidation programs are involved in the tBOOH response. Since β-oxidation can potentially produce lipid peroxides that can exacerbate oxidative stress, the lack of increase in β-oxidation activity is expected. Collectively, these experiments show that fatty acid desaturation and β-oxidation are not required for the tBOOH response, and are thus mechanistically separable functions of MDT-15 and its associated transcription factors.
It is important to note that the separation of lipid metabolism and oxidative stress responses may only apply to some types of ROS. As previously mentioned, Horikawa and Sakamoto found that Δ9 desaturases were required for resistance to paraquat, which causes oxidative damage primarily by generating superoxide (Horikawa and Sakamoto, 2009). In vitro, unsaturated fatty acids have been found to have superoxide scavenging ability, which may explain the requirement for Δ9 desaturases in paraquat resistance (Richard et al., 2008). However, evidence to support this in vivo has not been demonstrated. On the other hand, tBOOH acts by promoting lipid peroxidation, which favours unsaturated fatty acids, especially PUFAs, due to their carbon-carbon double bonds (Masaki et al., 1989). Under these conditions, it is likely that these fatty acids are, if anything, detrimental to survival – and in fact, I showed that a fat-6; fat-7 double mutant showed some resistance to tBOOH. Thus, the effects of differential lipid composition on oxidative stress, if any, are still mostly unclear.

What is the relevance of these findings to human disease? Taking cancer as an example, most tumour cells undergo high levels of oxidative stress, and thus have increased levels of antioxidants (Gorrini et al., 2013; Jaramillo and Zhang, 2013). In addition, stearoyl-coA desaturase (SCD), the human Δ9 fatty acid desaturase, is also upregulated in various types of cancers, due to the increased need for new membrane phospholipids (Igal, 2010). Understanding the relationship between lipid metabolism and oxidative stress resistance in tumours, if any, may help to refine therapeutic approaches in cancer.
4.5 Relevance to human health

As described in Section 1.1.1.1, oxidative stress plays a major role in many human diseases. While the role of human MED15 in oxidative stress responses is still unclear, the Mediator complex is known to interact with the homologs of both transcription factors that I described to bind MDT-15 - human Nrf2 binds MED16 to activate the antioxidant response, whereas PPARα binds MED1 (Sekine et al., 2016; Zhu et al., 1997).

Understanding the specific interactions between Mediator subunits and their partner transcription factors can aid in the design of small molecule drugs that target these interfaces. Zhao et al. identified a boron-containing compound that disrupts the interaction between SREBP and the MED15 KIX domain, which can prevent induction of SREBP target genes and improve lipid homeostasis in a mouse model of diet-induced obesity (Zhao et al., 2014). Nishikawa et al. also used this approach to disrupt the interaction between fungal Gal11/MED15 and the Pdr1 transcription factor, which mediates resistance to antifungal drugs such as ketoconazole (Nishikawa et al., 2016). Resistance of fungal pathogens such as Candida glabrata to these drugs is a major medical issue among immunocompromised patients. A similar strategy could be used to target other interactions between Mediator and its partner transcription factors in disease. For example, if the Mediator/Nrf2 interaction is critical for the expression of antioxidant genes that allow cancer cells to resist the high level of oxidative stress they encounter, either endogenously or during chemo/radiotherapy, disrupting that interface may allow for specific targeting of cancer cells.

The finding that NHR-49 regulates a SKN-1-independent oxidative stress response also has interesting implications for disease. Nuclear hormone receptors contain a ligand binding domain that can bind endogenous or exogenous ligands. Small molecules can be used to
modulate NHR activity; as such, NHRs are attractive drug targets. Many drugs that modulate NHR action have already been developed. For example glucocorticoids target the glucocorticoid receptor and are used to treat inflammatory diseases, while estrogen receptor modulators such as tamoxifen are used to treat breast cancer (Ottow and Weinmann, 2008). As explained in Section 3.4.5, there is some evidence that the functional homolog of NHR-49, PPARα, is also involved in regulating stress responses in humans (Anderson et al., 2004; Chen et al., 2000; Vellanat et al., 2010). PPARα agonists i.e. the fibrates, are widely used to treat hyperlipidemia (Tenenbaum and Fisman, 2012). Further studies could determine whether PPARα truly regulates stress responses in humans; if so, drugs that target PPARα activity could be used to modulate these responses in disease.

4.6 Future directions

Below I highlight several possible future directions for this research.

4.6.1 Identification of upstream regulators in the NHR-49-dependent tBOOH response

In this thesis, I identified NHR-49 as a regulator of the SKN-1-independent oxidative stress response. The mechanism by which tBOOH exposure activates NHR-49, however, is still unknown. In contrast, many upstream regulators of SKN-1 have been identified to date (An et al., 2005; Glover-Cutter et al., 2013; Hourihan et al., 2016; Inoue et al., 2005; Okuyama et al., 2010; Robida-Stubbs et al., 2012; Steinbaugh et al., 2015; Tullet et al., 2008; van der Hoeven et al., 2011; Wu et al., 2016). Many of these regulators are components of pathways that have been implicated in stress resistance and/or the regulation of longevity,
e.g. the IIS pathway, the TOR pathway, or the ER stress response pathway. It will be of interest to determine whether NHR-49 shares upstream activating signals and pathways with SKN-1, and if these regulators contribute to the distinct transcriptional responses induced by NHR-49 and SKN-1.

To identify regulators of the tBOOH response, a forward genetic screen could be performed by mutagenizing an integrated version of the \textit{fmo-2p::GFP} strain. This would allow identification of mutations that upregulate \textit{fmo-2} even under unstressed conditions. A secondary screen could then be carried out to test for mutations that require \textit{nhr-49} to upregulate \textit{fmo-2}. This screen might also uncover regulators of \textit{fmo-2} that do not act through NHR-49, which will contribute further to our understanding of the \textit{fmo-2} regulatory network.

\subsection*{4.6.2 Dissecting the \textit{fmo-2} regulatory network}

As detailed in (Section 3.4.4), \textit{fmo-2} is induced in response to many environmental stimuli, including oxidative stress (Oliveira et al., 2009), fasting (Leiser et al., 2015), hypoxia (Leiser et al., 2015; Shen et al., 2005), and \textit{S. aureus} infection (Visvikis et al., 2014). At least two regulatory axes of \textit{fmo-2} have been identified to date, one that is dependent on \textit{hlh-30} and one on \textit{nhr-49} (Leiser et al., 2015) (Figure 3.11). Whereas \textit{hlh-30} is required to induce \textit{fmo-2} in hypoxia (Leiser et al., 2015), whether or not \textit{nhr-49} is required for the hypoxia response is not known. Additionally, \textit{hlh-30} is not required for \textit{fmo-2} induction in a worm model of \textit{S. aureus} infection (Visvikis et al., 2014), leading to the hypothesis that \textit{nhr-49} may also be required for a HLH-30-independent response to \textit{S. aureus}. One future direction could be to determine whether \textit{nhr-49} is also required for \textit{fmo-2} induction in hypoxia and in \textit{S. aureus} infection, and whether it is required for resistance to these stresses.
HIF-1 also works upstream of HLH-30 in the hypoxia response (Leiser et al., 2015). Thus, future work could also include determining whether HIF-1 similarly regulates NHR-49 action. This could be accomplished using loss-of-function mutations in the von Hippel-Lindau (VHL-1) gene, which suppresses HIF-1 activity under normoxic conditions (Maxwell et al., 1999). Therefore, if HIF-1 induces fmo-2 through NHR-49, a vhl-1 LOF mutant should show increased expression of fmo-2 under normal conditions, which would be abrogated in a vhl-1; nhr-49 double LOF mutant.

Finally, tissue-specific RNAi strains and transgenic rescues would allow investigation of the spatial distribution of the fmo-2 regulatory network. As described in Section 4.2, NHR-49 acts in both the neurons and the intestine to regulate target genes in the intestine (Burkewitz et al., 2015). While neuronal NHR-49 is probably not critical for fmo-2 regulation, as discussed, components of upstream pathways may act in other tissues to activate NHR-49. Combined with identification of upstream regulators of NHR-49, this would further our understanding of how different environmental stimuli can converge on similar transcriptional outputs.

### 4.6.3 Determining the biological function of fmo-2

Despite being highly induced in response to several different stresses, the biological function of fmo-2 is still unknown. In this study, I showed that it is required for resistance to long-term starvation, but fmo-2 LOF causes resistance to tBOOH, whereas its overexpression causes tBOOH sensitivity. Leiser et al. found that FMO-2 overexpression conferred resistance to tunicamycin, dithiothreitol and heat stress, as well as extending lifespan (Leiser et al., 2015). In mammals, FMOs catalyze the oxygenation of many endogenous and
exogenous substrates (Phillips and Shephard, 2008; Ziegler, 2002). Therefore, it seems likely that fmo-2 performs important biological function(s) in C. elegans. As previously mentioned in Section 3.4.4, unbiased gene expression and metabolite analysis is likely necessary to determine pathways affected by fmo-2 activity, and may contribute to identifying the substrates and products of C. elegans FMO-2.

### 4.6.4 Conservation in mammals

It remains to be seen whether the functions of MDT-15 and NHR-49 in oxidative stress responses are conserved in mammals. While human MED15 interacts with SREBP, the orthologue of SBP-1, to regulate lipid metabolism, there is no evidence to date that it interacts with Nrf2 or nuclear hormone receptors to regulate stress/detoxification responses. The predicted functional orthologue of NHR-49, PPARα, appears to be involved in responses to various stresses, including oxidative stress (Abdelmegeed et al., 2009; Anderson et al., 2004; Chen et al., 2000; Vallanat et al., 2010). However, this has not been definitively tested. Genetic knockdown experiments could be performed in mammalian cell culture to determine whether MED15 and PPARα/HNF4 are involved in oxidative stress responses, and could potentially be followed up in vivo using mouse models. Protein-protein interaction studies such as yeast two-hybrid and co-immunoprecipitation could also be used to investigate whether Nrf2 and PPARα/HNF4 interact with MED15. Finally, ChIP-qPCR experiments could be used to determine whether MED15 and PPARα/HNF4 localize to regulatory elements upstream of oxidative stress response genes, which increases the likelihood that these genes are direct targets of MED15 and PPARα/HNF4 in the oxidative stress response.
Chapter 5: Conclusions

The Mediator subunit MDT-15/MED15 is required to regulate various physiological processes in *Caenorhabditis elegans*. Microarray analysis of *mdt-15*-dependent genes showed that *mdt-15* is required for expression of a number of oxidative stress response genes. Here, I have found that *mdt-15* is required for the response and resistance to oxidative stress in *C. elegans*. Many oxidative stress response genes are regulated by the cytoprotective transcription factor SKN-1/Nrf2. I found that MDT-15 regulates SKN-1-dependent genes, both in response to the oxidative stressor arsenite as well as in *wdr-23* loss-of-function backgrounds, where SKN-1 levels are upregulated. *mdt-15* was also required for survival on arsenite. Additionally, I found that MDT-15 physically binds SKN-1 in yeast two-hybrid assays. Thus, I conclude that MDT-15 is a coregulator of SKN-1 in the SKN-1-dependent oxidative stress response.

The transcriptional response to the oxidative stressor tBOOH was previously shown to be largely independent of SKN-1, even though *skn-1* is required for survival on tBOOH. I showed that *mdt-15* was also required for survival on tBOOH, and was required to induce SKN-1-independent tBOOH response genes, independently of MDT-15’s role in fatty acid desaturation. By conducting a candidate reverse genetic screen, I showed that the known MDT-15 partner NHR-49 was a novel regulator of the SKN-1-independent oxidative stress response. *nhr-49* was both necessary and sufficient to induce some tBOOH response genes, and is required for survival on tBOOH. I further showed that this NHR-49-dependent response was also induced in fasting, and that *nhr-49* is required for resistance to starvation. Additionally, these genes were also upregulated in the long-lived germline-less *glp-1* mutant background. *nhr-49* is known to be required for the long lifespan of *glp-1* mutants. Here, I
show that $glp-1$ mutants are also highly resistant to tBOOH, and that this resistance requires $nhr-49$. While another transcription factor, HLH-30, was previously shown to regulate the highly $nhr-49$-dependent gene $fmo-2$, I found that $hlh-30$ was only partially required for the tBOOH response. Thus, I conclude that NHR-49 is a regulator of a SKN-1-independent oxidative stress response in $C.~elegans$.

In summary, this thesis describes a novel function for the MDT-15 and NHR-49 in the oxidative stress response in $C.~elegans$. Further work is required to determine whether the human homologs of MDT-15 and NHR-49 also regulate oxidative stress responses, and if so, whether they could present drug targets for diseases where oxidative stress plays a major role.
Chapter 6: Materials and Methods

Nematode strains, growth conditions, and RNAi

*C. elegans* strains were cultured using standard techniques (Brenner, 1974) on nematode growth media (NGM)-lite (0.2% NaCl, 0.4% tryptone, 0.3% KH₂PO₄, 0.05% K₂HPO₄) agar plates supplemented with 5 μg/ml cholesterol. *E. coli* OP50 was used as food, except for RNAi experiments, for which *E. coli* HT115 was used. Worm strains are listed in Table 6.1. To avoid background effects, each mutant was crossed into our N2 strain; original mutants were backcrossed to N2 at least six times. All experiments were done at 20°C.

RNAi was performed on NGM-lite plates supplemented with 25 μg/mL carbenicillin, 1 mM IPTG, and 12.5 μg/mL tetracycline, and seeded with appropriate *E. coli* HT115 RNAi bacteria. RNAi clones were taken from the Ahringer library, unless otherwise noted. For custom-made RNAi clones, fragments of the targeted genes were amplified from genomic DNA using the primer sequences given in Table 6.2, which also added NheI to the 5’ end and HindIII (for nhr-112, nhr-114 and nhr-273) or XmaI (for nhr-28 and nhr-138) to the 3’ end. Gel purified PCR fragments were TOPO-cloned into pCR®-BluntII-TOPO® vectors using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen 45-0245) according to manufacturer’s protocol and sequenced. Sequence-verified clones were then restriction digested using NheI and HindIII/XmaI as appropriate, excised fragments gel purified, and ligated into the pL4440 RNAi vector. After ligation, plasmids were transformed into competent HT115 *E. coli* cells and cultured overnight at 37°C on Luria-Bertani media (10g/L bacto-tryptone, 5g/L yeast extract, 10g/L NaCl) containing 100 μg/mL ampicillin.

Sodium meta-arsenite (Sigma 71287), tBOOH (Sigma 458139), oleic acid (S-1120), linoleic acid (S-1127) and eicosapentanoic acid (S-1144) (Nu-Chek Prep) were added at
indicated concentrations. tBOOH-containing plates were made two days prior to each experiment (Chapter 2) or on the day of each experiment (Chapter 3).

For synchronized worm growths, populations of gravid adults were treated with 30% household bleach containing 70 mM KOH to release the embryos. Bleached embryos were hatched overnight on unseeded NGM-lite plates; hatched, synchronized L1 larvae were then grown to the desired stage, as indicated, and growths were adapted to ensure developmental synchronicity of slow-growing mutants. For the fmo-2p::GFP RNAi screen (Chapter 3), synchronized fmo-2p::GFP worms L1s were transferred to RNAi plates that had been seeded twice with the relevant RNAi clone and allowed to grow to the young adult stage. 10-15 transgenic worms (as assessed by the Roller transgenic marker) were transferred to NGM-lite plates containing 10 mM tBOOH for 3 hours. Fluorescence was assessed using a Leica M205 FA fluorescence stereo microscope. To harvest worms for gene expression analysis by qPCR in oxidative stress or fasting, worms were grown to the late L4 stage, as assessed by vulval morphology, and then transferred to either plates containing 7.5 mM tBOOH (Sigma 458139) or 5 mM sodium meta-arsenite (Sigma 71287) for 4 hours, or unseeded NGM-lite plates for 8 hours, as appropriate. Oxidant concentrations were chosen based on (Oliveira et al., 2009).

Table 6.1 List of C. elegans strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>(Brenner, 1974)</td>
</tr>
<tr>
<td>STE68 nhr-49(nr2041) I</td>
<td>(Van Gilst et al., 2005b)</td>
</tr>
<tr>
<td>RB1592 nhr-64(ok1957) I</td>
<td>(Liang et al., 2010)</td>
</tr>
<tr>
<td>wdr-23(tm1817) I</td>
<td>(Choe et al., 2009)</td>
</tr>
<tr>
<td>XA7702 mdt-15(tm2182) III</td>
<td>(Taubert et al., 2008)</td>
</tr>
<tr>
<td>BX156 fat-6(tm331) IV; fat-7(wa36) V</td>
<td>(Brock et al., 2006)</td>
</tr>
<tr>
<td>Strain</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>LD1171 (ldIs3[gcsl-1p::gfp + pRF4(rol-6(su1006))])</td>
<td>(Wang et al., 2010)</td>
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<tr>
<td>CL2166 (dvs19[PAF15(gsr-4::gfp::nls)])</td>
<td>(Leiers et al., 2003)</td>
</tr>
<tr>
<td>TJ356 (IsDAF-16::GFP)</td>
<td>(Henderson and Johnson, 2001)</td>
</tr>
<tr>
<td>LD1 (ldIs7[skn-1B/C::GFP + pRF4(rol-6(su1006))])</td>
<td>(An and Blackwell, 2003)</td>
</tr>
<tr>
<td>EB271 fmo-2p::GFP</td>
<td>J. Winter and E. Veal, unpublished</td>
</tr>
<tr>
<td>STE108 nhr-49(et7)</td>
<td>(Lee et al., 2016)</td>
</tr>
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<td>STE109 nhr-49(et8)</td>
<td>(Lee et al., 2016)</td>
</tr>
<tr>
<td>STE110 nhr-49(et13)</td>
<td>(Lee et al., 2016)</td>
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<td>nhr-49(et13); fmo-2p::GFP</td>
<td>This study</td>
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<td>STE71 nhr-13(gk796)</td>
<td>(Pathare et al., 2012)</td>
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<td>STE69 nhr-66(Ok940)</td>
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<td>STE70 nhr-80(tm1011)</td>
<td>(Goudeau et al., 2011)</td>
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<tr>
<td>VC1668 fmo-2(Ok2147)</td>
<td>(Leiser et al., 2015)</td>
</tr>
<tr>
<td>JIN1375 hih-30(tm1978)</td>
<td>(Lapiere et al., 2013)</td>
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<tr>
<td>MAH235 sqIs19[hlh-30p::hlh-30::GFP+rol-6(su1006)]</td>
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<td>sqIs19; nhr-49(nr2041)</td>
<td>This study</td>
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<tr>
<td>nhr-49(et13); hih-30(tm1978)</td>
<td>This study</td>
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<tr>
<td>KAE9 eft-3p::fmo-2::GFP</td>
<td>(Leiser et al., 2015)</td>
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<td>CF1903 glp-1(e2141)</td>
<td>(Arantes-Oliveira et al., 2002)</td>
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<tr>
<td>AGP22 glp-1(e2141); nhr-49(nr2041)</td>
<td>(Ratnappan et al., 2014)</td>
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</tbody>
</table>

Table 6.2 Sources of RNAi clones used.

For further details on the Ahringer RNAi library, see (Kamath et al., 2003). The nhr-12 RNAi clone was taken from the Vidal library (Rual et al., 2004).
<table>
<thead>
<tr>
<th>RNAi condition</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>nhr-49</td>
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<td>nhr-64</td>
<td>Ahringer library, Plate 2 Well G07</td>
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<tr>
<td>nhr-69</td>
<td>Ahringer library, Plate 16 Well G09</td>
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<td>nhr-86</td>
<td>(Arda et al., 2010)</td>
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<td>nhr-97</td>
<td>Ahringer library, Plate 108 Well C06</td>
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<td>nhr-112</td>
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<td>nhr-114</td>
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<tr>
<td>nhr-138</td>
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<td>nhr-273</td>
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<td>Ahringer library, Plate 179 Well A10</td>
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<td>hlh-8</td>
<td>Ahringer library, Plate 188 Well G02</td>
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<td>ztf-2</td>
<td>Ahringer library, Plate 11 Well E03</td>
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<tr>
<td>sbp-1</td>
<td>Ahringer library, Plate 86 Well B01</td>
</tr>
</tbody>
</table>

RNA isolation and quantitative PCR analysis

To isolate total *C. elegans* RNA, worm pellets were harvested and flash frozen, followed by addition of Trizol and sonication (Fisher Scientific Sonic Dismembrator Model 500) to increase RNA yield. Nucleic acids were then extracted using 1-bromo-3-chloropropane (Acros Organics 106862500) and precipitated by isopropanol. RNA was cleaned up using the RNeasy Mini kit (Qiagen 74106). 2 µg RNA was then reverse transcribed to generate cDNA using SuperScript III Reverse Transcriptase (Invitrogen 18080-044), random primers (Invitrogen 58875), 0.1 M DTT, dNTPs (Fermentas R0186) and RNaseOUT (Invitrogen 100000840).
qPCR was performed in a 96-well plate format. Primers (custom-made from IDT) were diluted with RNase/DNase-free water to a final concentration of 5 μM and tested prior to use on serial dilutions of *C. elegans* cDNA. To perform qPCR, in Chapter 2, a 30 μl reaction containing PCR buffer (final concentration 20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.67 mM MgCl₂, 0.02 μg cDNA, 0.15 mM dNTPs (Fermentas R0186), 0.2 μl ROX reference dye (Invitrogen 12223-012), 0.2 μl Taq (5U/μl) (Invitrogen 18038-042), and 0.04 μl SYBR Green I (100x) (Invitrogen S-7567) was added to each well. In Chapter 3, 1 μl of cDNA (diluted 1:10), 3 μl RNase/DNase-free water, and 5 μl FAST SYBR master mix (Applied Biosystems 4385612) was added to each well. All reactions were performed in triplicate on an Applied Biosystems StepOnePlus machine. Data were analyzed with the ΔΔCt method. For each sample, normalization factors were calculated by averaging the (sample expression)/(reference expression) ratios of three or four normalization genes, as indicated in the legends (Chapter 2), or *act-1*, *tba-1*, and *ubc-2* (Chapter 3); the reference sample was *control*(RNAi), WT, or untreated, as appropriate. Student’s t-test/non-parametric Mann-Whitney test was used to calculate the statistical significance of gene expression changes. Where appropriate, the Holm-Sidak method was used to correct for multiple comparisons (GraphPad Prism 7). Primers were tested on serial cDNA dilutions and analyzed for PCR efficiency (sequences in Table 6.3).

### Table 6.3 List of qPCR primers used in this chapter.

<table>
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<th>Forward primer</th>
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<td><em>ptps-1</em></td>
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<td>Gene</td>
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<td>Reverse primer</td>
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<td>daf-16</td>
<td>AACGTTCATCATCTTCCCG</td>
<td>GTTGCATCGATACAGCATTG</td>
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**DIC and fluorescence microscopy**

Worms were transferred onto 2% (w/v) agarose pads containing 10 mM sodium azide for microscopy. Images were captured on a CoolSnap HQ camera (Photometrics) attached to a Zeiss Axioplan 2 compound microscope, and MetaMorph Imaging Software with Autoquant 3D digital deconvolution was used for image acquisition.
**Microarray overlap analysis**

Overlaps in microarray datasets were identified using the ISNA(MATCH(lookup_value, lookup_array, [match_type])) function Microsoft Excel 2011. Statistical significance was calculated using Fisher’s exact test from the R Stats Package in RStudio version 0.98.

**Yeast-two-hybrid assays and immunoblots**

Yeast strains carrying the plasmids of interest were grown overnight in selective YPD media. 10 μl of each culture was transferred per well into a 96 well plate. 10 μl of permeabilization solution (40% 5x Z-buffer (0.3 M Na₂HPO₄*7H₂O, 0.3 M NaH₂PO₄*H₂O, 50 mM KCl, 5 mM MgSO₄*7H₂O), 5% CHAPS (Sigma 3023), 0.003% β-mercaptoethanol) was added to each well and incubated at room temperature for 5-15 minutes. 180 μl of pre-warmed substrate solution (20% 5x Z-buffer, 0.5 mM CPRG (Boehringer Mannheim 884308), 0.001% β-mercaptoethanol) was then added to each well and β-galactosidase activity was measured every 2 minutes for 60 minutes using an OmegaStar plate reader (BMG Labtech). β-galactosidase activity (OD₅₈₀) was normalized to yeast concentration (OD₆₆₀). Each assay included at least four technical replicates and was repeated three or more times. Yeast protein was extracted using a trichloroacetic acid extraction (Foiani et al., 1994) and Western blots to detect protein expression in yeast were done as described (Taubert et al., 2006) using an anti-Myc antibody (Santa Cruz sc-40).
**Oxidative stress resistance assays**

Worms were grown to the late L4 stage, as assessed by vulval morphology, and then transferred to normal or oxidant-containing plates. Dead and surviving worms were quantified twice a day for 96 hours or until all worms were dead, whichever came first. Death was assessed by prodding the animals with a platinum wire. GraphPad Prism 5 (Chapter 2) or GraphPad Prism 6 (Chapter 3) was used to generate survival curves and calculate statistical significance using the Log-rank (Mantel-Cox) test.

**Starvation resistance assays**

Worms were maintained for 2 generations in a non-starved state, bleached to collect embryos, and hatched overnight at 20°C on a rotator in S-basal medium (100 mM NaCl, 5 mM K2HPO4, 44 mM KH2PO4). Synchronized L1s were then transferred to NGM-lite plates containing OP50, allowed to grow to adulthood, and then bleached again. The embryos were allowed to hatch in the same way as the previous generation and then maintained at a concentration of 1-2 worms per μl of S-basal containing an antibiotic/antimycotic mix (Gibco 15240062) on a rotator. To assess viability, 300 μl aliquots of each genotype were taken every 2-3 days and transferred to NGM-lite plates. After 48 hours (72 hours for nhr-49(et13), to account for slow growth), the number of worms that reached the L4 stage versus those that did not were counted. The assay was stopped when none of the worms were able to reach the L4 stage after 48/72 hours.
Bibliography


Andersen, J.K. (2004). Oxidative stress in neurodegeneration: cause or consequence? , Published Online: 01 July 2004; | Doi:10.1038/Nrn1434 10, S18–S25.


Cullinan, S.B., Gordan, J.D., Jin, J., Harper, J.W., and Diehl, J.A. (2004). The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by
a Cul3-Keap1 ligase. Molecular and Cellular Biology 24, 8477–8486.


Dupuy, D., Bertin, N., Hidalgo, C.A., Venkatesan, K., Tu, D., Lee, D., Rosenberg, J.,


DNA polymerase alpha-primase complex in Saccharomyces cerevisiae executes an essential function at the initial stage of DNA replication. Molecular and Cellular Biology 14, 923–933.


Goh, G.Y.S., Martelli, K.L., Parhar, K.S., Kwong, A.W.L., Wong, M.A., Mah, A., Hou,


Schriner, S.E., Linford, N.J., Martin, G.M., Treuting, P., Ogburn, C.E., Emond, M., Coskun,


Sluder, A.E., Mathews, S.W., Hough, D., Yin, V.P., and Maina, C.V. (1999). The Nuclear Receptor Superfamily Has Undergone Extensive Proliferation and Diversification in Nematodes. Genome Res. 9, 103–120.


Neurochem. 71, 95–105.


Preimplantation Mouse Development. Molecular and Cellular Biology 27, 6177–6182.


Redox-Regulated Substrate Adaptor Protein for a Cul3-Dependent Ubiquitin Ligase Complex. Molecular and Cellular Biology 24, 10941–10953.


Zhang, Y., Crouch, D.H., Yamamoto, M., and Hayes, J.D. (2006). Negative regulation of the Nrf1 transcription factor by its N-terminal domain is independent of Keap1: Nrf1, but not Nrf2, is targeted to the endoplasmic reticulum. Biochem. J. 399, 373–385.


### Appendix

#### Appendix A

Supplementary data from Chapter 3.

**A.1 Detailed results of the RNAi screen for regulators of fmo-2.**

Results for individual repeats of the RNAi screen to identify transcriptional regulators of the *fmo-2p::GFP* reporter (see Section 3.3.1).

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<th>Reference</th>
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<th>Repeat 2</th>
<th>Repeat 3</th>
<th>Verified with Zeiss Axioplan 2 microscope</th>
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<td>-tBOOH</td>
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Fluorescence in -tBOOH condition very weak for Rpt 2, likely autofluorescence

No fluorescence in all 3 repeats
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### A.2 List of tBOOH response genes tested for dependence on *nhr-49*.

Full list of tBOOH response genes tested by qPCR for dependence on *nhr-49* (see Section 3.3.2). Values in green are statistically significant compared to N2 - tBOOH or N2 +tBOOH conditions, as indicated. *p*-values were calculated using unpaired Student’s t-test.

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