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## THE THIOREDOXIN TRX-1 REGULATES THE MAJOR OXIDATIVE STRESS RESPONSE TRANSCRIPTION FACTOR, SKN-1, IN CAENORHABDITIS ELEGANS

Katie C. McCallum

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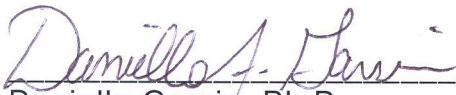
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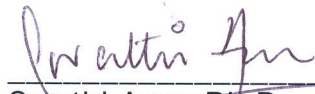
by

*Katie Carol McCallum, B.S.*

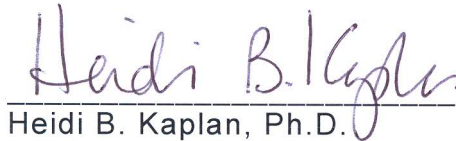
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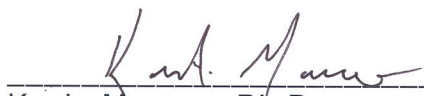
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A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
MD Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Katie Carol McCallum, B.S.  
Houston, Texas

May 2016

## **Dedication**

To my parents, Paul and Kathryn McCallum, who selflessly built the foundation  
for all that I am.

“If I have seen further than others, it is by standing upon the shoulders of giants.”

-Isaac Newton

## **Acknowledgements**

Pursuing my PhD has undoubtedly been the most difficult task I have undertaken, but the professional and personal growth I have attained during this challenging time will shape my future enormously. The work presented herein was conducted with the support, guidance, and mentorship of numerous individuals.

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Danielle fosters a uniquely motivating environment in her research lab, in which both independent critical thinking and collaborative teamwork are encouraged while exercising our scientific discipline. This environment was critical in my early years as a graduate student, as it placed me in the footsteps of Dr. Ransome van der Hoeven and Dr. Sruti DebRoy. These two individuals introduced me to numerous laboratory and scientific concepts and guided me through the early trenches of bench science (and only mildly picked on me for silly mistakes). I would especially like to thank Ransome, who has been an incredible scientific mentor, personal cheerleader, and friend. I sincerely hope the scientific community continues to appreciate your unique ability to both practice and teach science.

I would also like to thank the Microbiology and Molecular Genetics department as a whole, which has been a remarkable space to matriculate through. The collective atmosphere of this program is indescribable and has truly been a privilege to experience. At this time, I would also like to thank my fellow graduate students. Specifically, I would like to thank Jennifer Abrams, Kim Busiek, Veronica Garcia, Veronica Rowlett, Alex Marshall, Chris Evans, Elisa Vesely, and Robert Williams. Each one of you has impacted and motivated my research and will forever hold a very special place in my mind. A very sincere thank you to Elisa Vesely for editing my dissertation; you truly have a gift.

Although there is typically a sarcastic undertone to our dialogue, I would like to take a line or two to offer my sincere thanks to Dr. Kevin Morano. He has not only encouraged my growth as a scientist but also as a human; and has continuously demonstrated a genuine interest in my future, for which I am incredibly thankful. Additionally, on his own accord, Kevin graciously dragged me through the 5°F January winds of Evanston, Illinois, so that I could broaden my scientific breadth and network at a scientific meeting at Northwestern University. This is also where I tasted Moose Drool for the first, and only, time.

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lab bench, and for your helpful ideas and advice that navigated me through the intricacies of my thesis project.

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Katie Carol McCallum, B.S.

Advisory Professor: Danielle Garsin, Ph.D.

The ability to respond to hostile environmental conditions is critical for the survival of an organism. Oxidative stress is an adverse state in which reactive oxygen species (ROS) accumulate to a harmful level and, if left unresolved, can lead to cellular dysfunction and organismal disease. Sophisticated detoxification systems, characterized by a battery of enzymatic antioxidants, are utilized to neutralize ROS thereby reducing stress. However, ROS are also purposefully produced by designated cellular enzymes to facilitate the signaling and regulation of critical physiological processes. Therefore, both the production and neutralization of ROS must be tightly controlled. Indeed, the expression of detoxification enzymes is regulated by major oxidative stress response transcription factors, such as Nrf2 and SKN-1 in mammals and the model organism *Caenorhabditis elegans*, respectively. The activity of both Nrf2 and SKN-1 is highly regulated and many conserved mechanisms are used to facilitate proper control of these two transcription factors, making *C. elegans* a powerful tool in which to study the complex regulation of the major oxidative stress response transcription factors.

Herein, we uncover a novel mechanism of SKN-1 regulation, in which a thioredoxin, TRX-1, negatively impacts the intestinal subcellular localization of



this transcription factor in a cell non-autonomous manner from the ASJ neurons. This function of TRX-1 is specific, as SKN-1 regulation is not a common role for other *C. elegans* thioredoxins. Moreover, SKN-1 regulation is a redox-independent function of TRX-1 and does not impact transcriptional activation or previously characterized SKN-1-dependent protective responses, such as the oxidative or pathogen stress responses. Thioredoxins are understudied in *C. elegans*, but play an important role in worm lifespan. RNA Seq was used as an unbiased approach to determine potential physiological role(s) of TRX-1. Uncovered associations include changes in the expression of genes involved in collagen biosynthesis and lipid metabolism. In summary, TRX-1 regulates SKN-1 in a manner which dissects subcellular localization and transcriptional activation, emphasizing the importance of strict regulation of SKN-1 activation.

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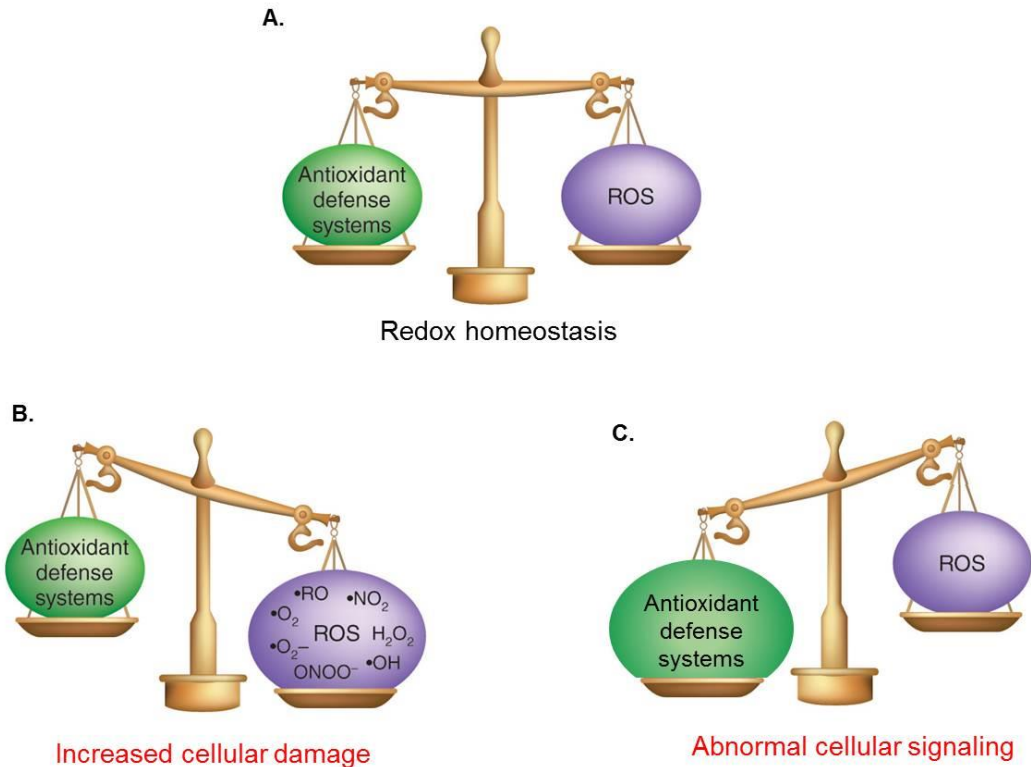
## **Chapter 1: Background and Significance**

## Reactive Oxygen Species: A Physiological Paradox

Reactive Oxygen Species (ROS) are reactive molecules that have the potential to elicit extreme biological damage, yet they are required for the maintenance of proper cellular function (63). For instance, excess ROS can lead to several disease states and are an underlying pathology of a majority of neurodegenerative diseases (111). Conversely, ROS are required to mediate critical signaling pathways, such as those coordinating development and immunity (9, 140, 157, 213, 229). The contrasting physiological and pathological outcomes that ROS are capable of eliciting highlight the paradoxical nature of these molecules, in which both their existence and elimination can be detrimental to an organism. Ultimately, this necessitates the maintenance of physiologically appropriate levels of ROS, termed redox homeostasis (Figure 1.1) (181).

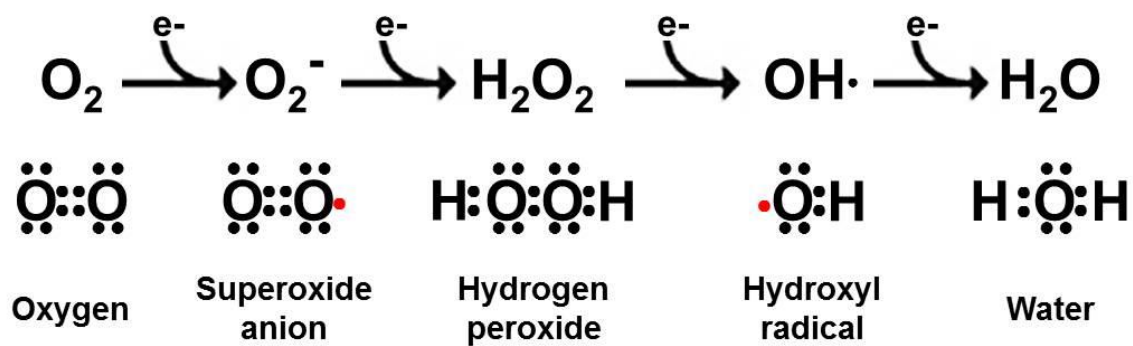
Specifically, ROS are partially reduced derivatives of molecular oxygen that include radical species, such as superoxide anion ( $O_2^-$ ) and hydroxyl radical ( $HO\cdot$ ), and nonradical species, such as hydrogen peroxide ( $H_2O_2$ ) (80). The complete reduction of oxygen requires the addition of four electrons and results in the production of water (Figure 1.2). The incomplete reduction of oxygen, however, results in biologically reactive species, which are characterized by either unpaired electrons or highly unstable oxygen-oxygen chemical bonds, both of which are capable of causing oxidative modification of cellular components. At physiologically relevant levels of ROS, reversible oxidative modification of biological molecules is regulated and can even be required for the function of essential proteins (41, 63, 166). However, if allowed to accumulate, ROS-induced oxidative modification can occur non-specifically, leading to irreversible biological damage.

The free radical superoxide anion is a moderate oxidant and considered to be the progenitor species of reactive oxygen, as it is generated upon the addition of a single



**Figure 1.1: Redox homeostasis is governed by the balance of ROS and antioxidants.**

**A)** Balanced levels of both antioxidant defense systems and ROS results in proper cellular redox homeostasis. **B)** ROS accumulation is a consequence of increased production of ROS (or decreased levels of antioxidant defense systems), resulting in increased cellular damage. **C)** Overproduction of antioxidants (or decreased production of ROS) results in reduced ROS-dependent cellular signaling. Both the increased cellular damage and reduced cellular signaling that results from imbalance of redox homeostasis results in cellular dysfunction and organismal disease. This schematic was reproduced and adapted from: Tomaselli, G. F., and A. S. Barth. 2010. Sudden cardio arrest: oxidative stress irritates the heart. *Nature Medicine* 16:648-649 (207). Permission to use this figure was granted by Copyright Clearance Center (License #: 3833640898208).



**Figure 1.2: Stepwise reduction of molecular oxygen.**

Molecular oxygen ( $\text{O}_2$ ) requires the addition of four electrons ( $\text{e}^-$ ) to reach its fully reduced form, water. Note: This is not a balanced reaction and the addition of protons is not shown.

Free electrons denoted in red.

electron to molecular oxygen and therefore the primary intermediate that gives rise to more potent forms of ROS (37, 149, 215). As a relatively unstable species, superoxide anion rapidly dismutates, either spontaneously or enzymatically, to produce the fairly stable and abundant hydrogen peroxide species, which plays critical roles as both a signaling molecule and antimicrobial (45, 63, 78). While hydrogen peroxide is a biologically poor oxidant, it can react with metals, such as iron, or excess superoxide ion to elicit the generation of hydroxyl radical through the Fenton and Haber-Weiss reactions, respectively (166). The hydroxyl radical is extremely reactive, short-lived, and capable of modifying most biological components, eliciting extensive cellular damage.

ROS can be generated endogenously, either as a byproduct of enzymatic function or purposefully by dedicated enzymes, or as a consequence of exposure to xenobiotic compounds. For most cell types, a majority of endogenously generated ROS are produced in the mitochondria as an inadvertent consequence of aerobic respiration, a vital process by which cellular energy is produced (16, 108, 149). ROS are generated during the final step of aerobic respiration, which utilizes the electron transport chain to drive a proton gradient required for ATP production. In this process, molecular oxygen is used as the final electron acceptor of the electron transport chain, generating water. However, the complete reduction of  $O_2$  requires the addition of four electrons through separate reactions, which occasionally results in the partially reduced, reactive forms of oxygen (80, 149, 215). Given the necessity for all aerobic organisms to produce energy in this way, generation of ROS byproducts is continuous and unavoidable. Other inadvertent sources of endogenous ROS as a byproduct of enzymatic function include xanthine oxidase, lipoxygenases, cytochrome P450, and cyclooxygenases (78, 120). ROS production can also occur in response to exogenous insult, such as exposure to xenobiotics or ionizing radiation (108, 144, 230). For instance, arsenic is a potent ROS-inducing xenobiotic that can be consumed as a result of agriculture

being associated with contaminated soil and groundwater (227). Arsenic elicits toxicity by reacting with the thiol groups of redox buffering proteins, altering cellular signaling pathways, and increasing cellular heavy metal levels, all of which cause increased ROS (64, 92). Regardless of their origin, their extreme reactivity and potential for eliciting extensive damage had previously encouraged the belief that ROS production was synonymous with adverse cellular and physiological conditions. However, it is now appreciated that both free radical and non-radical species of oxygen, namely superoxide anion and hydrogen peroxide, respectively, are purposefully produced and play crucial physiological roles that are required for a diverse array of cellular processes (37, 63, 78).

The ROS that are generated as byproducts of enzymatic function are not the sole contributors to redox homeostasis. Rather, enzymes dedicated to the 'professional' production of superoxide anion significantly contribute to the ROS pool of an organism. NADPH oxidases (NOXs) are enzyme complexes that reduce molecular oxygen to superoxide via the transport of electrons from NADPH across a membrane. These enzymes are stimulated to produce ROS in response to a variety of specific factors, such as growth factors, hormones, and inflammatory mediators; examples of which are epidermal growth factor, insulin, and tumor necrosis factor, respectively (103). NOX2 was the first enzyme of this family to be characterized, and its beneficial role in pathogen elimination will be discussed later (10). The NOX family has been expanded to include four other NOXs and two Dual oxidases (DUOXs), all of which share the common function of ROS generation (162). These enzymes exhibit varying tissue distributions and physiological outputs, underscoring the broad impact ROS production has on varying organismal functions. For instance, NOXs and DUOXs have been shown to impact blood pressure regulation, renal function and absorption, remodeling of airway vasculature, and serotonin biosynthesis in an ROS-dependent manner (11, 12, 56, 70, 110).

## **The Biological Costs of ROS Accumulation**

Oxidative stress is the adverse cellular state that is characterized by the sustained accumulation of ROS. Once generated and regardless of their origin, if ROS are allowed to accumulate they can be promiscuous and potent oxidizers of major cellular macromolecules, resulting in DNA mutagenesis, lipid peroxidation, and protein damage/dysfunction (45, 80, 95). Both genomic DNA and the cellular nucleotide pool are susceptible to oxidative damage. Specifically, ROS-induced damage of DNA is characterized by the formation of abasic sites, single and double strand DNA breaks, aberrant base modification, and inappropriate DNA-protein crosslinking (40, 102). Moreover, RNA is also susceptible to ROS attack and is more susceptible to ROS than DNA as it lacks the dedicated repair pathways afforded to rectifying DNA damage (115). ROS can also react with lipids, resulting in lipid peroxidation. This peroxidation causes loss of membrane function, changes in membrane fluidity, dysfunction of membrane-anchored proteins (including transporters, receptors, and enzymes), and production of potent lipid peroxyl radicals, such as malondialdehyde (MDA) and 4-hydroxynonenol (4-HNE) (15, 23, 138). Lastly, proteins are the most common cellular targets of ROS, accounting for around 70% of the oxidative modifications that occur in the cell (41). Accumulation of ROS results in the indiscriminate oxidation of amino acid residues, generating a slew of altered amino acid products which adversely affect protein function (28, 44, 48). Overall, the ROS-induced damage of DNA, lipids, and proteins during states of unresolved oxidative stress leads to extensive disruption of cellular function and results in adverse physiological conditions.

Although the cellular implications of ROS accumulation are substantial, the diverse organismal consequences highlight the indiscriminate biological damage these molecules inflict. ROS accumulation is an underlying hallmark of a range of diseases, including numerous neurodegenerative diseases, cancers, atherosclerosis, obesity, aging, and Type-



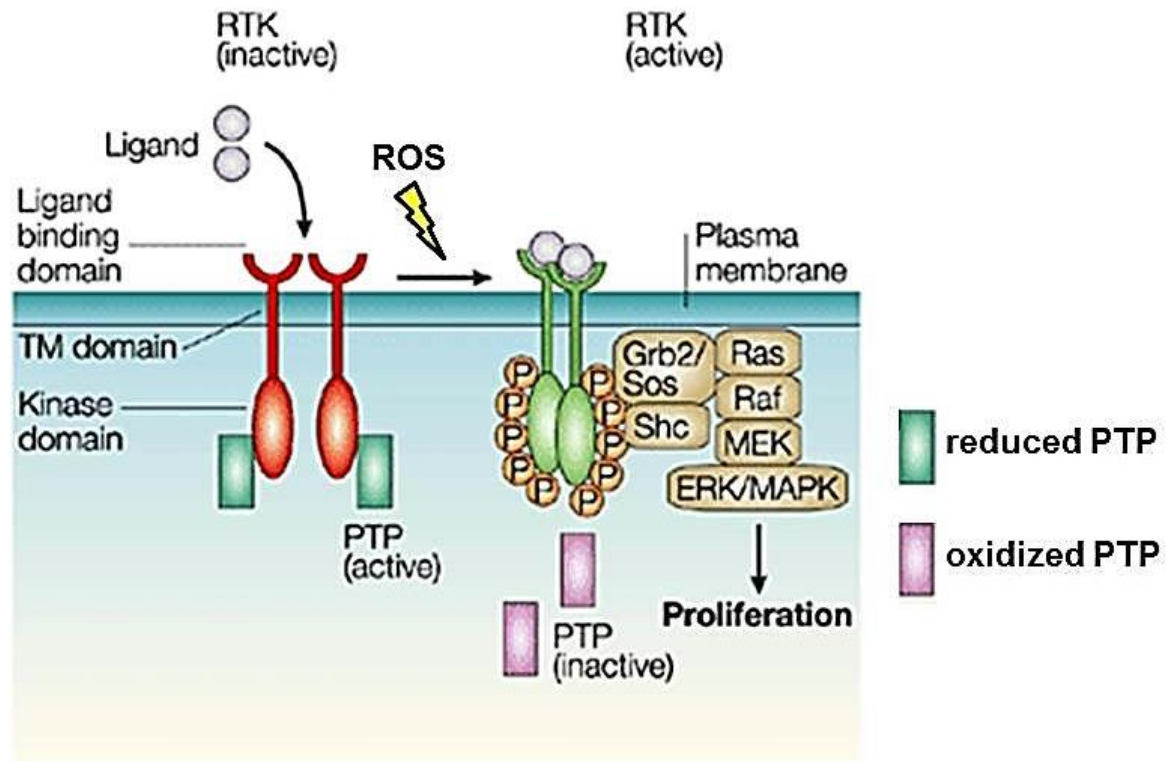
2 diabetes (47, 51, 217). As an example, the accumulation of ROS is representative of several chronic neurodegenerative diseases, including Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), and amyotrophic lateral sclerosis (ALS) (186). The prevalence of oxidant-associated pathologies of the brain can, in part, be attributed to the increased levels of ROS the brain encounters as a result of the increased oxygen consumption needed to power the central nervous system. The abundance of lipids sensitive to ROS, particularly polyunsaturated fatty acids, in neuronal cell membranes and the lack of a robust antioxidant defense system throughout the central nervous system also contribute to the prevalence of oxidant-associated pathologies underlying neurodegenerative diseases (216).

### **The Biological Benefits of ROS**

The toxic effects of ROS accumulation are severe, yet organisms require ROS to survive. The beneficial roles ROS play in vital physiological processes serve as the crux for understanding this biological paradox. Far less-studied than its reactive, 'darker' side, the positive impacts that ROS have on biological processes are essential and range from immunity to development (9, 157). Probably the most well understood and direct benefit of ROS is in immune defense. As mentioned previously, NOX2 is a NADPH oxidase that generates ROS as a mechanism for pathogen elimination. Specifically, neutrophils engulf pathogens into isolated compartments, called phagosomes, and utilize NOX2-generated ROS to destroy the pathogen. Neutrophils also employ NOX2-generated ROS to attack pathogens that are too large to be engulfed, such as hypha-forming yeast pathogens (55). Accordingly, Chronic Granulomatous Disease (CGD), a disorder characterized by defective NOX enzymes, and therefore a lack of ROS generation in immune cells, renders patients highly susceptible to pathogen infection. Currently, no permanent treatment for CGD exists and patients are given lifelong antibiotics and antifungals to prevent life-threatening infection

(38). While the purposeful utilization of ROS to eliminate pathogens was postulated over forty years ago, it would take twenty-two years before ROS would garner attention as critical signaling molecules capable of coordinating physiological functions, termed redox signaling (9, 176, 199). A somewhat more trivial indicator of the growing importance of redox-dependent signaling can be seen in a simple literature survey, in which the few, broad scientific reviews of ROS-dependent signaling (circa 1995) have now shifted to an expansive number of detailed review articles illuminating the intricacies and importance of redox signaling in a single cell type, such as endothelial cells (164, 176).

ROS are able to impact a diverse array of biological processes as a result of their signaling capacity (41, 78). Specifically, reversible oxidation of thiol-containing target proteins is the primary mechanism by which redox changes are utilized to modulate signal transduction pathways, and is the foundation of redox signaling (166, 177). In this way, ROS signaling molecules and redox signaling pathways modulate a variety of target molecules, including enzymes, receptors, kinases, transcription factors, and proteases. This modulation can influence wide-ranging cellular processes such as growth, autophagy, apoptosis, aging, and proliferation (175). For instance, activation of MAPK pathways (examples being the ERK and JNK pathways), is regulated by redox-sensitive sensor proteins such as protein tyrosine phosphatases (PTPs) (Figure 1.3) (41). Briefly, ROS-dependent oxidation of the cysteine residues inactivates the ability of PTPs to inhibit MAPK signaling activation, which results in the initiation of MAPK signaling and concurrent activation of a variety of physiological responses. The oxidative modification of PTPs is reversible, whereby reduction of oxidized PTPs restores the ability of these enzymes to inhibit kinase activity. In this way, redox signaling serves as a reversible mechanism of modulating kinase signaling activation in an ROS-dependent manner (208).



**Figure 1.3: The reversible oxidation of PTPs modulates the activity of ERK/MAPK-dependent activation of proliferation.**

Receptor tyrosine kinase (RTK) activation is inhibited by 'active' (or reduced) PTPs. ROS-induced oxidation of PTPs leads to their dissociation from RTKs and results in ERK/MAPK-dependent activation of proliferation. This schematic was reproduced and adapted from: Wetzker, R., and F. D. Bohmer. 2003. Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nature Reviews* 4:651-657 (225). Permission to use this figure was granted by Copyright Clearance Center (License #: 3833891186636).

## **Cysteine: The Redox Sensitive Amino Acid**

As mentioned above, oxidative modification of amino acid residues is required to modulate the activity of regulatory proteins, highlighting the mechanism by which ROS impacts diverse physiological processes. Cysteine is the most physiologically relevant redox sensitive amino acid residue, due to its thiol functional group, and is the primary amino acid residue that accounts for the overall redox sensitivity of a protein (169). However, both the surface exposure of a cysteine and the surrounding amino acids greatly impact its reactivity. The location of cysteine within the folded structure of a protein dictates its redox sensitivity, as only solvent-exposed amino acids are susceptible to oxidative modification (166). Interestingly, surface exposed cysteines are not inherently redox reactive and rely on surrounding amino acids to increase their susceptibility to oxidative modification. This can be attributed to the high pKa of cysteine (approximately 8.2), which results in its sulfur-containing functional group existing in the less reactive, protonated thiol state at physiological pH. Surrounding amino acids, such as positively charged amino acids, and folding structures can greatly reduce the pKa of a particular cysteine to around 3 or 4. This reduction in pKa allows the thiol groups of specific cysteines to exist in their deprotonated thiolate anion form, increasing their redox sensitivity dramatically and resulting in residues that are more readily modified by ROS (41, 166).

The most functionally and physiologically relevant result of the oxidation of cysteine's thiolate functional group is the formation of reversible disulfide bonds in proteins. Upon oxidation, the thiolate group of cysteine is transformed into a sulfenic acid intermediate, which then readily reacts with neighboring thiols to generate a reversible disulfide bond between two cysteines. Disulfide bonds can be formed intra- or inter-molecularly and greatly impact the structure and stability of proteins (43, 166, 169). For instance, disulfide bonds often influence the structure of proteins, such that hydrophobic residues are buried within

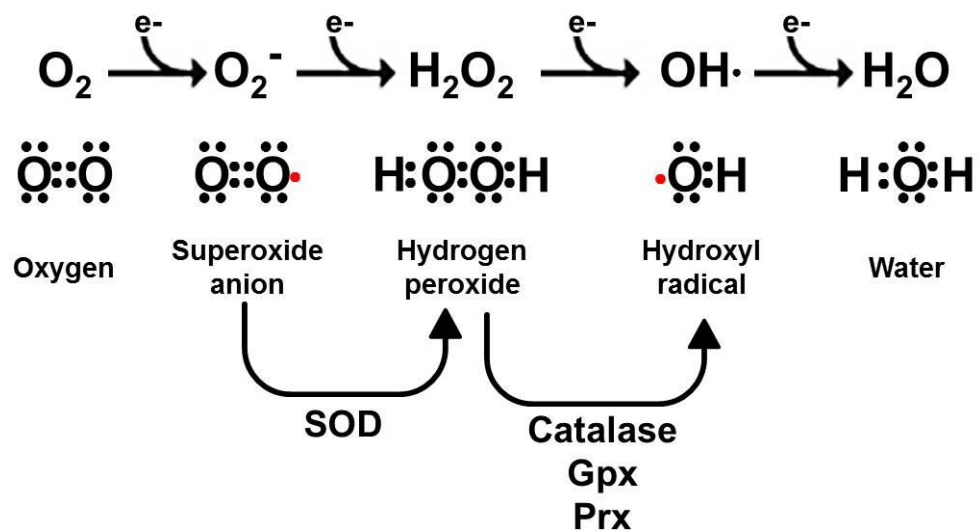
the interior core of a folded protein. If a protein requires disulfide bond formation to achieve a folded state, the lack of disulfide bond formation can result in a dysfunctional protein (14). Therefore, some proteins require oxidative-dependent disulfide bond formation to result in a properly folded, functional protein. While disulfide bond formation is required for the structural stability of specific proteins, another intriguing property of disulfide bond formation relies on the dynamic and reversible nature of this covalent modification (43, 166). Given the dramatic impact disulfide bond formation can have on the structure of proteins, and the reversible nature of this modification, the function of some proteins is impacted by the oxidation status of its cysteines. Unlike proteins that require disulfide bond formation for structural integrity, these redox sensitive proteins require reversible oxidative modification to transiently change their folding status in a way that impacts their cellular function (130). For example, the oxidation of PTP cysteines results in the formation of an intramolecular disulfide bond that changes the conformation of the protein thereby resulting in an altered function (41). In summary, whether disulfide bond formation serves to permanently stabilize the folding structure or transiently affect the activity of a protein, disulfide bond formation is of great physiological impact and highlights the importance of oxidative modification as a mechanism of redox signaling.

### **The Dual Role of Antioxidants: Detoxification and Reversible Modification**

As their name implies, antioxidants are the antithesis to oxidants such as ROS. Given that ROS impose both biological consequences and benefits, it is not surprising that antioxidants also exhibit dual functions. During periods of excess ROS accumulation, antioxidants serve to neutralize ROS and repair inadvertently-oxidized molecules, thereby restoring redox homeostasis. The loss of antioxidant activity is an underlying factor in the progression of numerous diseases, underscoring the importance of antioxidant-dependent detoxification. For instance, dysfunction of a critical antioxidant, SOD1, contributes to the

manifestation of late-onset ALS (105). Conversely, antioxidants are essential components of redox signaling, as oxidized signaling targets and redox regulators must be reduced, or reversibly modified, in order to inactivate redox-dependent signaling outcomes and restore redox signaling capacity, respectively.

Detoxification results in ROS being neutralized to non-reactive products. Numerous enzymatic antioxidants, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and peroxiredoxins are employed to neutralize ROS (Figure 1.4). This is also true for non-enzymatic antioxidants, such as vitamins C and E, carotenoids, and flavonoids (80, 120, 192, 210). SOD and catalase work in concert to detoxify excess superoxide anion. First, two molecules of superoxide anion are converted into hydrogen peroxide and molecular oxygen by SOD. This is then followed by a second reaction in which the toxic hydrogen peroxide intermediate is further dismutated into oxygen and water by catalase. Hydrogen peroxide can also be reduced via glutathione peroxidase, using glutathione as a substrate. Peroxiredoxins catalyze the reduction of hydrogen peroxide and its reactive hydroperoxide derivatives, such as alkyl hydroperoxides, using thioredoxin as an electron donor. Furthermore, glutathione S-transferases conjugate glutathione to reactive groups of endogenous or exogenous oxidized products, in a process called glutathionylation, to mitigate their reactivity (142). Regardless of the method of detoxification used to neutralize accumulated ROS, the level of redundancy of antioxidants within a cell highlights their importance. The sensors and signaling pathways used to coordinate the expression of these detoxification enzymes will be addressed in detail later. The reversible modification of oxidized signaling targets and redox regulators is critical for redox homeostasis and redox signaling and, therefore, cellular physiology (43). Recall that PTPs are redox sensitive regulators that, when oxidized, can no longer inhibit MAPK activation. Once MAPK



**Figure 1.4: Enzymatic antioxidants neutralize ROS.**

SOD: Superoxide dismutase; Gpx: Glutathione peroxidase; Prx: Peroxiredoxin. Note: Not a balanced reaction and the addition of protons is not shown.

activation has sufficiently impacted the cellular environment, reduction of PTP is required to restore inhibition of MAPK. Without reduction-dependent restoration of PTP inhibition, the MAPK will become constitutively activated and, depending on the response being affected, can lead to exaggerated cellular outcomes such as autoimmunity (82). While PTP-dependent regulation is one of many mechanisms of MAPK regulation, however its redox-sensitive mechanism of regulation highlights the importance of the reversibility of redox modifications. Reduction of oxidized PTP and other oxidized redox targets is achieved by antioxidants such as glutathione and thioredoxin (46, 49).

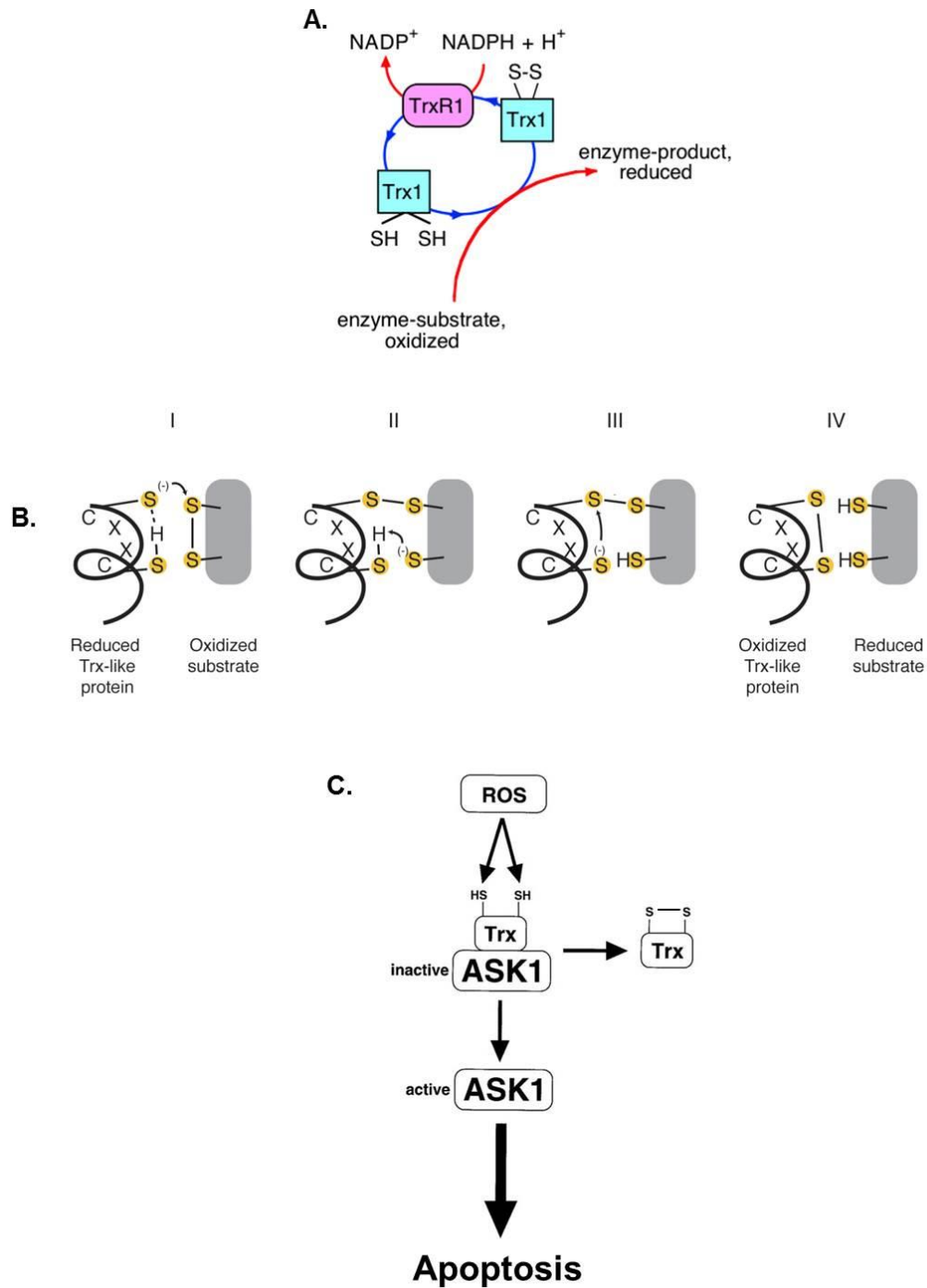
Glutathione is a highly abundant non-protein thiol that, in the cytosol, is commonly found in its reduced form (GSH), rather than its oxidized form (GSSG). Glutathione is the major redox buffering system and the abundance of GSH largely dictates the redox environment of the cell or subcellular compartment. The critical cysteine residues of GSH enable it to reversibly switch between its reduced and oxidized states, via glutathione reductase-catalyzed reduction of GSSG using NADPH as a reducing equivalent. Using this system, GSH is responsible for the reduction of target proteins and, arguably most importantly, several major antioxidant enzymes, including glutathione peroxidase, glutaredoxin, and peroxiredoxins. As reversible modification of these scavengers is required to recycle their activity and restore their antioxidant capacity, this is a critical role of glutathione. Aside from the ability to reversibly modify several major antioxidants, glutathione can itself act as a powerful antioxidant, during glutathionylation as mentioned previously. In summary the glutathione and thioredoxin systems, through their roles in reducing target substrates and other enzymatic antioxidants, allow oxidative modifications to be reversible and are critical components of the maintenance of redox homeostasis within an organism.



## Thioredoxins

The thioredoxin system is comprised of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, with Trx being the major player in this system (Figure 1.5A). Trx is a small, 12 kDa protein that is highly conserved from bacteria to humans (135). Throughout all forms of life, this protein uses its Cys-Gly-Pro-Cys active site, in which the cysteine residues are sensitive to thiol oxidation, to catalyze disulfide bond exchange between itself and target proteins (Figure 1.5B) (170). Moreover, its reactive cysteines are utilized to reduce and recharge antioxidants, most notably peroxiredoxins. The resulting oxidized dithiol moieties of Trx are then reduced via the TrxR-dependent addition of NADPH-derived electrons, thereby recycling the reductive capacity of Trx. While the thioredoxin system aids in the maintenance of redox homeostasis, the more intriguing role of Trx is its ability to modulate a wide variety of oxidized target proteins that impact a diverse array of biological processes, such as apoptosis, signal transduction, gene regulation, the inflammatory response, and immunomodulation (36, 74, 112, 147, 185).

From bacteria, to yeast, to humans, Trxs play important regulatory roles in various cellular processes, the majority of which rely on the ability of Trx to modulate the oxidation status of target proteins. First discovered in *Escherichia coli*, Trx is required for the reduction of ribonucleotide reductase, an enzyme critical for deoxyribonucleotide (dNTP) production and, therefore, DNA synthesis (122). The functions of Trxs increase in complexity as their roles in higher eukaryotes are considered. In the budding yeast *Saccharomyces cerevisiae*, a transcription factor controlling the response to oxidative stress, Yap1p, is regulated in a redox-dependent manner by Trx (50, 118, 119). Specifically, Yap1p accumulates in the nucleus upon oxidation of two critical cysteine residues and subsequently initiates the expression of antioxidants. Nuclear accumulation of Yap1p is modulated by Trx, as Trx-dependent reduction of oxidized Yap1p is required for Yap1p export from the nucleus (100).



**Figure 1.5: The activity, mechanism, and outcomes of the redox function of thioredoxins.**

**A)** The thioredoxin system is comprised of a thioredoxin (Trx1), thioredoxin reductase (TrxR1), and NADPH. In its reduced form, Trx1 reduces an oxidized substrate, resulting in

oxidation of Trx1. TrxR1 then utilizes electrons from NADPH to recycle oxidized Trx1 to its reduced, active form so that it may reduce another target protein. This schematic was reproduced and adapted from: Schmidt, E. E. 2011. DNA Replication in Animal Systems Lacking Thioredoxin Reductase I, DNA Replication-Current Advances (189). Permission to use this figure is granted by the Creative Commons CC BY-NC-SA 3.0 license and no permission is required from the authors or the publishers. **B)** A schematic depicting the four-step mechanism by which the sulfhydryl moieties of the conserved cysteine residues of thioredoxin are utilized to reduce an oxidized substrate. This schematic was reproduced and adapted from: Atkinson, H. J., and P. C. Babbitt. 2009. An atlas of the thioredoxin fold class reveals the complexity of function-enabling adaptations. PLoS Computational Biology 5:e1000541 (8). Permission to use this figure is granted by the PLOS applied Creative Commons Attribution (CC BY) license and no permission is required from the authors or the publishers. **C)** A model depicting the outcome of the redox activity of thioredoxin. Reduced thioredoxin (Trx) binds the mammalian p38 MAPKKK, ASK1, inhibiting its ability to autophosphorylate and, subsequently, activate. Oxidation of Trx via ROS dissociates the Trx1/ASK interaction, thereby relieving repression of ASK, which ultimately results in activation of apoptosis. This schematic was reproduced and adapted from: Saitoh, M., H. Nishitoh, M. Fujii, K. Takeda, K. Tobiume, Y. Sawada, M. Kawabata, K. Miyazono, and H. Ichijo. 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. The EMBO Journal 17:2596-2606 (183). Permission to use this figure was granted by Copyright Clearance Center (License #: 3833700510046).

The ability of Trxs to modulate the activity of transcription factors is not unique to yeast. NFκB is a mammalian transcription factor that responds to a wide variety of stress signals, including ROS accumulation (123). The DNA binding efficiency of NFκB is greatly enhanced upon Trx-dependent reduction of this transcription factor (81, 141). Trx not only modulates the activity of redox sensitive proteins. One of the most well-studied examples of Trx-dependent regulation does not involve a redox sensitive target and relies, rather, on Trx itself acting as a redox sensitive regulator to convert cellular redox changes into an activated kinase signaling program. The mammalian MAPKKK, ASK1, is a serine/threonine protein kinase that ultimately regulates p38 MAPK activation, a conserved signaling kinase that is important for the activation of several physiological pathways (96, 155). ASK1-dependent regulation of p38 MAPK activation controls stress-induced apoptosis in a redox-dependent manner, independent of the redox status of ASK1 (96). Kinase activation of ASK1 requires homodimerization at both its N- and C-terminus and while dimerization at the C-terminus occurs intrinsically, N-terminal dimerization is inhibited in a redox-dependent manner by TRX1 (65, 206). Upon ROS-induced oxidation, a conformational change in TRX1 coordinates the dissociation of the TRX1/ASK1 heterodimer, thereby allowing ASK1 homodimerization to occur, which stimulates the ability of this kinase to autophosphorylate, ultimately resulting in p38 MAPK-dependent induction of apoptosis (Figure 1.5C) (65, 183). Therefore, TRX1 acts as a redox sensor to modulate the activity of MAPK pathway activation. Ultimately, the evolutionarily conserved ability of Trxs to modulate the activity of enzymes, transcription factors, and kinases underscores the overall role of Trxs as mediators of both redox-dependent processes and redox signaling.

### **Oxidative Stress Response and its Regulation**

The oxidative stress response is a dynamic cellular program by which an organism restores redox homeostasis during excess ROS accumulation and oxidative stress. This

response is characterized by the increased production of antioxidants through the ROS-dependent activation of the sensors, signaling pathways, and transcription factors controlling antioxidant expression.

Of central importance to the regulation of the oxidative stress response are the transcription factors that control antioxidant gene expression upon increased oxidative stress, such as nuclear factor-erythroid-2-related factor 2, Nrf2 (98, 221). Nrf2 is a member of the Nrf/CNC (cap'n'collar) protein family, made up of Nrf1, Nrf2, Nrf3, and p45-NFE2, which control developmental pathways and various adaptive stress responses (31, 32, 114, 200). Nrf2 is the most well-studied of these factors, with its major role being a master regulator of antioxidant and xenobiotic defense (98, 221). Given this, it is not surprising that Nrf2 activity is important for the prevention of numerous diseases and disorders (94). Several layers of regulation govern the activation of Nrf2-dependent expression of antioxidants, many consisting of overlapping signal transduction pathways, which ultimately adds to the complexity of studying Nrf2. Moreover, Nrf2 has been implicated in diabetes and lipid accumulation in mammals, however the detailed mechanism by which this is achieved remains unclear (3, 231). Therefore, the existence of a functional homolog of Nrf2 in the simple multicellular model organism, *Caenorhabditis elegans*, provides a unique opportunity to garner a deeper understanding of Nrf2 regulation. Additionally, due to the simplicity of *C. elegans* it is possible to uncover novel biological processes previously masked by the complexity of overlapping pathways and responses of mammals (4).

### **Utilizing *C. elegans* as model to understand the maintenance of redox homeostasis**

The nematode *Caenorhabditis elegans* is a soil dwelling organism that is naturally found on rotting fruit, root structures, and in compost piles. In the laboratory setting, *C. elegans* is a sophisticated model organism for many biological fields of study, including

genetics and genomics, cell and molecular biology, developmental biology, and neurobiology. Advantages of such a model organism include *i.*) the many conserved genes with higher eukaryotes, which allows for the study of complex problems using a simple system (84), *ii.*) the ease of genetic manipulation, transparency of the animal, and short life cycle, and *iii.*) the vast number of tools and methodologies available for use in this system. Importantly, because *C. elegans* is a whole animal model organism, it allows for the study of cellular and organismal processes, functions, and disorders.

The oxidative stress response is among the cellular and organismal processes that can be assessed using the model organism *C. elegans*. The worm encounters endogenously generated ROS, typically byproducts of mitochondrial respiration and professionally generated via enzymes, as well as exogenously generated ROS (34, 148, 204). The age-related accumulation of ROS-induced damage can be visualized via the autofluorescence of the oxidative damage-induced pigment, lipofuscin (24, 35). As previously mentioned, a functional homolog of Nrf2, called SKN-1, is encoded in the worm genome and, similarly to mammals, induces the expression of antioxidants in response to oxidative stress (154). Antioxidants similar to those found in mammals are employed by *C. elegans* to restore redox homeostasis, such as superoxide dismutase, catalase, glutathione S-transferases, and peroxiredoxins (146).

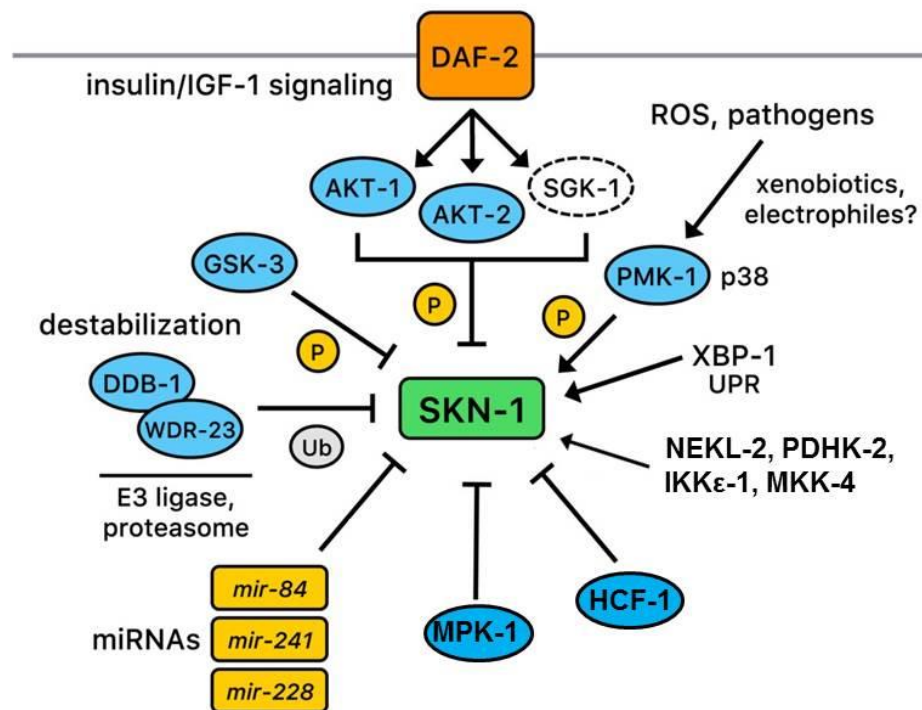
### **SKN-1: A critical regulator of antioxidant and xenobiotic defense**

As previously mentioned, SKN-1 is the functional ortholog of Nrf2 and is the major oxidative stress transcription factor in *C. elegans*, but its sequence and mechanism of DNA binding differ from its mammalian counterpart. Similarly, both Nrf2 and SKN-1 possess a CNC domain followed by a basic region (223). However, Nrf2 also contains a basic leucine zipper (bZIP) domain and requires a Maf cofactor to bind DNA, whereas SKN-1 lacks the

bZIP domain and binds DNA as a monomer (18). SKN-1 DNA binding is supported by a small peptide region that is responsible for binding the DNA minor groove (29, 116). Both Nrf2 and SKN-1 possess a DIDLID sequence at their amino terminus, which is important for transcriptional activation. This conserved domain was first discovered in *C. elegans*, and represents an example of the power of using the worm to uncover relevant characteristics of Nrf2/SKN-1 (223). Regardless of the differences in DNA binding between Nrf2 and SKN-1, the analogous functionality and similarities in regulation between the two are striking. However, it should be noted that functional differences between the two do exist. A majority of these differences, however, are likely a consequence of SKN-1 being the sole Nrf/CNC homolog in *C. elegans* and likely fulfilling a subset of the ancient roles of the Nrf/CNC family as a whole. For example, SKN-1 plays an important role in development, unlike its Nrf/CNC protein kin (21, 33).

SKN-1 is activated in response to several oxidative stressors, including hydrogen peroxide, sodium azide, paraquat, juglone, sodium arsenite, and hyperbaric oxygen (107, 154). Exposure to oxidative stress activates SKN-1 to induce the expression of a battery of antioxidants and detoxification enzymes, many of which correspond to Nrf2-regulated targets (154). Concordantly, *skn-1* mutants are hypersensitive to the aforementioned oxidative stressors. Divergent from canonical Nrf2 functions, SKN-1 plays a role in proteostasis, the unfolded protein response (UPR), and immunity (73, 85, 126, 151, 154, 168). Interestingly, Nrf2 induces expression of proteasomal subunits during oxidative stress, suggesting that some of the extended roles of SKN-1 may be conserved functions of Nrf2 (167, 168).

SKN-1 regulation is complex and relies on several conserved signaling pathways to stimulate and inhibit its activation (Figure 1.6). The mechanisms of SKN-1 activation are both direct and indirect, and the majority of these mechanisms rely on post-translational



**Figure 1.6: The many layers of SKN-1 regulation.**

Depicted are the positive and negative layers of SKN-1 regulation, as described in the text.

This schematic was reproduced and adapted from: Blackwell, T. K., M. J. Steinbaugh, J. M.

Hourihaan, C. Y. Ewald, and M. Isik. 2015. SKN-1/Nrf, stress responses, and aging in

*Caenorhabditis elegans*. *Free Radical Biology & Medicine* 88:290-301 (19). Permission to

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modifications, such as phosphorylation, subcellular localization, and degradation. Post-translational modification is a common form of regulation for proteins necessary for the acute stress response, such as oxidative stress, which enables the ready availability of functional protein without the delay that arises when generating new protein by regulation at the transcriptional or translational level. Many of the signaling pathways that control SKN-1 activation are conserved methods of Nrf2 regulation; however, both variant and novel mechanisms are also utilized.

A classic example of Nrf2 regulation, cofactor-directed ubiquitination and degradation, is functionally conserved in *C. elegans*. Nrf2 localization is restricted to the cytosol as a result of Kelch ECH associating protein 1 (Keap1) binding. Moreover, as an ubiquitin ligase component, Keap1 facilitates the degradation of cytosolic Nrf2 by targeting it for proteasomal degradation. In addition, as its reactive cysteines are susceptible to oxidation, Keap1 functions as a redox sensor. When oxidized, it has been suggested that a conformational change in Keap1 relieves its ability to bind and inhibit Nrf2 (99, 235). While no ortholog of Keap1 exists in the worm, a similar mechanism of proteasome-dependent degradation of SKN-1 is utilized. The E3 ubiquitin ligase substrate adaptor, WDR-23, binds SKN-1 and facilitates its ubiquitin-mediated degradation via the proteasome. Accordingly, *wdr-23* mutants exhibit increased *i.)* SKN-1 protein levels and nuclear accumulation, *ii.)* *skn-1*-dependent target gene expression, and *iii.)* resistance to oxidative stress. While it is less clear how SKN-1 repression is relieved, the enrichment of cysteine residues in WDR-23 similar to the enrichment seen in Keap1, suggests that oxidation of this inhibitor reduces its ability to facilitate SKN-1 degradation (39, 124). However, the evidence demonstrating that ROS-dependent Keap1 oxidation relieves Nrf2 repression completely is not entirely convincing. Specifically, it has yet to be conclusively demonstrated that oxidation of Keap1 cysteine residues is required for relief of Nrf2 repression. For instance, while Keap1 contains

27 cysteine residues, many of which are predicted to be reactive due to neighboring basic amino acids, point mutations specific to cysteine residues and combinatorial mutations do not clearly affect Nrf2 activation (25). The elucidation of the molecular mechanism by which WDR-23 regulates SKN-1 activation could provide insight into a more detailed mechanism for redox sensitive Nrf2 regulation. Interestingly, WDR-23 is evolutionarily conserved in mammals and exhibits some parallel functions, such as its role as an ubiquitin-ligase adaptor (39). Given this and that Keap1 is not the sole facilitator of Nrf2 degradation, a potential role for WDR-23 in regulating Nrf2 seems feasible (6).

Other direct mechanisms of SKN-1 inhibition include the inhibitory phosphorylation modifications by both glycogen synthase kinase-3 (GSK-3) and the Insulin/IGF-1-like signaling (IIS) kinase, AKT. Specifically, GSK-3 is a serine/threonine kinase that phosphorylates SKN-1 at serine residues 483 and 522, which serves to prevent SKN-1 nuclear accumulation and activation of target genes (5). This mechanism of regulation is indeed conserved in mammals, as GSK-3 $\beta$  phosphorylation of Nrf2 inhibits its nuclear accumulation and activation of antioxidant gene expression (173, 180). Furthermore, activation of the *C. elegans* IIS receptor, DAF-2, signals the downstream kinase, AKT, to directly phosphorylate SKN-1 at serine residue 12, thereby preventing nuclear accumulation and activation of target gene expression (214). In mammals, no direct role for AKT-dependent inhibition of Nrf2 has been identified. SKN-1 nuclear accumulation and target gene expression is also inhibited by host cell factor-1 (HCF-1), however the mechanism by which this is accomplished remains unclear (178). Lastly, *skn-1* is also subject to transcriptional regulation, as microRNAs, such as *mir-228*, inhibit its expression and the transcription factor XBP-1 promotes its expression (73, 193).

While the mechanisms of SKN-1 repression are extensive, the p38 MAPK pathway is essential for activation of this transcription factor. The p38 MAPK pathway is a highly

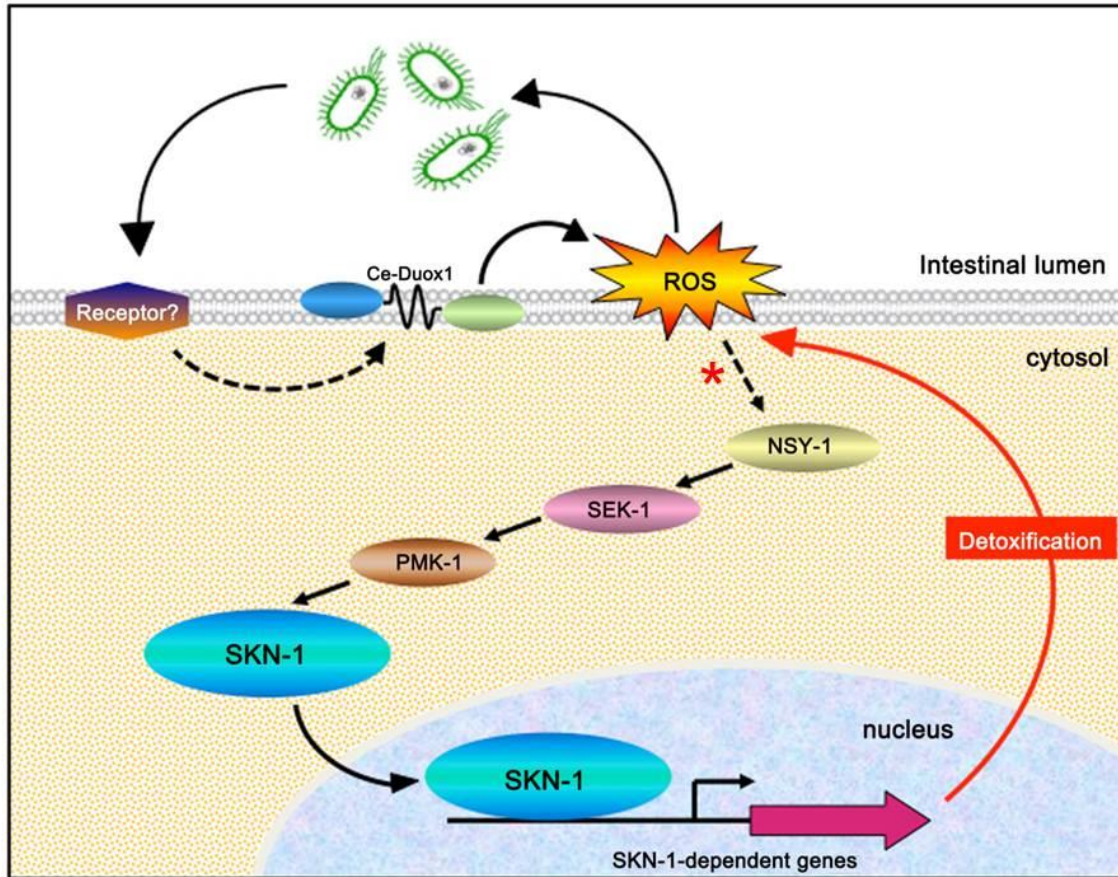
conserved Mitogen Activated Protein Kinase (MAPK) pathway that consists of the MAPKKK NSY-1, MAPKK SEK-1, and MAPK PMK-1. This kinase pathway is activated in a cascade fashion, with PMK-1 being the most downstream kinase that ultimately catalyzes the phosphorylation of the pathway's target proteins, such as SKN-1, at critical serine or threonine residues. In eukaryotes, the p38 MAPK pathway is activated in response to oxidative stressors, including hydrogen peroxide (79, 97). Disconcertingly, the p38 MAPK pathway has been reported to play contradictory roles in Nrf2 regulation, ranging from positive, to negative, to no role (156, 234, 239). These conflicting results warrant the utilization of *C. elegans* as a simple model organism to aid in the elucidation of the true molecular and *in vivo* role of the p38 MAPK pathway in regulating Nrf2. In *C. elegans*, p38 MAPK pathway activation of SKN-1 is well-defined. The p38 MAPK pathway components are required for SKN-1 nuclear accumulation and target gene expression in an oxidative stress and pathogen stress-dependent manner (85, 97). Furthermore, in response to oxidative stress, PMK-1 directly phosphorylates serine residues 164 and 430 of SKN-1, which is required for both nuclear accumulation and transcriptional activation of SKN-1. Additionally, these two serines of SKN-1 are also phosphorylated by the ERK MAPK, MPK-1, to facilitate nuclear accumulation. Interestingly, MPK-1-dependent activation of SKN-1 results in the induction of a transcriptional program enriched in expression of insulin-like peptides, rather than antioxidants (153). Together, these data suggest that phosphorylation at serine residues 164 and 430 is essential for SKN-1 nuclear accumulation, but there may be unknown post-translational modifications required to determine which transcriptional program of SKN-1 is induced once it accumulates in the nucleus. On this note, a large scale RNAi screen identified four other kinases that activate SKN-1 nuclear accumulation and target gene expression, *nekl-2* (cell cycle kinase), *ikke-1* (ikb-kinase ortholog), *mkk-4* (neuron specific kinase), and *pdhk-2* (pyruvate dehydrogenase kinase), however the mechanism by which they do so remains elusive (109).

Overall, the variety of mechanisms by which SKN-1 activation is regulated highlights the importance of ensuring tight control of this central regulator of antioxidant and xenobiotic defense. In support of this, while the modest overexpression of SKN-1 results in lifespan extension and resistance to oxidative stress, the complete loss or dramatic overexpression of *skn-1* are detrimental to lifespan (4, 214).

### **Significance of this study**

As mentioned, both Nrf2 and SKN-1 are activated in response to oxidative stress to induce the expression of antioxidant genes. Moreover, the stress-dependent activation of both of these transcription factors is regulated by conserved MAPK signaling pathways. What is less clear is the mechanism by which these conserved MAPK signaling pathways are themselves activated by oxidative stress. Two potential mechanisms of ROS-induced activation of MAPK signaling pathways include *i.*) the direct oxidation of the signaling kinases and *ii.*) redox regulators governing the activation of signaling kinases, with precedence existing for each of these mechanisms. While MAPKs have not been demonstrated to be sensitive to oxidative modification, other serine/threonine signaling kinases have. For instance, the Protein Kinase C (PKC) family of serine/threonine kinases are directly governed in a redox-dependent manner. Both the cysteine-rich regulatory domain and presence of sulfhydryls in the catalytic domain render PKCs sensitive to oxidative modification, in which oxidation transiently controls their activation (1, 42). As a second possibility, redox regulators may govern MAPK activation. This is particularly intriguing as one of the MAPKKK upstream of p38 MAPK is regulated in a redox-dependent manner by a thioredoxin, as previously mentioned. While thioredoxin-dependent regulation of ASK1 activation ultimately impacts apoptosis, rather than Nrf2-dependent expression of antioxidants, it is tempting to speculate whether this could be a common mechanism of regulation of p38 MAPK to elicit a variety of downstream responses. Redox-dependent

regulation of the *C. elegans* ASK1 homolog, NSY-1, has not been elucidated. However, ROS-dependent activation of p38 MAPK pathway controls SKN-1 nuclear accumulation and antioxidant gene expression (Figure 1.7)(85). Furthermore, the *C. elegans* genome encodes several thioredoxins, which, in this system, are understudied and may exhibit important roles as redox regulators. The mechanism by which ROS induce p38 MAPK pathway activation remains unclear and, given the previously mentioned parallels, it seems plausible that NSY-1 may also be redox regulated. Given this, the aim of this study is to address whether SKN-1 activation and function are governed in redox-dependent manner by thioredoxins.



**Figure 1.7: Mechanism by which ROS activates SKN-1-dependent oxidative stress response is unknown.**

Several stressors and regulators have been implicated in governing SKN-1 activation, including pathogen-induced generation of ROS, which inadvertently activates SKN-1 in a p38 MAPK-dependent manner. However, the mechanism by which ROS activates the p38 MAPK pathway (red star) remains elusive. Similarly, the mechanism by which ROS activates the other known SKN-1 regulators is unclear (not shown). Elucidating the mechanism by which ROS stimulate or inhibit SKN-1 activators and repressors, respectively, is the primary goal of this study. This schematic was reproduced and adapted from: Hoeven, R., K. C. McCallum, M. R. Cruz, and D. A. Garsin. 2011. Ce-Duox1/BLI-3 generated reactive oxygen species trigger protective SKN-1 activity via p38 MAPK signaling during infection in *C. elegans*. *PLoS Pathogens* 7:e1002453 (85). Permission to use this figure is granted by the

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## **Chapter 2: Materials and Methods**

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## Strains and Maintenance

The *C. elegans* strains used in this study (Table 2.1) were maintained as previously described (88). The following bacterial strains were used in this study: *E. coli* OP50, *E. faecalis* OG1RF, *P. aeruginosa* PA14, and *E. coli* HT115.

### *C. elegans* strain construction

In general, all mutant strains were backcrossed to wild type N2 6x. To generate the VZ27, VZ26, and VZ157 strains, *Is007 [skn-1b/c::gfp; rol-6(su1006)]* worms were crossed with *trx-1(ok1449)*, *trx-2(tm2720)*, and *trx-3(tm2820)* mutants, respectively. To visualize TRX-1 localization, the OE3381 strain was generated by injecting pPD95.77 plasmid containing *trx-1* (1kb promoter + gene) fused to *gfp* with the *trx-1* 3' UTR (rather than the *unc 54* 3'UTR native to this vector). To generate the VZ472, VZ458, and VZ461 strains, the *trx-1* promoter was replaced with tissue specific promoters (*ssu-1*; ASJ, *ges-1*; intestinal, *daf-7*; ASI) in the above mentioned plasmid. These constructs were then injected into wild type N2 worms along with the *Punc-122::DsRed* coinjection marker which causes red fluorescence in the coelomocytes. Finally, the arrays were transferred into the *trx-1(ok1449)*; *Is007* background to generate GF92, GF93 and GF94 strains, respectively. To determine p38 MAPK signaling dependence, *nsy-1*, *sek-1*, and *pmk-1* mutants were crossed into the *trx-1(ok1449)*; *Is007* background to generate the GF96, GF97, GF98 strains, respectively. *Ex060[skn-1(S74,340A)b/c::gfp;rol-6(su1006)]* was also crossed into the *trx-1(ok1449)*; *Is007* background to further verify p38 MAPK signaling dependence (GF95). To visualize *gst-4* expression in the *trx-1* background, the VZ433 strain was made by crossing *trx-1(ok1449)* mutants with *dvIs19 [Pgst-4::gfp::NLS; rol-6(su1006)]* worms. To generate GF99 and GF100, OE4064 and OE4067 were crossed into the *trx-1(ok1449)*; *Is007* background

Strain Name	Genotype	Reference
N2	Wild type Bristol	
VZ1	<i>trx-1(ok1449) II</i>	(145)
CL2166	<i>dvls19 [Pgst-4::gfp::NLS; rol-6(su1006)] III</i>	(127)
LD007	<i>Is007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	(4)
LD1782	<i>Ex060 [skn-1(S74,340A)b/c::gfp; rol-6(su1006)]</i>	(97)
OE4064	<i>trx-1(ok1449) II; daf-28(sa191) V; ofEx416 [Ptrx-1::trx-1::gfp; Punc-122::DsRed]</i>	(61)
OE4067	<i>trx-1(ok1449) II; daf-28(sa191) V; ofEx419 [Ptrx-1::trx-1(SGPS)::gfp; Punc-122::DsRed]</i>	(61)
VC390	<i>nsy-1(ok593) II</i>	CGC
KU4	<i>sek-1(km4) X</i>	CGC
KU25	<i>pmk-1(km25) IV</i>	CGC
OE3381	<i>lin-15(n765ts) X ; ofEx284 [Ptrx-1::trx-1::gfp::trx-1 3'-UTR; lin-15ab (+)]</i>	This study; Miranda-Vizuete lab, Spain
VZ27	<i>trx-1(ok1449) II; Is007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	This study; Miranda-Vizuete lab, Spain
VZ26	<i>trx-2(tm2720) V; Is007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	This study; Miranda-Vizuete lab, Spain
VZ157	<i>trx-3(tm2820) IV ; Is007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	This study; Miranda-Vizuete lab, Spain
VZ472	<i>trx-1(ok1449) II; vzEx168 [Pssu-1::trx-1::trx-1 3'-UTR; Punc-122::DsRed]</i>	This study; Miranda-Vizuete lab, Spain
VZ458	<i>vzEx163 [Pges-1::trx-1::trx-1 3'-UTR; Punc-122::DsRed]</i>	This study; Miranda-Vizuete lab, Spain
VZ461	<i>vzEx166 [Pdaf-7::trx-1::trx-1 3'-UTR; Punc-122::DsRed]</i>	This study; Miranda-Vizuete lab, Spain
VZ433	<i>trx-1(ok1449) II; dvls19 [Pgst-4::gfp::NLS; rol-6(su1006)] III</i>	This study; Miranda-Vizuete lab, Spain
GF92	<i>trx-1(ok1449) II; Is007 [skn-1b/c::gfp; rol-6(su1006)] X; vzEx168 [Pssu-1::trx-1::trx-1 3'-UTR; Punc-122::DsRed]</i>	This study; generated in Garsin lab
GF93	<i>trx-1(ok1449) II; Is007 [skn-1b/c::gfp; rol-</i>	This study; generated in Garsin lab

	<i>6(su1006)] X; vzEx163 [Pges-1::trx-1::trx-1 3'-UTR; Punc-122::DsRed]</i>	
GF94	<i>trx-1(ok1449) II; ls007 [skn-1b/c::gfp; rol-6(su1006)] X; vzEx166 [Pdaf-7::trx-1::trx-1 3'-UTR; Punc-122::DsRed]</i>	This study; generated in Garsin lab
GF95	<i>trx-1(ok1449) II; Ex060 [skn-1(S74,340A)b/c::gfp; rol-6(su1006)]</i>	This study; generated in Garsin lab
GF96	<i>nsy-1(ok593) II; trx-1(ok1449) II; ls007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	This study; generated in Garsin lab
GF97	<i>trx-1(ok1449) II; sek-1(km4) X; ls007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	This study; generated in Garsin lab
GF98	<i>trx-1(ok1449) II; pmk-1(km25) IV; ls007 [skn-1b/c::gfp; rol-6(su1006)] X</i>	This study; generated in Garsin lab
GF99	<i>trx-1(ok1449) II; ls007 [skn-1b/c::gfp; rol-6(su1006)] X; ofEx416 [Ptrx-1::trx-1::gfp; Punc-122::DsRed]</i>	This study; generated in Garsin lab
GF100	<i>trx-1(ok1449) II; ls007 [skn-1b/c::gfp; rol-6(su1006)] X; ofEx419 [Ptrx-1::trx-1(SGPS)::gfp; Punc-122::DsRed]</i>	This study; generated in Garsin lab

**Table 2.1: Strains used in this study**

and maintained at 25°C for four generations to remove the *daf-28(sa191)* mutation. See 'C. *elegans* strain building' for method of crossing for strains with GFxx(x) nomenclature.

### **C. *elegans* strain building**

In general, LD007 and VZ27 worms were knocked down for their roller phenotype (*rol-6*) and non-rolling males were generated (5 hours heat shock at 30C) and maintained. Corresponding males were allowed to mate with corresponding mutant L4 hermaphrodites at a 2:1 male to female ratio (typically 10:2) for two days on *rol-6* seeded RNAi plates. F1 male progeny were then moved to fresh NGM plates and allowed to mate with mutant L4 hermaphrodites at a 2:1 ratio for two days. From this, 20-40 hermaphrodite roller F1 progeny (corresponding to presence of the *skn-1b/c::gfp* background) were selected at random and transferred to fresh plates (1 worm per plate) and allowed to lay eggs for two days. Immediately post-egg laying, hermaphrodites were subjected to Single Worm Lysis PCR (described below) for genotype analysis at either one or two alleles, depending on the strain. Two plates with positive genotyping results were selected and 10 progeny of each positively genotyped hermaphrodite were themselves genotyped to ensure mutant population confluency. Genomic isolation of DNA (described below) was used for all generated strains to verify proper alleles.

### **Genotyping via Single Worm Lysis PCR**

Lysis of adult hermaphrodites that had been allowed to lay eggs was performed as follows. 3 µl of Single Worm Lysis Buffer (10mM Tris-Cl, pH 8.3; 50 mM KCl; 2.5 mM MgCl<sub>2</sub>; 0.45% Tween 20; 0.05% gelatin, 100 µg/ml Proteinase K) was placed in PCR tubes. Individual parents (P0) were placed into each PCR tube containing buffer. To lyse worms and subsequently deactivate Proteinase K, PCR tubes were then heated at 65°C (1.5 hr) and then 95°C (15 min), respectively. Following lysis, 17 µl of PCR mix (1X PCR Buffer,

dNTPs, corresponding gene-specific primers, Taq polymerase) was placed in each tube. The following PCR program was used to amplify gene-specific segments: 94°C for 2 min, [94°C for 10 sec, 55°C for 12 sec, 72°C for x minutes (x = 60s/kb)]X35, 72°C for 2 min. Upon amplification of the gene of interest, amplified product was separated using gel electrophoresis and the band size was compared to wild type and null controls. For verification of double mutants, the 3 µl of lysed worm product was split into two 1.5 µl samples and the genotype checked at each allele using above method.

### **Genomic DNA Isolation**

Isolated genomic DNA was used to verify proper genotype at a whole population level for every strain generated. About 500 progeny of the generated strain were harvested from plates into a 1.5 ml tube and washed four times to ensure removal of bacteria. After the last wash, supernatant was aspirated to achieve a 100 µl pellet, which was then flash frozen in dry ice/ethanol. Five volumes of Worm Genomic DNA Lysis Buffer (0.1 M NaCl, 10mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 1% β-mercaptoethanol, and 100 µg/ml Proteinase K) was added and lysis was achieved by incubating at 65°C for 1.5 hrs. Proteinase K was deactivated by incubating at 95°C for 20 min. RNase A was added to a concentration of 0.1 mg/ml and incubated for 1 hr at 37°C. Next, one volume of phenol/chloroform buffered with Tris was added and the tubes were mixed gently. The samples were then centrifuged at 4,000 rpm for 5 min at room temperature. The aqueous phase was transferred to a fresh tube and two more rounds of phenol/chloroform extraction were conducted. After three extractions, 0.1 volume of 3M sodium acetate and at least two volumes of 100% EtOH were added and mixed. The samples were incubated at -20°C for 1 hr to several days. The following steps were carried out at 4°C. DNA was pelleted by centrifugation at 13,000 rpm for 20 min and the supernatant was carefully aspirated off. The pellet was washed with 1 ml of 70% EtOH and gently mixed (by inverting tube) ten times and the samples were then

centrifuged for 5 min at 13,000 rpm. Supernatant was aspirated away and samples were dried in the fume hood for 10 minutes, followed by resuspending in 50  $\mu$ l ddH<sub>2</sub>O. DNA content was measured for concentration and purity and subjected to PCR amplification using gene-specific primers and DNA gel electrophoresis to analyze the altered allele of interest.

### **RNA Isolation**

Isolated RNA was used for generation of RNAi constructs, qRT-PCR, and RNA Seq. About 1,000 worms were harvested by centrifugation to generate a 100  $\mu$ l pellet in a 1.5 ml tube. RNA was extracted using TRIzol (ThermoFisher Scientific), in which 900  $\mu$ l was added and the sample was briefly vortexed. The samples were then subjected to four freeze/thaw cycles in liquid nitrogen and then incubated at room temperature for 5 min. Next, 200  $\mu$ l of chloroform was added and tubes were inverted 15 times and incubated at room temperature for 2-3 min. After 15 min of centrifugation at 12,000 X g at 4°C, the supernatant was dispensed into a fresh tube. RNA was precipitated by adding 500  $\mu$ l of isopropanol and inverting the samples five times, followed by a 10 min incubation at room temperature. RNA was pelleted by centrifuging at 12,000 X g at 4°C and the isopropanol-containing supernatant was discarded. The pellet was washed by resuspending in 1 ml of 75% EtOH and centrifuged at 4°C for 5 min at 7,500 X g. The EtOH was decanted and the RNA pellets were air dried. The samples were resuspended in 25  $\mu$ l of nuclease free water. RNA to be used for qRT-PCR or RNA Seq were DNase treated (Turbo DNA Free, ThermoFisher). The quantity and purity of RNA were measured.

### **Generation of RNAi constructs**

Total cDNA was synthesized from the isolated RNA using the ThermoFisher Scientific SuperScript® II Reverse Transcriptase kit. Synthesized cDNA was used as a substrate for PCR with gene-specific primers. PCR fragments were digested and ligated into

pL4440, which was subsequently transformed into electrocompetent *E. coli* HT115. The generated strain produces dsRNA to the specific target genes, which can be used to direct the host RNAi machinery to degrade gene-specific transcripts.

### **Knockdown of genes using RNA Interference**

Bacterial RNAi strains were streaked on LB agar plates containing 100µg/ml ampicillin and grown overnight at 37°C. Single colonies were inoculated in 5 ml liquid LB broth containing 100µg/ml ampicillin and grown shaking at 37°C overnight. Turbid culture was spotted onto RNAi plates (NGM plates containing 100µg/ml ampicillin and 1mM IPTG) and incubated at 37°C overnight. Day old adult worms were allowed lay eggs overnight on the appropriately seeded RNAi plates and progeny were allowed to develop using indicated RNAi strain as a food source until the appropriate age was reached (usually young adult depending on type of experiment).

### **Fluorescence Microscopy**

To visualize SKN-1B/C::GFP and TRX-1::GFP localization and *gst-4:gfp* expression, young adults were washed from plates and anesthetized with 1 mM levamisole. Anesthetized worms were mounted on 2% agarose pads and visualized and imaged using an Olympus IX81 automated inverted microscope and Slidebook (version 5.0) software. For SKN-1B/C::GFP localization quantification the percent intestinal SKN-1 nuclear localization was categorically scored as follows: none = no localization, low = posterior **or** anterior intestinal localization, medium = posterior and anterior intestinal localization, high = localization throughout the entire intestine (97). For GF99 and GF100, the percent intestinal SKN-1 nuclear localization was categorically scored in an area limited to the anterior intestine of animals as follows: none = no localization, low = weak fluorescence, medium = moderate fluorescence, or high = bright fluorescence. Chi square and Fisher's exact tests

(GraphPad Prism version 5.0) were used to calculate significance of SKN-1 nuclear localization averaged between three biological replicates of  $N \geq 50$  worms.

## **Western Blotting**

About 1,000 worms were washed from NGM plates and collected in a 100  $\mu$ l pellet using protein extraction buffer (50 mM Tris pH 7.5, 50 mM NaCl, protease inhibitor cocktail (Roche, 11873580001), PhosStop (Roche, 04906845001). The pellet was sonicated (on ice) for 10 sec at Level 5 and 50% duty. The suspensions were incubated in 1% SDS on ice for five min and then centrifuged in the cold at 14,000 rpm for 10 min. The supernatants were transferred to fresh microfuge tubes and the total protein concentration was measured using the BCA assay (Pierce, 23227). Sample buffer was added and protein lysates were boiled for 5 min. For SKN-1C detection, total protein was separated using a 10% SDS-PAGE gel with 15  $\mu$ g of total protein per well. The gel was transferred to a nitrocellulose membrane for 60 min at 4°C. The membrane was blocked in 5% milk + TBST for 1 hr at room temperature. The membrane was then incubated with 1:200 monoclonal SKN-1 antibody (FC4) overnight at 4°C (20). The blot was washed eight times for five min intervals with TBST. The blot was then incubated with 1:1000 secondary HRP conjugated anti-mouse antibody for 30 min and subsequently washed 8 times for 5 min intervals. Blots were developed using SuperSignal West Dura Extended Duration Substrate (Pierce, 37071) and visualized using an ImageQuant LAS 4000 imager (GE Healthcare Life Sciences). Note that we were unable to detect SKN-1C::GFP and there are presently no examples in the literature of successful detection of the fusion protein with this antibody. For phospho-NSY-1 detection, total protein was separated using an 8% SDS-PAGE gel with at least 70  $\mu$ g of total protein per well. The gel was transferred to a nitrocellulose membrane for 75 min at 4°C. The membrane was blocked in 5% BSA + TBST overnight at 4°C. The membrane was then incubated with 1:1000 Phospho-ASK1 (Thr845) Antibody (Cell Signaling, #3765) or Phospho-p38 MAPK



(Thr180/Tyr182) Antibody (Cell Signaling, #9211) for 5.5 hrs at 4°C. The blot was washed four times for five minutes intervals with TBST. The blot was then incubated with 1:3000 secondary HRP conjugated anti mouse antibody (for anti-ASK1 blot) or 1:5000 secondary HRP conjugated anti-rabbit antibody (for anti-p38 blot) for 30 min and subsequently washed four times for five minute intervals. Blots were developed as above. All blots were repeated at least three times, obtaining similar results. Anti- $\alpha$ -tubulin was used as a loading control with an hour incubation in 1:1000 primary antibody (Sigma, T9026) concentration and a 30 min incubation in 1:3000 goat-anti-rabbit HRP conjugated secondary antibody, with similar washing procedures as described above.

### **Quantitative Real Time PCR (qRT-PCR) Analysis**

RNA was extracted (as described above) from young adult worms exposed or left unexposed to sodium arsenite and analyzed via qRT-PCR, as previously described (85). The average gene expression of biological triplicates was graphed and error bars represent the standard error of the mean (SEM). A paired Student's t-test was used to determine significance where indicated. Primers are listed in Table 2.2.

### **Oxidative Stress and Killing assays**

To assess sensitivity to oxidative stress, sodium arsenite ( $\text{NaAsO}_2$ ) was added to NGM plates to a final concentration of 10 mM. An overnight *E. coli* OP50 culture was generously seeded (~400  $\mu\text{l}$ ) and incubated for growth (at 37°C) on  $\text{NaAsO}_2$  plates. For microscopy, qRT-PCR, and western blotting, about 1,000 worms were incubated on  $\text{NaAsO}_2$  plates for 5 hrs at 20°C. For survival assays, 90 worms were added to three replicate plates and scored hourly for survival. To assess sensitivity to pathogen stress, killing assays were performed as previously described, with slight modification (68, 134). *E. faecalis* OG1RF grown in BHI for 5 hrs was seeded onto BHI plates and grown overnight at 37°C. *P. aeruginosa* PA14 grown in LB for 8 hrs was seeded onto SK plates and grown overnight at

<i>snb-1</i> F	CCGGATAAGACCATCTTGACG
<i>snb-1</i> R	GACGACTTCATCAACCTGAGC
<i>gst-4</i> F	CGTTTTCTATGGAAGTGACGC
<i>gst-4</i> R	TCAGCCCAAGTCAATGAGTC
<i>gst-7</i> F	GGACAAGACTTCGAGGACAAC
<i>gst-7</i> R	AACTGACGAGCCAAGTAACG
<i>gst-9</i> F	TCTCGGTGACCAATTCAAGG
<i>gst-9</i> R	AAGCCGGAACGAATAAATCTTTG
<i>gst-10</i> F	AAGAGATTGTGCAGACTGGAG
<i>gst-10</i> R	AGAACATGTGCGAGGAAGGTTG
<i>gst-12</i> F	TTTTGGAGATGGAAGCTGGG
<i>gst-12</i> R	TTTTCCAGCGAACCCGAA
<i>gst-14</i> F	TTGAGGATGAACGGGTGAAC
<i>gst-14</i> R	TCTAGCAAGGTAGCGATTTATGG
<i>gst-19</i> F	TGATTGCCCGTTTAAAGATGAAC
<i>gst-19</i> R	ATTCAGAGCAAGGTAGCGG
<i>gst-36</i> F	GTTTTGAAATCCGAGATGCCG
<i>gst-36</i> R	ATATCCAAGCGAGCACAGTC
<i>gst-38</i> F	TCAACGGAAAGAGCAGATGG
<i>gst-38</i> R	CGTCTCCTTCTGTGTAACCAAG
<i>gst-44</i> F	GCAGAAAGTCTACTGGAAGGAG
<i>gst-44</i> R	AAGTTGTCCGATGGAAGTGG
<i>vit-4</i> F	AGAGCATCACACCATTGAGAG
<i>vit-4</i> R	GATTGGGCGAGTTGGATAAGA
<i>vit-5</i> F	CTACGAGAGCAACTACGATGAAA
<i>vit-5</i> R	CTCCTTGATGAGGGTCTTCTTG
<i>col-81</i> F	TAGCAACTCAGTGGGCTATTG
<i>col-81</i> R	ATGCTGGAATTGAGAGGGATATT
<i>col-137</i> F	ACGTCATACCGGACAAATGAA
<i>col-137</i> R	CATTCCCATCCTCGTCAACATA
<i>col-140</i> F	GATACCTGGAACCGTGTTGT
<i>col-140</i> R	GCGAGATCTACGAATACCGAAA
<i>skpo-1</i> F	GGACAACGTGTATCGTACACCAG
<i>skpo-1</i> R	CCATCACGGAGTCTCTCAA
<i>lbp-8</i> F	TCAGTCCTTCCTTATGGTTTCC
<i>lbp-8</i> R	AGACCAACTCCGATTTCTTTCA
<i>lips-6</i> F	TGCCAACTATCCACGACAATAC
<i>lips-6</i> R	TGTCCCAAACAGTCAACTTACA
<i>lips-11</i> F	TTCAGCATAGACAAGCGAGTAG
<i>lips-11</i> R	GTATGGAATGGTGTAGGGATCTG

**Table 2:2 Primers used for qRT-PCR**

37°C. For 'full lawn' killing assay plates with *E. faecalis*, 150 ul of culture was spotted and spread onto plates and allowed to grow overnight at 37°C. Only plates that grew a confluent, full lawn were used for pathogen avoidance killing assays. A total of 90 worms were added to three replicate plates of each pathogen and scored for survival at various times points over the course of the assay. Kaplan-Meier log rank analysis was used to compare the significance of the survival curves using median survival.

## **RNA Sequencing**

Young adult animals were incubated on NGM plates with and without 10 mM sodium selenite for 5 hrs, at which point total RNA was extracted (as described above) and sent for RNA Sequencing. There were an average 101,140,446 reads/sample generated from the RNA-seq. The average percentage of the bases with  $\geq$ Q30 reads was 90.1%. We used the *Caenorhabditis elegans* genome (version ce10) as the reference that can be downloaded from the iGenome databases ([ftp://igenome:G3nom3s4u@ussd-ftp.illumina.com/Caenorhabditis\\_elegans/UCSC/ce10/Caenorhabditis\\_elegans\\_UCSC\\_ce10.tar.gz](ftp://igenome:G3nom3s4u@ussd-ftp.illumina.com/Caenorhabditis_elegans/UCSC/ce10/Caenorhabditis_elegans_UCSC_ce10.tar.gz)). The sequencing reads were mapped to the genome with the application TopHat (211, 212), which utilizes the high throughput sequence aligner bowtie (121). The application Cufflinks further processed the transcripts assembly and the abundance estimation. The fragments per kilobase of transcript per million mapped reads (FPKM) were calculated for the gene differential expression analysis. To visualize the results generated by Cufflinks an R/Bioconductor package CummeRbund (<http://bioconductor.org>) was used. Venn diagrams generated by R programming enabled the affected gene comparison between samples. To explore the functional groups for the differentially expressed genes, we further carried out GO (Gene Ontology) analysis with the application The Database for Annotation, Visualization and Integrated Discovery (DAVID)(89, 90). The three sub-

ontologies (MF: molecular function, BP: biological process and CC: cellular component) were assessed.

### **Oil-Red-O Staining and Analysis**

Oil-Red-O staining, visualization, and analysis was performed as previously described (152, 196), with modification. Briefly, day-old adult worms were harvested and washed three times with PBS. Worm pellets were then resuspended in 2% PFA and subject to three freeze thaw cycles using a dry ice ethanol bath. The worm pellet was then washed with PBS to remove PFA. Oil-Red-O stain (60%, filtered, and freshly prepared) was then added to the worm pellet and allowed to incubate with gentle shaking at room temperature for three hrs. The worms were then mounted on 2% agarose pads and visualized and imaged at 40x using an Olympus IX81 automated inverted microscope and Slidebook (version 5.0) software. To quantify Oil-Red-O staining, the Slidebook software was used to analyze the intensity in the FITC channel, as previously described but with minor modifications (232). Briefly, Oil-Red-O absorbs light at 510 nm, enabling visualization through the FITC channel. Directly beneath the pharynx, the FITC intensity throughout a circular area with a 40 pixel radius was quantified. The background was measured similarly and subtracted. Moreover, the TRITC intensity was measured in each area to remove any worm autofluorescence. At least 20 animals were scored and the experiment was repeated twice. The error bars represent the standard error of the mean (SEM). A paired Student's t-test was used to determine significance.

### **Chapter 3: TRX-1 regulates SKN-1 subcellular localization**

Portions of this chapter are based on my first author publication. I have received permission to reproduce data or text from this article through license number 3830380085023 as assigned by Copyright Clearance Center (CCC), per Genetics Society of America (GSA) journal of Genetics instructions. I have contributed significantly to this publication. The article is listed for reference: McCallum, K. C., B. Liu, J. C. Fierro-Gonzalez, P. Swoboda, S. Arur, A. Miranda-Vizuite, and D. A. Garsin. 2016. TRX-1 Regulates SKN-1 Nuclear Localization Cell Non-autonomously in *Caenorhabditis elegans*. *Genetics* (143).

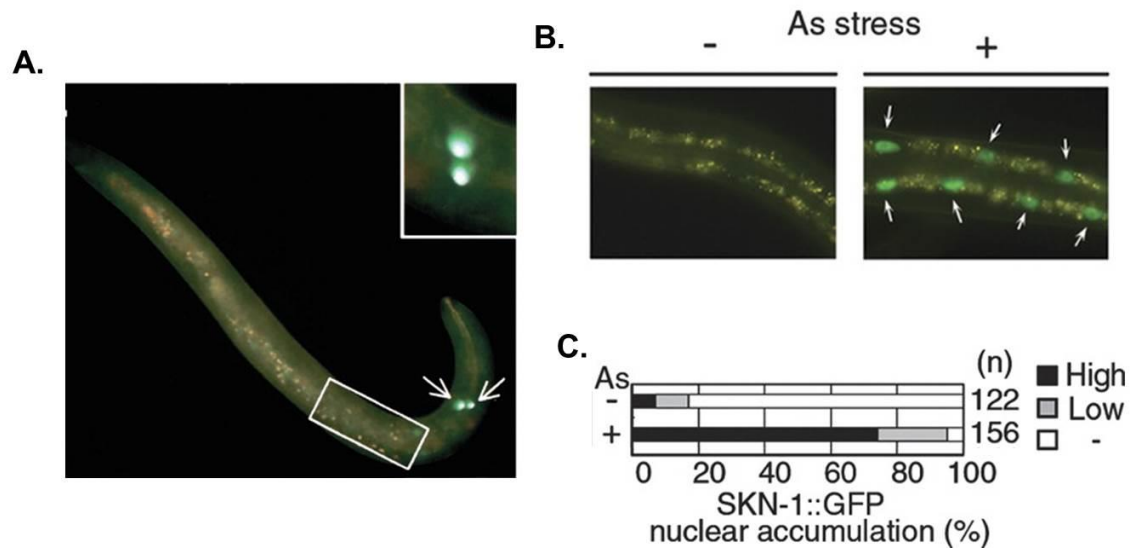
## Introduction

The nematode *Caenorhabditis elegans* utilizes a functional ortholog of mammalian Nrf proteins, SKN-1, to coordinate its oxidative stress response (4, 223). More recently, a role for SKN-1 has been found in the regulation of the Unfolded Protein Response (UPR) and the maintenance of lipid homeostasis (73, 132, 196). Similar to Nrf2, SKN-1 regulation is also well studied and overlapping mechanisms of regulation exist between mammals and worms. In general, both Nrf2 and SKN-1 seem to be regulated at the level of nuclear accumulation. Specifically, both mammals and worms employ a cysteine-rich adaptor protein, Keap1 and WDR-23, respectively, to facilitate the degradation of these transcription factors by the proteasome, thereby preventing their nuclear accumulation (39, 137). Furthermore, both mammalian and worm glycogen synthase kinase 3 phosphorylate Nrf2 and SKN-1, respectively, in a manner that impacts the subcellular localization of these transcription factors (5, 184). In *C. elegans*, additional mechanisms of SKN-1 regulation were elucidated. SKN-1 isoform C is antagonized by insulin/IGF-1-like signaling and is positively regulated by the p38 MAPK-pathway, via phosphorylation of Serines 74 and 340 (97, 214). Exposure to oxidative stressors, such as sodium arsenite, impact these positive and negative regulators governing intestinal SKN-1, resulting in increased nuclear localization and transcriptional activation, thereby maintaining redox homeostasis (97). However, while many factors and mechanisms of regulating SKN-1 are known, how these signaling pathways initially sense oxidative imbalance remains unclear.

A powerful tool to analyze SKN-1 regulation is a well characterized strain expressing GFP-tagged SKN-1 protein (SKN-1B/C::GFP) that was generated by the Blackwell lab in 2003 (4). Since its generation, this strain has been utilized as an indicator of intestinal SKN-1 activation in a majority of the studies that have examined novel mechanisms of SKN-1 regulation (5, 39, 85, 97, 109, 126, 178, 179, 214). Under normal, unstressed conditions,

SKN-1c::GFP does not localize to any one tissue or organelle in this reporter strain, with the exception of the ASI neurons, where SKN-1b::GFP is constitutively localized to the nuclei (white arrows, Figure 3.1A) (4, 17). Upon exposure to the oxidative stressor sodium arsenite, intestinal SKN-1::GFP strongly accumulates in the nuclei of intestinal cells throughout the body of the worm (white arrows, Figure 3.1B)(4). Intestinal SKN-1::GFP nuclear accumulation is promoted by the p38 MAPK pathway and inhibited by other oxidative stressors, including hydrogen peroxide, and *gsk-3*, *daf-2*, and *wdr-23*, highlighting the clout of this strain as a formidable, reliable tool for the analysis of SKN-1 regulation (5, 39, 107, 214).

Thioredoxins are small proteins that, due to their inherent amino acid chemistry, are redox reactive (7, 26, 171). While thioredoxins can act as antioxidants via their ability to reduce oxidized proteins, they play a prominent role in the regulation of signaling pathways in several organisms (66, 233). While the redox activity of thioredoxin is important for a majority of its cellular functions, it has important, redox-independent cellular roles. In mammals, TRX1 promotes ASK1 ubiquitination and degradation irrespective of its redox activity (128). Moreover, a *C. elegans* thioredoxin, TRX-1, modulates DAF-28 signaling during dauer formation in a redox- independent fashion (61). However, no specific role for thioredoxins in signaling have been characterized in the worm. Given the general ability of thioredoxins to act as both redox-dependent and redox-independent regulators and for mammalian TRX1 to regulate the p38 MAPK pathway, we reasoned that a thioredoxin may regulate SKN-1 and/or the *C. elegans* oxidative stress response.



**Figure 3.1: Oxidative stress triggers intestinal SKN-1::GFP nuclear accumulation.**

**A)** SKN-1::GFP is constitutively localized to the nuclei of the ASI neurons (white arrows). In unstressed conditions, intestinal SKN-1::GFP does not accumulate in intestinal nuclei (white box.) **B)** Worms harboring SKN-1::GFP were stressed with 5 mM sodium arsenite (As) for 1 hr or left unstressed. Specifically in response to oxidative stress, SKN-1::GFP accumulates in intestinal nuclei (white arrows). **C)** Quantification of intestinal SKN-1::GFP nuclear accumulation shown in A. Worms were categorically scored 'high,' 'low,' or none ('-') for percent SKN-1::GFP nuclear accumulation throughout the population with and without exposure to sodium arsenite. Number of worms scored is indicated by (n). This figure was reproduced and adapted from two Blackwell lab publications: 1.) An, J. H., and T. K. Blackwell. 2003. SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. *Genes & Development* 17:1882-1893 & 2.) An, J. H., K. Vranas, M. Lucke, H. Inoue, N. Hisamoto, K. Matsumoto, and T. K. Blackwell. 2005. Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. *Proceedings of the National Academy of Sciences* 102:16275-16280 (4, 5).



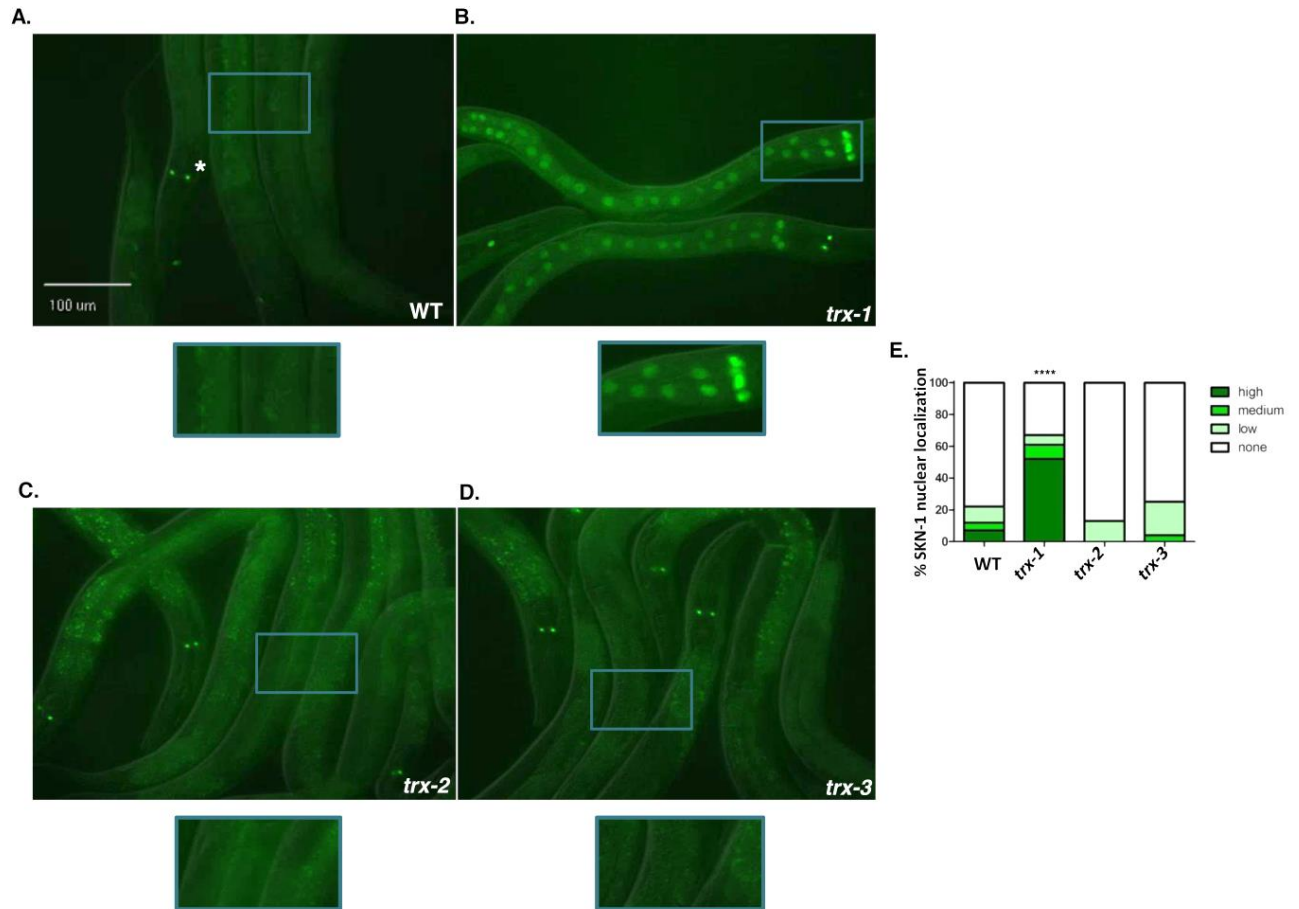
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## Results

### TRX-1 negatively regulates SKN-1 subcellular localization

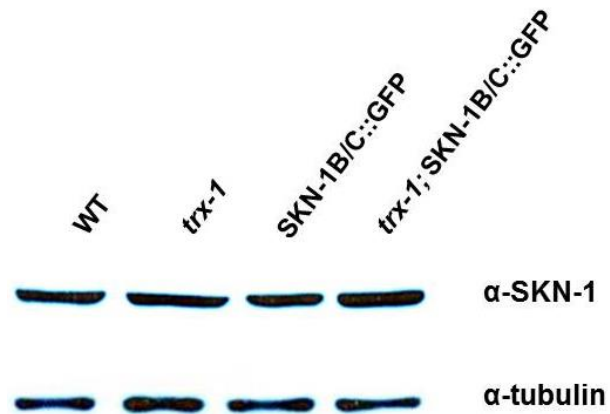
Previous studies in our laboratory identified a role for SKN-1 in the *C. elegans* immune response. Much like the oxidative burst of macrophages, we showed that the *C. elegans* dual oxidase, Ce-Duox1/BLI-3 is responsible for the purposeful production of ROS as a means of combatting bacterial infection (34). In a follow-up study, we showed that ROS produced by BLI-3 during infection activates SKN-1-dependent expression of antioxidants in a p38 MAPK pathway-dependent manner as a means to protect the host from the inadvertent consequences of protective ROS production (85). However, the mechanism(s) by which the p38 MAPK pathway is activated to stimulate SKN-1 remained elusive.

Given the ability of thioredoxins to regulate a variety of signaling pathways, specifically p38 MAPK pathway activation in mammals, we wondered whether thioredoxins can regulate SKN-1. Using fluorescence microscopy, we examined the ability of TRX-1, TRX-2, TRX-3, three thioredoxins encoded by the *C. elegans* genome, to regulate SKN-1 nuclear localization using the well characterized strain expressing GFP-tagged SKN-1 protein (SKN-1B/C::GFP), described previously (4, 27, 104, 145). Interestingly, SKN-1 localizes to the nuclei of intestinal cells in *trx-1* null mutants, even in the absence of stress (Figure 3.2 A-B). This effect was specific to *trx-1*. Loss of either *trx-2* or *trx-3* does not affect intestinal SKN-1 nuclear localization, compared to the parent background (Figure 3.2 A, C-D). Quantification demonstrated that loss of *trx-1* increases the percentage of intestinal SKN-1 nuclear localization three-fold (Figure 3.2E). The increase in nuclear localization of intestinal SKN-1::GFP is not due to a general increase in protein levels. We assayed the protein levels of intestinal SKN-1, isoform SKN-1c, via western analysis and found that the SKN-1c levels remain unaltered in *trx-1* null mutants compared to wild type (Figure 3.3).



**Figure 3.2: TRX-1 negatively regulates nuclear localization of intestinal SKN-1.**

**A-D)** Fluorescence microscopy was used to analyze the intestinal nuclear localization of SKN-1 (SKN-1B/C::GFP) upon the loss of *trx-1*, *trx-2*, or *trx-3*. Only upon the loss of *trx-1* did SKN-1::GFP accumulate in intestinal nuclei. Asterisk in panel A depicts constitutive SKN-1B/C::GFP localization in the nucleus of the ASI neurons. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes below each micrograph. **E)** Percent SKN-1::GFP nuclear localization was categorically scored and quantified as described in Materials and Methods. The percent SKN-1 nuclear localization increased three-fold upon loss of *trx-1* ( $P$ -value < 0.0001 as compared to wild type). Percentages are an average of three biological replicates (N=100 worms per replicate).



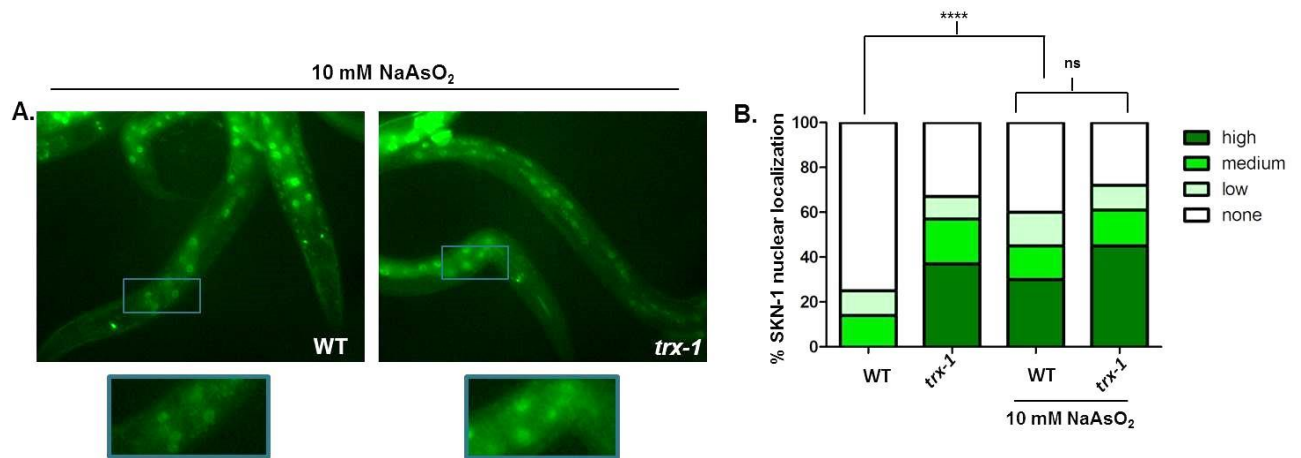
**Figure 3.3: SKN-1 protein levels remain unchanged in *trx-1* mutants.**

Western blotting was used to analyze endogenous SKN-1C protein levels in wild type, *trx-1*, *skn-1b/c::gfp*, and *trx-1; skn-1b/c::gfp* worms. SKN-1C protein levels remain unchanged upon loss of *trx-1* in both the wild type and *skn-1b/c::gfp* backgrounds. α-tubulin served as a loading control.

Moreover, the degree to which intestinal SKN-1::GFP nuclear localization increases upon loss of *trx-1* is similar to that seen upon exposure to the oxidative stressor, sodium arsenite (Figure 3.4). From this, we conclude that TRX-1 suppresses the nuclear localization of intestinal SKN-1 and therefore may be a novel negative regulator of this transcription factor.

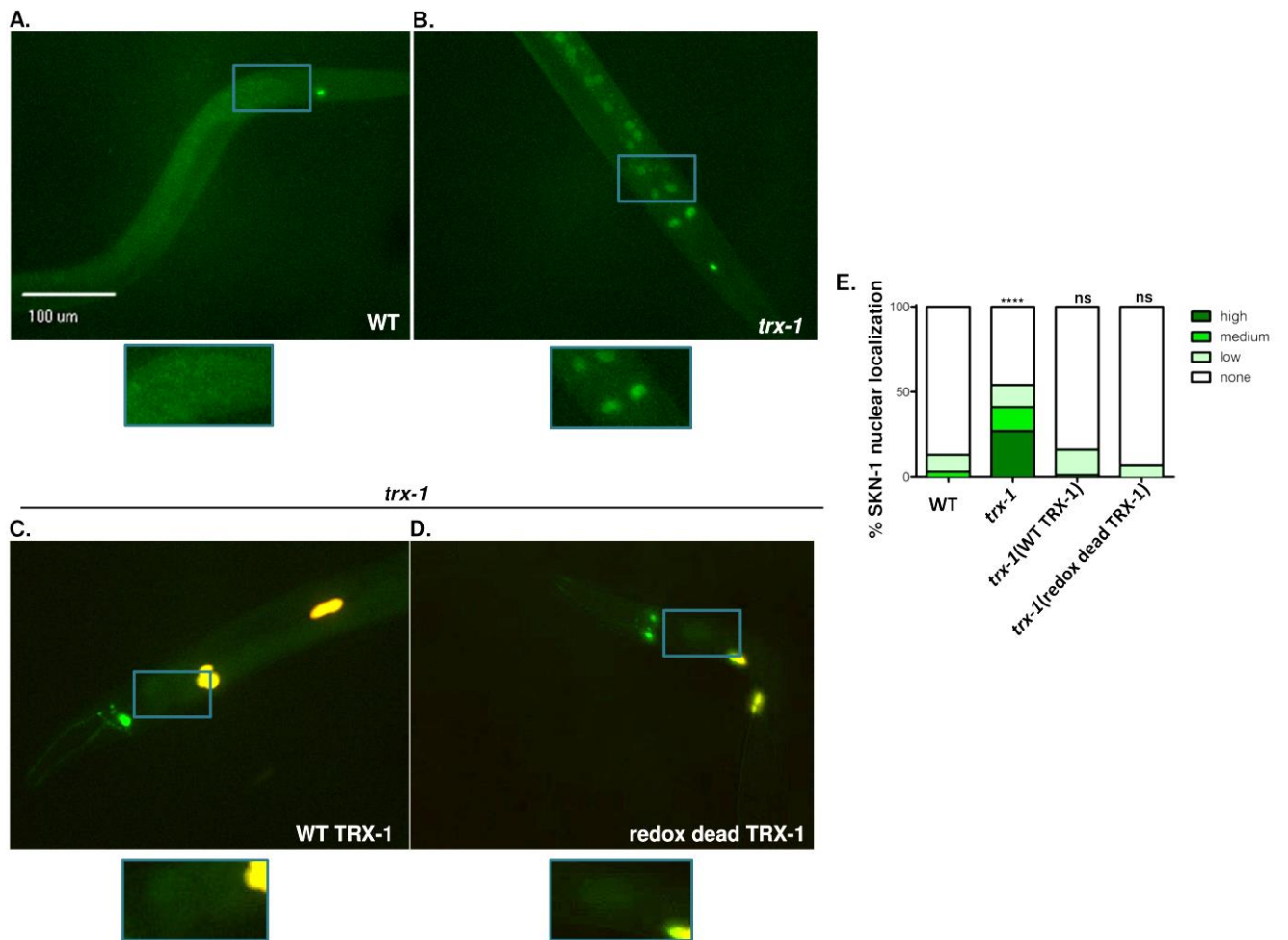
### **TRX-1 regulates intestinal SKN-1 nuclear localization in a redox-independent fashion**

Thioredoxins utilize a highly conserved CGPC (Cys-Gly-Pro-Cys) redox active site to reduce disulfide bonds of protein substrates (87). *C. elegans* TRX-1 has been shown to utilize this redox reactive capability to reduce insulin *in vitro* (101). However, thioredoxins also have redox-independent functions, notably in the promotion of protein folding and turnover (13). Given the ability of thioredoxins to elicit both redox-dependent and redox-independent functions, we investigated whether the redox reactive residues of TRX-1 were required for its regulation of intestinal SKN-1 nuclear localization. *trx-1* null mutants were complemented with either wild type *trx-1* or 'redox dead' *trx-1*, in which the redox reactive cysteines of *trx-1* within the CGPC motif are replaced with non-reactive serine residues (61). The transgenes were tracked using *Punc122::DsRed* as a co-injection marker, which labels coelomocytes (scavenger cells of the pseudocoelomic cavity) with red fluorescence (129). Some bleed-through into the green channel resulted in the marker appearing more yellow than red in the resulting pictures (Figure 3.5 C and 3.5 D). When SKN-1 nuclear localization was analyzed under non-stressed conditions, we observed that complementation with wild type *trx-1* restores proper SKN-1 localization, similar to that seen in the SKN-1B/C::GFP parent background (Figure 3.5 A,C,E). Interestingly, complementation with 'redox dead' *trx-1* also restores proper SKN-1 localization, indicating that TRX-1 regulates intestinal SKN-1 nuclear localization in a redox-independent fashion (Figure 3.5 D,E). Upon exposure to oxidative stress, an increase in SKN-1 nuclear localization was observed in both the wild



**Figure 3.4: Intestinal SKN-1 nuclear localization is increased as a result of exposure to Sodium Arsenite and it is not further increased upon *trx-1* loss.**

**A)** Fluorescence microscopy was used to analyze the intestinal nuclear localization of SKN-1 (SKN-1B/C::GFP) in wild type and *trx-1* worms after being exposed to 10mM NaAsO<sub>2</sub> for three hours at 20°C. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes below each micrograph. Control animals not exposed to NaAsO<sub>2</sub> are shown in Figure 1A and B. **B)** Percent SKN-1 nuclear localization was categorically scored and quantified as described in Materials and Methods. Intestinal SKN-1 nuclear localization significantly increased during oxidative stress (*P*-value < 0.0001). The degree to which intestinal SKN-1 nuclear localization increases upon loss of *trx-1* is similar to that seen upon exposure to the oxidative stressor, sodium arsenite (*P*-value 0.64, as compared to wild type) and their effects on SKN-1 nuclear localization are not additive.



**Figure 3.5: TRX-1 regulates intestinal SKN-1 nuclear localization in a redox-independent fashion .**

Fluorescence microscopy was used to analyze the intestinal nuclear localization of SKN-1::GFP in (A) wild type, (B) *trx-1* mutants, *trx-1* mutants complemented with either (C) wild type *trx-1* or (D) 'redox dead' *trx-1*. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes below each micrograph. E) Percent SKN-1::GFP nuclear localization was categorically scored and quantified as described in Materials and Methods. While the percent SKN-1::GFP nuclear localization increased over two-fold upon loss of *trx-1* ( $P$ -value < 0.0001), complementation

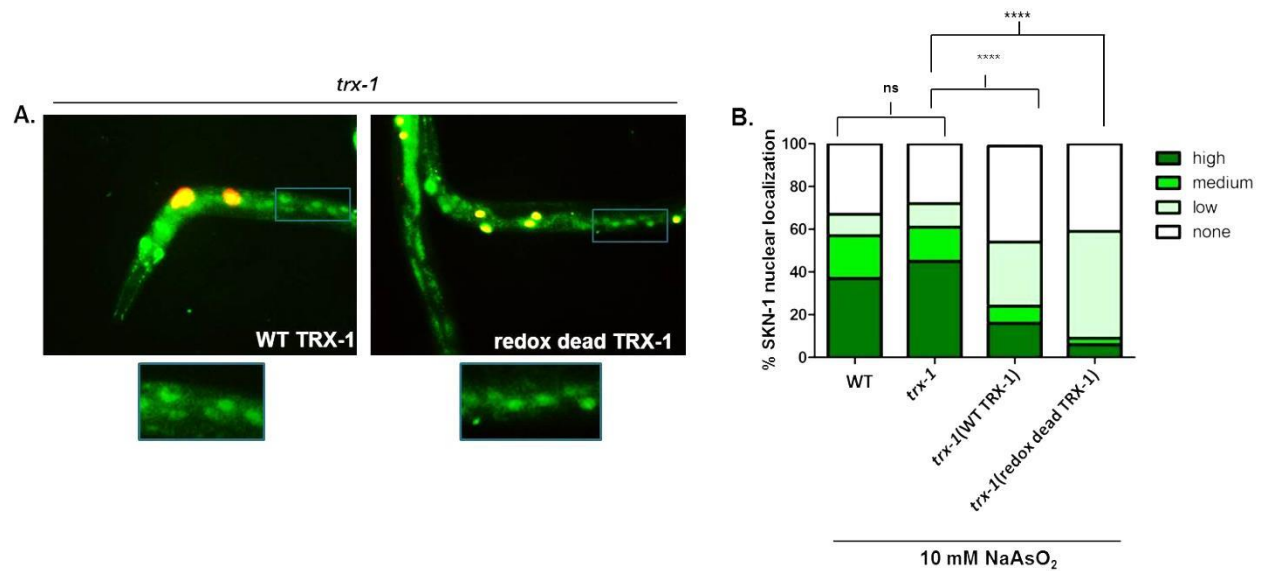
with either wild type or 'redox dead' *trx-1* restored proper SKN-1 localization, ( $P$ -value = 0.3843 and  $P$ -value = 0.1931, as compared to wild type, respectively). Percentages are an average of three biological replicates (N=40 worms per replicate).



type and 'redox dead' complements of *trx-1*, indicating that intestinal SKN-1 nuclear localization is indeed inducible in these strains (Figure 3.6). However, stress-induced SKN-1 nuclear localization was only partially complemented upon the complementation of both wild type and 'redox dead' TRX-1, as compared to the non-transgenic background. As complementation with transgenes often leads to overexpression, this may indicate that overproduction of TRX-1 causes it to maintain its role as a negative regulator of intestinal SKN-1 nuclear localization, even during stress.

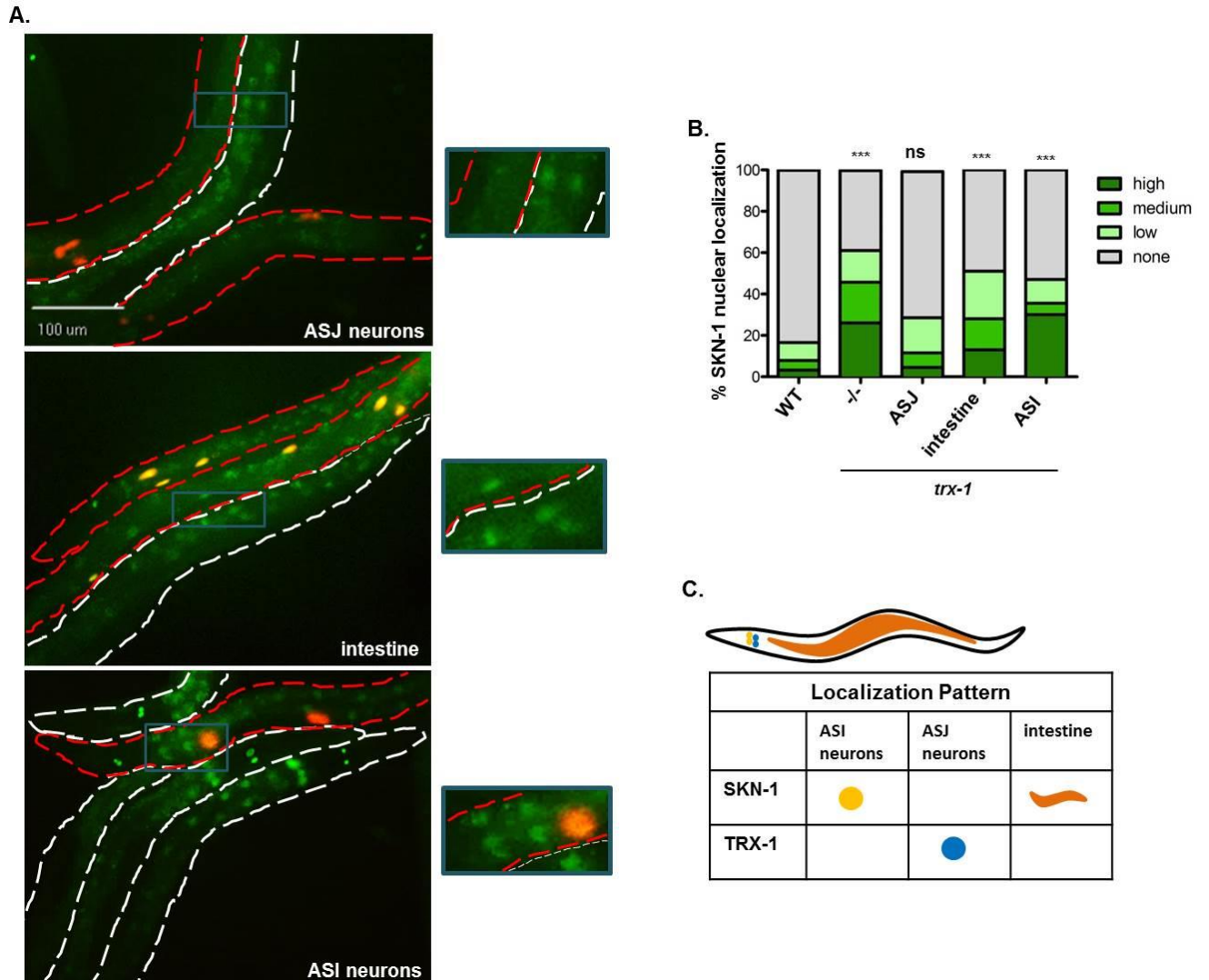
### **TRX-1 regulates SKN-1 localization cell non-autonomously from the ASJ neurons**

*skn-1* is constitutively expressed and localized to the nuclei of the ASI neurons and conditionally becomes localized to intestinal nuclei when worms are exposed to stress (4). *trx-1* is expressed solely in the ASJ neurons and impacts worm longevity (62, 76). Given that the expression of *trx-1* is restricted to the ASJ neurons, but is able to impact intestinal SKN-1 localization, we aimed to address the possibility that TRX-1 may regulate intestinal SKN-1 cell non-autonomously. To assess this, we complemented the *trx-1*;SKN-1B/C::GFP strain with *trx-1* expressed under the control of three separate tissue specific promoters, *ssu-1* (ASJ neurons), *ges-1* (intestine) and *daf-7* (ASI neurons) (30, 57, 187). To track tissue-specific rescue throughout the population, *Punc122::DsRed* was again used as a co-injection marker (129). In Figure 3.7A, worms outlined in red express *trx-1* with the indicated tissue specificity, while worms outlined in white do not carry the tissue specific rescue and display increased intestinal SKN-1 nuclear localization, thus serving as an internal control for the experiment. As evident in Figure 3.7A, the increased intestinal SKN-1 nuclear localization seen upon loss of *trx-1* was abolished upon specific expression of *trx-1* in the ASJ neurons, while rescue of *trx-1* expression to the intestine or ASI neurons could not restore proper intestinal SKN-1 nuclear localization.



**Figure 3.6: Intestinal SKN-1 nuclear localization upon both wild type and redox dead *trx-1* complementation is increased upon exposure to Sodium Arsenite.**

**A)** Fluorescence microscopy was used to analyze the intestinal nuclear localization of SKN-1 (SKN-1B/C::GFP) upon complementation of wild type or 'redox dead' *trx-1* after being exposed to 10mM NaAsO<sub>2</sub> for three hours at 20°C. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes below each micrograph. Non-transgenic controls can be seen in Supplementary Figure 3.5A. **B)** Percent SKN-1 nuclear localization was categorically scored and quantified as described in Materials and Methods. Both wild type and 'redox dead' complement of *trx-1* partially restored proper intestinal SKN-1 nuclear localization upon exposure to oxidative stress (*P*-value < 0.0001 and *P*-value < 0.0001, respectively, as compared to *trx-1*; *skn-1b/c::gfp*), indicating that intestinal SKN-1 nuclear localization is indeed inducible in this strain, however the ability to properly promote SKN-1 localization during stress is dampened by the overexpression of *trx-1*.



**Figure 3.7: TRX-1 regulates SKN-1 nuclear localization cell non-autonomously.**

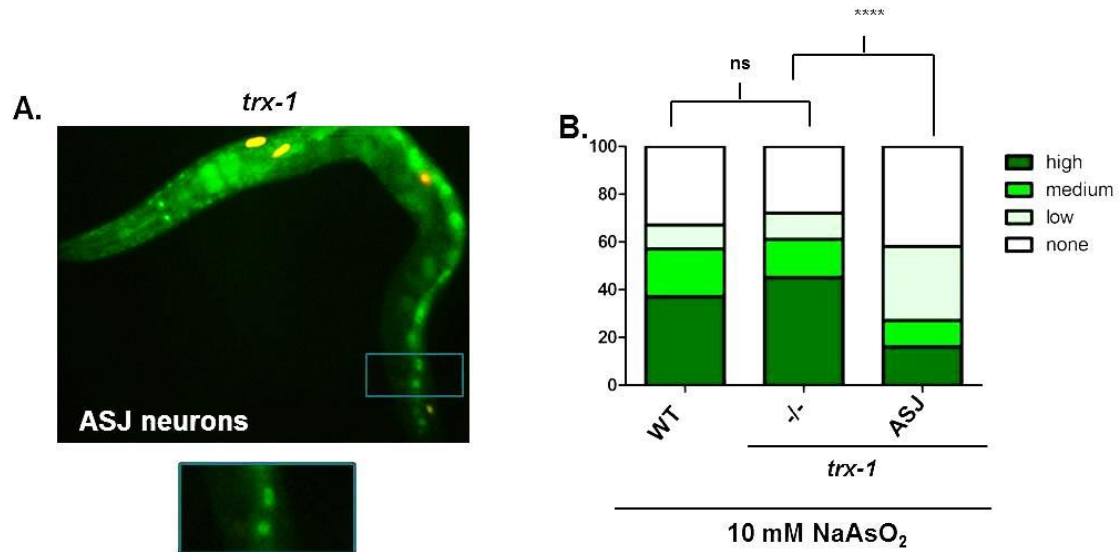
**A)** Fluorescence microscopy was used to analyze intestinal SKN-1 nuclear localization upon rescue of *trx-1* expression in specific tissues in *trx-1; skn-1b::gfp* animals. Worms outlined in red expressed wild type *trx-1* under the regulation of a promoter specific for the designated tissue. Non-transgenic worms (outlined in white) had a *trx-1; skn-1b::gfp* genotype, and served as internal controls. Only expression of *trx-1* in the ASJ neurons rescued proper intestinal SKN-1::GFP nuclear localization. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes to the right of each micrograph. **B)** Percent SKN-1::GFP nuclear localization was

categorically scored and quantified as described in Materials and Methods. Percentages are an average of three biological replicates ( $N \geq 35$  worms per replicate). The *trx-1* mutant exhibited a three-fold increase in the percentage of intestinal SKN-1::GFP nuclear localization ( $P$ -value  $< 0.0001$  as compared to wild type). The increased SKN-1::GFP nuclear localization seen upon loss of *trx-1* could be fully rescued with specific expression of *trx-1* in the ASJ neurons ( $P$ -value = 0.2377, as compared to wild type). Rescue of *trx-1* expression in the intestine and ASI neurons did not restore proper SKN-1 nuclear localization ( $P$ -value  $< 0.0001$  &  $P$ -value  $< 0.0001$  as compared to wild type, respectively).

**C)** A schematic depicting the tissue localization of TRX-1 and SKN-1, emphasizing the absence of overlapping tissue expression between these two proteins. The ability of ASJ-expressed TRX-1 to regulate intestinal SKN-1 nuclear localization indicates a mechanism of cell non-autonomous regulation.

To more quantitatively assess these observations, the percentage of SKN-1 nuclear localization was categorically scored and analyzed (Figure 3.7B). The loss of *trx-1* significantly increased SKN-1 localization about three-fold, as shown in Figure 3.2E. Restoring *trx-1* expression specifically in the ASJ neurons, reduced SKN-1 localization to levels seen in the SKN-1B/C::GFP parent background. This suggests that TRX-1 regulates intestinal SKN-1 nuclear accumulation cell non-autonomously from the ASJ neurons. To show that intestinal SKN-1 nuclear localization is still inducible in the ASJ-specific complement of *trx-1*, the strain was exposed to oxidative stress. Intestinal SKN-1 nuclear localization was partially restored (Figure 3.8). This finding further supports the notion that when overexpressed TRX-1 dampens the ability of oxidative stress to fully induce intestinal SKN-1 nuclear localization (Figure 3.8). In contrast to the ASJ complement, restoring *trx-1* expression to the intestine or the ASI neurons did not result in a rescue of the increased SKN-1 localization caused by loss of *trx-1*. It is interesting that artificially driving expression of *trx-1* in the intestine, the very same tissue in which SKN-1 localization is exhibited, could not restore proper SKN-1 localization. This further suggests that a critical action required for TRX-1-dependent SKN-1 regulation must occur from the distal site of the ASJ neurons. Furthermore, while SKN-1 is constitutively localized to the nuclei of the ASI neurons, expression of *trx-1* in these neurons does not abrogate SKN-1 nuclear localization. This suggests that TRX-1 specifically affects intestinal SKN-1 localization cell non-autonomously.

The model in Figure 3.7C summarizes the expression pattern of both *trx-1* and *skn-1*. *skn-1* is expressed in both the ASI neurons and intestine. *trx-1* is expressed solely in the ASJ neurons. Proper intestinal SKN-1 localization occurs only when *trx-1* null mutants are complemented with ASJ-specific *trx-1* expression. Therefore, we conclude that TRX-1 negatively impacts intestinal SKN-1 nuclear localization in a cell non-autonomous manner from the ASJ neurons.



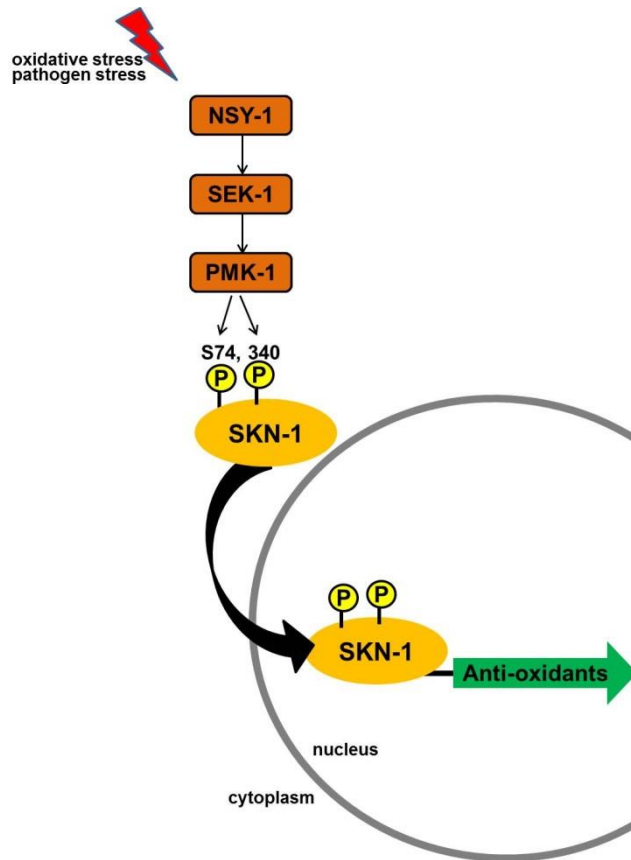
**Figure 3.8: Intestinal SKN-1 nuclear localization upon ASJ-specific rescue of *trx-1* is increased upon exposure to Sodium Arsenite.**

**A)** Fluorescence microscopy was used to analyze the intestinal nuclear localization of SKN-1 (SKN-1B/C::GFP) upon rescue of *trx-1* animals with ASJ-specific expression of wild type *trx-1* after being exposed to 10mM NaAsO<sub>2</sub> for three hours at 20°C. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes below each micrograph. Non-transgenic controls can be seen in Supplementary Figure 1A. **B)** Percent SKN-1 nuclear localization was categorically scored and quantified as described in Materials and Methods. ASJ-specific rescue of *trx-1* resulted in partial intestinal SKN-1 nuclear localization upon exposure to oxidative stress; a statistically significant difference of *P*-value < 0.0001, was apparent when the ASJ complement was compared to the parent background of *trx-1*; *skn-1b/c::gfp*.

### **TRX-1-dependent regulation of SKN-1 localization requires the p38 MAPK pathway**

It has previously been shown that the p38 MAPK pathway is an important regulator of SKN-1 localization and activation (97). The p38 MAPK pathway is comprised of three kinases, NSY-1 (MAPKKK), SEK-1 (MAPKK), and PMK-1 (the p38 MAPK). One outcome of the stimulation of this pathway by oxidative stress is the phosphorylation of SKN-1 at two serine residues, S74 and S340, ultimately leading to the nuclear translocation and transcriptional activation of this protein (Figure 3.9) (97). To highlight the importance of this signaling pathway, it has been shown that in the absence of a functional p38 MAPK pathway or upon alanine substitution at these critical residues of SKN-1, intestinal SKN-1 nuclear localization and activation does not occur, even during stress (97). Given that TRX-1 negatively impacts intestinal SKN-1 nuclear localization, we sought to address the dependence of this regulation on the p38 MAPK pathway.

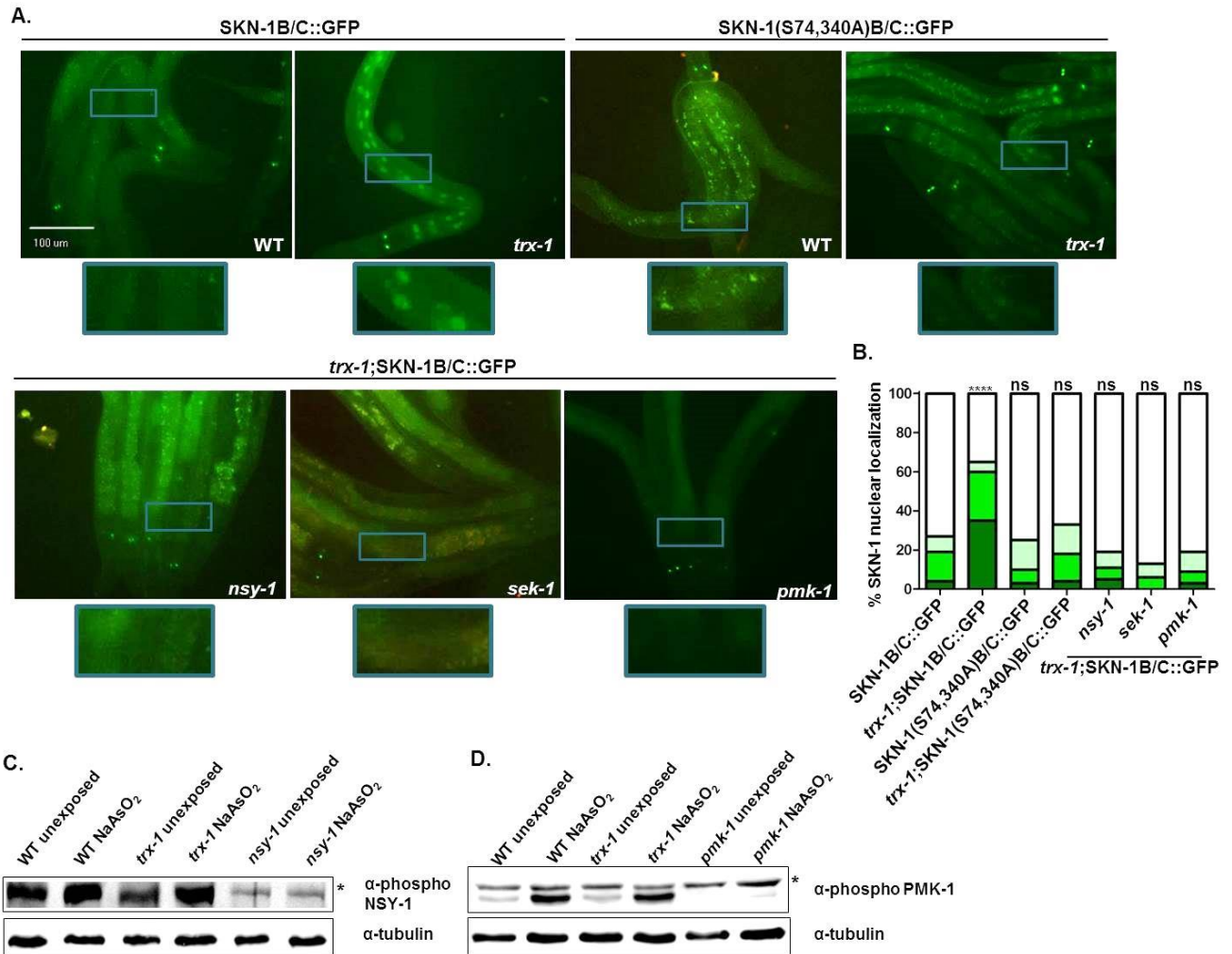
First, we examined the importance of SKN-1 phosphorylation on TRX-1-dependent intestinal SKN-1 nuclear localization. To do this, we generated a *trx-1*; SKN-1(S74,340A)B/C::GFP strain (97). While this strain manifested higher background fluorescence in the intestine, the fluorescent micrographs in Figure 3.10A and quantification in Figure 3.10B demonstrate reduced intestinal localization of this non-phosphorylatable version of SKN-1 as compared to SKN-1B/C::GFP, even upon loss of *trx-1*. To further address the necessity of TRX-1-dependent SKN-1 regulation on the p38 MAPK pathway, we crossed the *trx-1*;SKN-1B/C::GFP strain with null mutants of each p38 MAPK components (*nsy-1(ok593)*, *sek-1(km4)*, and *pmk-1(km25)*). Fluorescent micrographs in Figure 3.10A and quantification in Figure 3.10B show that while loss of *trx-1* alone causes a significant, three-fold increase in intestinal SKN-1 nuclear localization compared to wild type, the additional loss of any component of the p38 MAPK significantly abrogates this phenotype. These data suggest that TRX-1-dependent regulation of intestinal SKN-1 nuclear



**Figure 3.9: The p38 MAPK pathway promotes intestinal SKN-1 nuclear accumulation and SKN-1-dependent antioxidant expression in response to stress.**

The p38 MAPK pathway is required for SKN-1 activation in response to exposure to oxidative stressors, such as sodium arsenite, or pathogen stress, such as *Enterococcus faecalis* infection. Activated PMK-1 phosphorylates cytosolic SKN-1 at serine residues 74 and 340, facilitating intestinal nuclear accumulation and transcriptional activation. In the absence of *sek-1* and *pmk-1*, SKN-1 cannot translocate to the nucleus and activate the expression of antioxidants. Moreover, substitution of the PMK-1 sensitive serine residues 74 and 340 of SKN-1 with alanines suppresses the ability of SKN-1 to translocate to the nucleus and activate the expression of antioxidants.



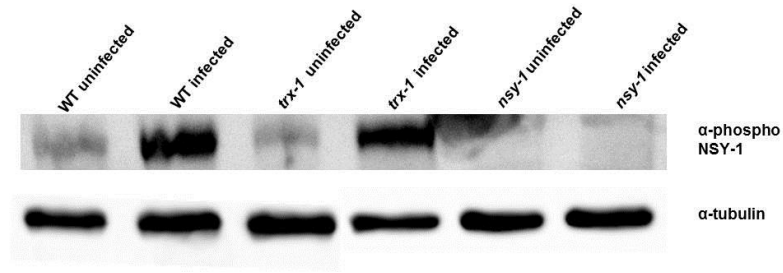


**Figure 3.10: TRX-1-dependent regulation of SKN-1 nuclear localization is dependent on the p38 MAPK pathway.**

**A)** Fluorescence microscopy was used to analyze *trx-1* worms that express a mutant form of SKN-1 (SKN-1(S74,340A)B/C::GFP), which cannot be phosphorylated by PMK-1. The increased percentage of intestinal SKN-1 nuclear localization seen upon loss of *trx-1* was abrogated upon mutation of Serines 74 and 340 of SKN-1. Mutants of p38 MAPK pathway components (*nsy-1*, *sek-1*, and *pmk-1*) were crossed into *trx-1*; *skn-1b/c::gfp* animals and viewed by fluorescence microscopy. The increased percentage of intestinal SKN-1 nuclear localization seen upon loss of *trx-1* was abrogated upon the additional loss of each p38 MAPK signaling component. Wild type and *trx-1* mutants with a *skn-1b/c::gfp* background

are shown as controls. Worms were visualized using a 20X objective. Blue boxes indicate the portion of the micrograph field that is magnified in the boxes below each micrograph. **B)** Percent SKN-1::GFP nuclear localization was categorically scored and quantified as described in Materials and Methods. Percentages are an average of three biological replicates (N≥50 worms per replicate). *trx-1* mutants expressing the mutated form of SKN-1 did not exhibit increased nuclear SKN-1 localization (*P*-value = 0.4895, as compared to wild type). *trx-1(ok1449)*; *nsy-1(ok593)*, *trx-1(ok1449)*; *sek-1(km4)* and *trx-1(ok1449)*; *pmk-1(km25)* mutants did not exhibit the increased SKN-1 localization seen in *trx-1* single mutants (*P*-value = 0.9629, *P*-value = 0.2322, and *P*-value = 0.8863, as compared to wild type, respectively). **C)** Western blotting was used to analyze the level of phosphorylation (at residue Thr829) of NSY-1 in wild type and *trx-1* mutants with and without exposure to the oxidative stressor sodium arsenite. NSY-1 phosphorylation was not increased in *trx-1* mutants, as compared to wild type animals, regardless of sodium arsenite exposure. *nsy-1* mutants served as a negative control and  $\alpha$ -tubulin as a loading control. **D)** Western blotting was used to analyze the level of phosphorylation (at residues Thr180 & Thr182) of PMK-1 in wild type and *trx-1* mutants with and without exposure to the oxidative stressor sodium arsenite. PMK-1 phosphorylation was not increased in *trx-1* mutants, as compared to wild type animals, regardless of sodium arsenite exposure. *pmk-1* mutants served as a negative control and  $\alpha$ -tubulin as a loading control. Black asterisks indicate non-specific bands. Both of the blots shown are representative of three biological replicates.

localization requires p38 MAPK-dependent phosphorylation of SKN-1 at serine residues 74 and 340. While we were able to show that TRX-1-dependent regulation of intestinal SKN-1 nuclear localization was dependent on the p38 MAPK pathway, we wished to address whether the dependence was direct or indirect, i.e. in the same or a parallel pathway. One mechanism by which TRX-1 could modulate SKN-1 localization in a direct, p38 MAPK-dependent manner would be to regulate the level of activity of a p38 MAPK component. To address this, we sought to determine whether *trx-1* null mutants exhibit increased activation of NSY-1 and/or PMK-1, which can be measured by determining the levels of phosphorylation on the relevant residues in these kinases. In mammals, autophosphorylation of ASK, the mammalian NSY-1 homolog, at threonine 845 activates the kinase (206). In *C. elegans*, this threonine residue is found at position 829 of the amino acid sequence, and the surrounding amino acids are fully conserved between mammals and worms. We took advantage of a peptide-derived antibody specific for mammalian ASK1 phosphorylation at threonine 845 that has previously been shown to cross-react with phosphorylated NSY-1 (139). To analyze PMK-1 activation, we obtained an antibody specific to PMK-1 phosphorylated at threonine 180 and 182. (188). Using Western Blot analysis, we tested NSY-1 and PMK-1 phosphorylation in wild type and *trx-1* null mutants which demonstrated no increase in active NSY-1 (Figure 3.10C) or active PMK-1 (Figure 3.10D) in a *trx-1* null mutant, as compared to wild type. Exposure to sodium arsenite stress modestly increased NSY-1 activation and dramatically increased PMK-1 phosphorylation, but to a similar extent in both wild type animals and *trx-1* null mutants (Figure 3.10C-D). Previously, *Pseudomonas aeruginosa* infection had been shown to increase NSY-1 phosphorylation dramatically (139). Similarly, we saw a dramatic increase in NSY-1 phosphorylation upon *P. aeruginosa* infection, however the level of increased NSY-1 phosphorylation of *trx-1* null mutants did not differ from wild type animals (Figure 3.11). These data indicate that TRX-1 does not increase signaling through the p38 MAPK



**Figure 3.11: *Pseudomonas aeruginosa* infection increases NSY-1 phosphorylation to a similar extent in both wild type animals and *trx-1* mutants.**

Western blotting was used to analyze the level of phosphorylation (at residue Thr829) of NSY-1 in wild type and *trx-1* mutants after being infected with *Pseudomonas aeruginosa* (as compared to uninfected worms) for 3 hours. NSY-1 phosphorylation was increased upon infection, however this increase did not differ between *trx-1* mutants and wild type animals. *nsy-1* mutants served as a negative control and  $\alpha$ -tubulin as a loading control.

pathway, as measured by NSY-1 and PMK-1 phosphorylation levels, to cause the observed increase in SKN-1 nuclear localization. Overall, these experiments suggest that while regulation of SKN-1 nuclear localization depends on p38 MAPK signaling, TRX-1 likely does not achieve this regulation by affecting the phosphorylation levels of the kinases. These data support a model in which TRX-1 acts in a pathway parallel to the p38 MAPK pathway, but is only able to regulate after SKN-1 has been phosphorylated by the p38 MAPK pathway.

## Discussion

In this chapter, I demonstrate a novel mechanism of SKN-1 regulation in which TRX-1 specifically regulates intestinal SKN-1 nuclear localization in a redox-independent, cell non-autonomous fashion from the ASJ neurons (Figures 3.3-3.8). This method of regulation requires the p38 MAPK pathway, however may occur through a parallel, dependent pathway (Figure 3.10, 3.11).

The ability of TRX-1 to impact intestinal SKN-1 nuclear localization is not shared by all thioredoxins, as loss of TRX-2 and TRX-3 had no effect (Figure 3.2). TRX-2 is a component of the mitochondrial thioredoxin system in several tissues and is 54% similar and 32% identical to TRX-1 (27). TRX-3 is localized to the cytoplasm and nuclei of intestinal cells and is important for resistance to certain bacterial and fungal pathogens and is 42% similar and 26% identical to TRX-1 (104). Given the differences in cellular expression patterns and subcellular localization, we were not surprised to find that regulation of intestinal SKN-1 nuclear localization was not common to the thioredoxin family as a whole. However, given that TRX-3 is localized to the same tissue as SKN-1, we were surprised to find that loss of *trx-3* does not affect intestinal SKN-1 nuclear localization. These data suggest that a critical step in TRX-1-dependent SKN-1 regulation occurs from the ASJ neurons, specifically. Alternatively, regions of sequence unique to *trx-1*, specifically those not shared with *trx-3*, may be critical for the ability of TRX-1 to regulate SKN-1 nuclear localization.

Thioredoxins utilize a pair of conserved, redox reactive cysteine residues at their active site to fulfill a variety of oxidoreductase-related functions, including maintenance of cellular homeostasis and regulation of transcription factors [reviewed in (86, 87)]. However, thioredoxins also facilitate important cellular processes independently of their oxidoreductase functions. Recently, thioredoxins have been demonstrated to exhibit redox-independent chaperone-like functions (52). Specifically in *C. elegans*, TRX-1 has been shown to modulate DAF-28 signaling during dauer formation in a redox-independent fashion (61). 'Redox dead' complementation of *trx-1* demonstrated that TRX-1 regulates SKN-1 in a manner independent of its redox status, expanding upon the list of redox-independent functions of TRX-1. This is further supported by the fact that there is no significant difference in the ability of stressed *trx-1* null mutants, as compared to both stressed wild type animals and unstressed *trx-1* null mutants, to impact intestinal SKN-1 nuclear localization. These data suggest that *trx-1* negatively regulates intestinal SKN-1 nuclear localization regardless of the redox ability of *trx-1* or the presence of an oxidative stressor.

Cell non-autonomous regulation is a classic method of coordinating an organism-wide response in a predictive and adaptive fashion. In *C. elegans*, cell non-autonomous signaling has been shown to regulate several stress responses including the heat shock response, the unfolded protein response, pathogen stress response, and longevity (172, 198, 203, 218, 219, 236). Overall, knowledge regarding the signals that coordinate cell non-autonomous regulation is very limited. However, numerous biological components have been implicated as trans-tissue signaling molecules, examples being lipids and free fatty acids, 'mitokines,' neuropeptides, and neurohormones (202, 218). In this work, we expand the list of stress pathways governed cell non-autonomously to include the oxidative stress transcription factor, SKN-1.

The requirement of the p38 MAPK pathway for TRX-1-dependent SKN-1 regulation was not surprising, given that the p38 MAPK signaling pathway has been found to be

essential in several previously identified mechanisms of SKN-1 regulation, including *daf-2*, *gsk-3*, and *wdr-23* (5, 124, 214). Whether this is a direct or indirect dependence is still unknown, and several models can be postulated. First, it is possible that TRX-1 acts in the same pathway as p38 MAPK signaling to regulate SKN-1 localization. While we ruled out the possibility that loss of *trx-1* increases NSY-1 or PMK-1 phosphorylation, there could be, and likely are, novel post-translational modifications of either kinase that facilitates proper SKN-1 regulation in a TRX-1-dependent manner (private communication, Keith Blackwell, Harvard Medical School). Another possibility is that TRX-1 regulates intestinal SKN-1 nuclear localization in a pathway parallel to, but still dependent on, the p38 MAPK pathway. For instance, TRX-1 may only be able to regulate SKN-1 after it has been phosphorylated by PMK-1 at serines 74 and 340.

In conclusion, we report that in *C. elegans* the major oxidative stress transcription factor, SKN-1, is cell non-autonomously regulated by the ASJ neurons via the thioredoxin TRX-1. This further expands the list of stress responses that are modulated from distant tissues at the organismal level.

## **Chapter 4: TRX-1-dependent SKN-1 regulation does not result in activation of SKN-1 or an increase in any previously characterized protective responses**

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## Introduction

A classic hallmark of previously identified mechanisms of SKN-1 regulation is the correlation between intestinal SKN-1 nuclear localization and activation of the transcription factor, as exhibited by *i)* resistance to both oxidative and pathogen stressors and *ii)* the increased expression of phase II antioxidants (4, 5, 85, 97, 124, 214). Table 4.1 depicts the previously identified mechanisms of SKN-1 regulation and serves to highlight the preponderance of SKN-1 nuclear accumulation resulting in SKN-1 transcriptional activation. Given that loss of *trx-1* dramatically increases intestinal SKN-1 nuclear localization, we predicted that *trx-1* mutants would exhibit increased expression of SKN-1-dependent antioxidants and demonstrate resistance to previously characterized oxidative and pathogen stressors (85, 154, 224).

## Results

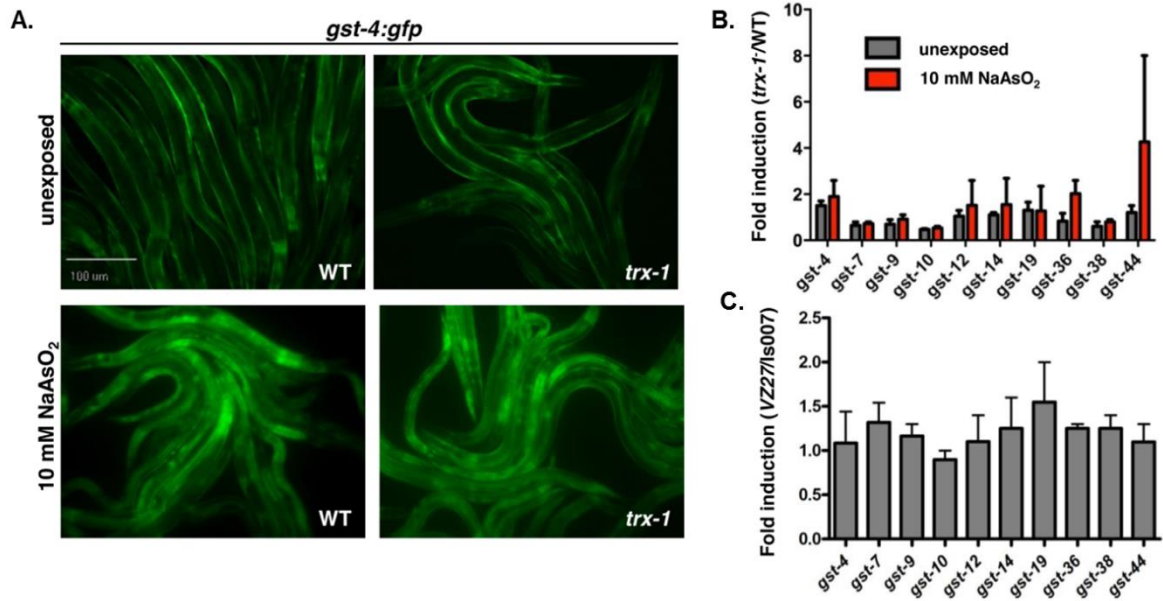
### **TRX-1-dependent SKN-1 regulation does not result in transcriptional activation of SKN-1**

We examined the transcriptional activity of SKN-1 in wild type and *trx-1* null mutants under both stressed and unstressed conditions. As previously mentioned, increased intestinal SKN-1 nuclear localization typically results in increased transcriptional activation of SKN-1 (5, 97, 124, 214). A transcriptional *gst-4::gfp* reporter strain, which has been well characterized to act as a readout of SKN-1-dependent transcriptional activation, was crossed with the *trx-1* null mutant and analyzed via fluorescence microscopy (165). As shown in Figure 4.1A, loss of *trx-1*, as compared to wild type, did not significantly increase *gst-4* expression, as determined by GFP fluorescence in intestinal cells under unstressed conditions. Furthermore, upon exposure to sodium arsenite, induction of this reporter in the

<b>Mechanism of SKN-1 regulation</b>	<b>Activation/Repression</b>	<b>NA</b>	<b>TA</b>	<b>Ref.</b>
oxidative stressors (sodium arsenite, paraquat, hydrogen peroxide, <i>t</i> -BOOH, sodium azide, jugalone)	Activates	+	+	(97, 107)
p38 signaling	Activates	+	+	(97)
<i>gsk-3</i> signaling	Repress	+	+	(5)
IIS ( <i>daf-2</i> ) signaling	Repress	+	+	(214)
WDR-23 facilitated degradation	Repress	+	+	(39)
<i>nekl-1</i> , <i>ikke-1</i> , <i>mkk-4</i> , <i>pdhk-2</i>	Activates	+	+	(109)
loss of chaperonin complex	Activates	-	+	(107)
loss of ubiquitin ligases	Activates	+	-	(107)
loss of core proteasomal subunits	Activates	+	+	(107)
loss of regulatory proteasomal subunits	Activates	+	-	(107)
unfolded proteins/ER stress	Activates	n/a	+	(73)
mTOR signaling	Represses	+	+	(179)

**Table 4.1: Mechanisms of SKN-1 regulation**

Table lists the stimulatory and regulatory mechanisms of SKN-1 activation and repression (denoted by activates or represses). The majority of mechanisms elicit both SKN-1 nuclear accumulation and transcriptional activation. Only the loss of regulatory proteasomal subunits elicits SKN-1 nuclear accumulation without concomitant transcriptional activation. In one case (loss of the chaperonin complex) SKN-1 transcriptional activation is elicited without nuclear accumulation. NA: nuclear accumulation of SKN-1; TA: transcriptional activation of SKN-1. \*Nuclear accumulation of the oxidative stressor jugalone could not be visualized.



**Figure 4.1: Loss of *trx-1* does not impact SKN-1-dependent gene expression.**

**A)** Fluorescence microscopy was used to analyze *gst-4::gfp* expression in wild type and *trx-1* mutant animals with (bottom panel) and without (top panel) five hr exposure to 10mM sodium arsenite. *gst-4* expression was not induced in the intestine of *trx-1* mutants as compared to wild type. Worms were visualized using a 10X objective. **B)** qRT-PCR was used to determine the fold change in SKN-1-dependent gene expression in *trx-1* mutants (as compared to wild type animals) with (red bars) and without (black bars) 5 hour exposure to 10 mM sodium arsenite. Expression of SKN-1-dependent genes did not significantly change in *trx-1* mutants, regardless of stress. **C.** qRT-PCR was used to determine the fold change in SKN-1-dependent gene expression in *trx-1*;SKN-1B/C::GFP (VZ27) animals as compared to SKN-1B/C::GFP (Is007) control animals. The expression of SKN-1-dependent genes did not significantly change upon the loss of *trx-1* in the SKN-1B/C::GFP parent background. The average gene expression of biological triplicates is shown and the error bars represent the standard error of the mean (SEM).

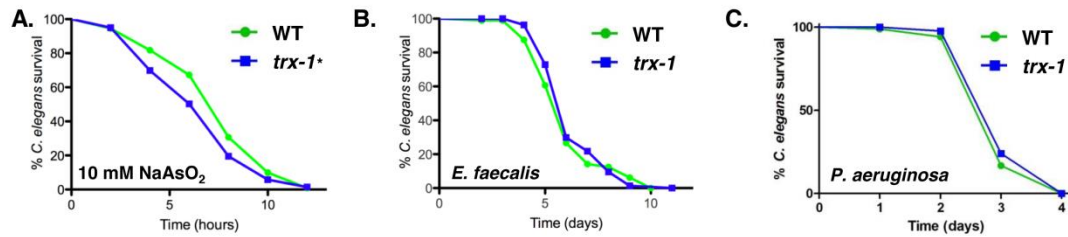
intestine of *trx-1* mutants was comparable to wild type (Figure 4.1A). To enable the examination of additional SKN-1 regulated genes in a quantitative manner, qRT-PCR was utilized. In agreement with the *gst-4::gfp* reporter strain, loss of *trx-1* did not significantly increase gene expression of any of the ten SKN-1-dependent genes analyzed as compared to wild type worms, under unstressed conditions or after exposure to sodium arsenite (Figure 4.1B) (154). Furthermore, there is no significant difference in SKN-1-dependent transcripts in *trx-1*;SKN-1B/C::GFP worms as compared to SKN-1B/C::GFP animals (Figure 4.1C). These data suggest that while intestinal nuclear localization of SKN-1 is increased, loss of *trx-1* may not affect the classical antioxidant transcriptional activity of SKN-1.

### **TRX-1-dependent SKN-1 regulation does not result in previously characterized protective SKN-1-dependent responses**

As previously shown with other oxidative stressors (101), *trx-1* mutants were sensitive to 10 mM sodium arsenite (oxidative stress) (Figure 4.2A). However, this may be explained by the intrinsic short-lived phenotype of *trx-1* worms (101, 145). Furthermore, *trx-1* mutants exhibit no significant increase in survival upon *Enterococcus faecalis* (Figure 4.2B) or *Pseudomonas aeruginosa* (Figure 4.2C) infection (pathogen stress), indicating that loss of *trx-1* does not impact previously characterized *skn-1*-dependent protective stress responses (4, 85).

## **Discussion**

In this chapter, I demonstrated that while TRX-1 regulates nuclear accumulation of intestinal SKN-1, it does not impact SKN-1 transcriptional activity or previously characterized SKN-1-dependent protective responses (Figures 4.1, 4.2).



**Figure 4.2: Loss of *trx-1* does not promote previously characterized SKN-1-dependent protective responses.**

Resistance to several stressors, including **(A)** 10 mM sodium arsenite, **(B)** *Enterococcus faecalis* infection, and **(C)** *Pseudomonas aeruginosa* infection was examined. *trx-1* mutants were sensitive to oxidative stress induced by sodium arsenite ( $P$ -value = 0.02). Loss of *trx-1* did not significantly alter the ability to resist either pathogen infection ( $P$ -value = 0.3261 and  $P$ -value=0.1527, respectively).

In my opinion, the TRX-1-dependent dissociation of SKN-1 nuclear localization from transcriptional activation is one of the more intriguing findings of this study. As previously mentioned, other mechanisms that affect nuclear localization of SKN-1, such as regulation via *gsk-3*, *wdr-23* and the insulin and p38 MAPK signaling pathways, are characterized by congruence between the degree of nuclear localization of intestinal SKN-1 and the degree of SKN-1-associated gene expression (5, 39, 97, 124, 214). Dissociation of these layers of regulation has been previously described. For example, several proteasome regulatory subunits and ubiquitin hydrolases stimulate increased intestinal SKN-1 nuclear localization but not increased *gst-4* expression. However, it is not clear how dissociation of intestinal SKN-1 nuclear localization and transcriptional activation occur in this circumstance. Furthermore, loss of certain chaperonins and a proteasomal protease, *pas-6*, elicits an opposite effect, in which increased *gst-4* expression is not accompanied by detectable intestinal SKN-1 nuclear localization (107). Additionally, loss of TOR signaling or treatment with rapamycin, which results in increased autophagy and decreased translation, causes an increase in SKN-1-associated gene expression without concomitant nuclear localization (179).

The possibility remains, however, that both SKN-1-dependent transcription and/or protective responses may be affected by the loss of *trx-1*. While we examined the expression of ten *skn-1*-dependent antioxidants and two stress-related (oxidative and pathogenic) protective responses, *skn-1* controls the expression of a battery of antioxidants and plays an important role in numerous physiological processes (19, 154). Additionally, *skn-1* regulates the expression of transcriptional programs that are not characterized by the increased expression of antioxidants (73). Therefore, it is possible that the expression of an alternative set of SKN-1-dependent genes and physiological processes are affected in *trx-1* null mutants. These possibilities are addressed in Chapters 5 and 6.

In conclusion, TRX-1 is now one of several factors that affect intestinal SKN-1 nuclear localization in a manner independent of its degree of transcriptional activation. Understanding the basis of SKN-1 activity, or lack of activity, under these incongruous circumstances is an area ripe for future investigation. While these findings increase the complexity of intestinal SKN-1 regulation, they highlight the importance of utilizing overlapping layers of regulation to ensure tight control of SKN-1 activation.

## **Chapter 5: Cuticle components and lipid transport gene expression altered in *trx-1* null mutants**

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## Introduction

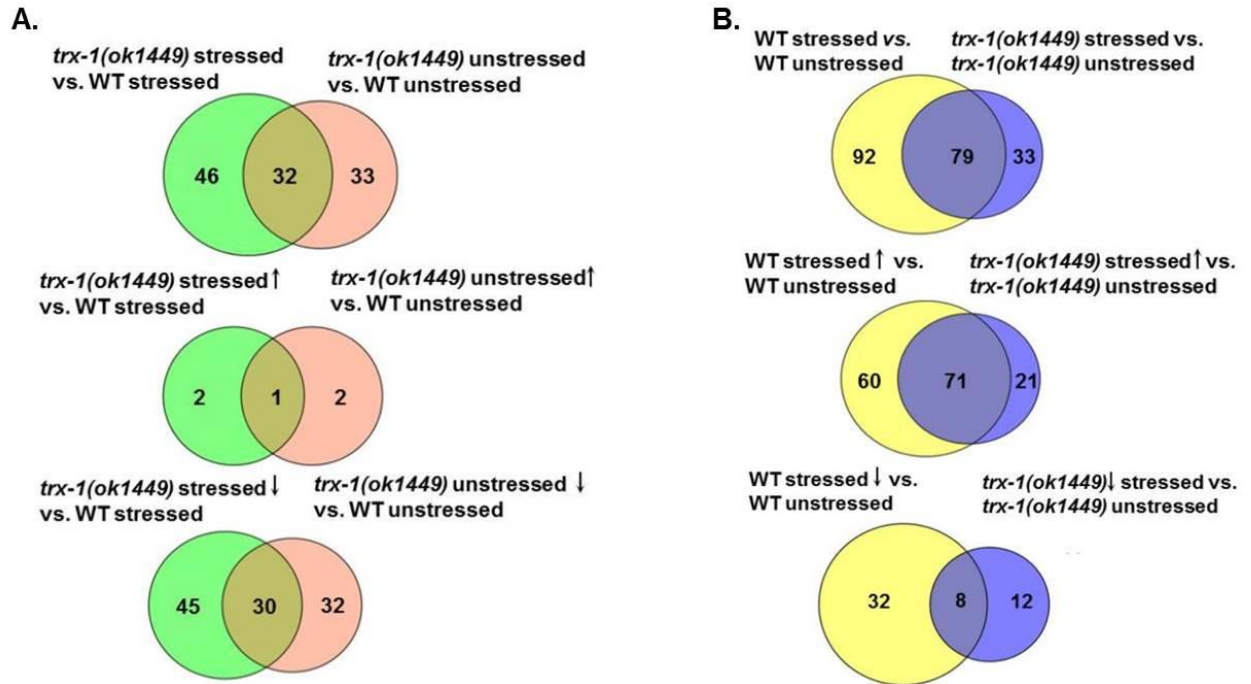
Thioredoxins are an evolutionarily conserved family of proteins that are responsible for a variety of cellular functions including both those dependent on its oxidoreductase ability, such as in disulfide bond reduction and redox regulation, and those independent of its oxidoreductase ability, such as its involvement with increasing the processivity of the T7 DNA polymerase (7, 91, 182). In *C. elegans*, thioredoxins are understudied and limited roles for these proteins are known. Although loss of *trx-1* results in decreased longevity, overexpression of TRX-1 extends lifespan and *trx-1* null mutants exhibit sensitivity upon exposure to the oxidative stressor paraquat (101). However, despite *trx-1* null mutants exhibiting a dramatic lifespan defect, the physiological process or processes in which *trx-1* is involved remain unknown. Translation fusion of GFP to full length TRX-1 protein demonstrates that TRX-1 expression is restricted to the ASJ neurons, a pair of amphid chemosensory neurons that control entry and exit from the stress-resistant dauer state of larva, among other processes (145). Concordantly, TRX-1 represses the insulin-like signaling peptide, *daf-28*, which affects dauer arrest in a *daf-2*-dependent manner (125). Interestingly, the oxidoreductase function of *trx-1* is not required for this function. However, the interaction between DAF-28 and TRX-1 does not explain the lifespan defect of *trx-1* mutants. Furthermore, TRX-1 regulates dietary restriction-dependent lifespan extension. Specifically, dietary restriction can be modeled genetically using *eat-2* mutants, which, due to a pharyngeal pumping defect, demonstrate decreased food consumption. The *eat-2* mutants are extremely long-lived, but concurrent loss of *trx-1* is sufficient to fully suppress this lifespan extension, indicating that *trx-1* regulates *eat-2*-dependent promotion of longevity. However, while this *trx-1*-dependent phenotype is more closely related to aging, it still does not offer an explanation for the longevity defect of *trx-1* null mutants.

Two perplexing findings were uncovered in the last two chapters: *i.*) the cell non-autonomous regulation of SKN-1 via TRX-1 expressed from the ASJ neurons and *ii.*) the lack of SKN-1 transcriptional activation seen in *trx-1* null mutants, given the increased intestinal nuclear accumulation. Although this is indeed an interesting finding, it remains entirely unclear how TRX-1 regulates SKN-1 from the ASJ neurons. There are numerous neurohormones, neuropeptides, insulin-like peptides, and lipid signals that impact cell non-autonomous regulation (218). Furthermore, a limited bank of antioxidants was assessed by qRT-PCR to analyze the transcriptional activation of SKN-1, which may not accurately represent SKN-1 transcriptional activation as a whole. With this in mind, and compounded by the lack of knowledge regarding the physiological relevance of the *trx-1*-dependent lifespan defect, we reasoned that examination of the global transcriptional changes in *trx-1* mutants, as elucidated upon transcriptional profiling via RNA Sequencing, may potentially answer one or all of the ambiguities surrounding the role of *trx-1*.

## Results

### Transcriptome analysis of *trx-1* mutants

Because TRX-1-dependent SKN-1 nuclear localization did not appear to activate expression of the SKN-1 regulon in a limited analysis (Figure 4.1A-C), we utilized a more unbiased approach to identify SKN-1-regulated genes in a *trx-1* mutant background. Furthermore, we wished to better understand how loss of *trx-1* impacts the transcriptome as a whole. We used RNA-Seq to examine global transcriptional changes in *trx-1* mutants as compared to wild type worms, with and without exposure to sodium arsenite. Interestingly, loss of *trx-1* results in a decrease in gene expression, with 75 (with stress) and 62 (without stress) genes being down regulated, whereas only three genes are up regulated regardless of stress (Figure 5.1A). Specifically, upon loss of *trx-1*, there is a significant enrichment in the down regulation of genes encoding cuticle components and lipid localization and



**Figure 5.1: Transcriptome analysis of *trx-1* mutants.**

**A)** Top: Number of shared genes significantly changed in *trx-1* animals, as compared to wild type animals, in both stressed (10 mM sodium arsenite) and unstressed conditions. Middle: Number of shared, over-expressed genes. Bottom: Number of shared, under-expressed genes. **B)** Top: Number of shared genes significantly changed in stressed (10 mM sodium arsenite), as compared to unstressed, conditions in both wild type and *trx-1* animals. Middle: Number of shared, over-expressed genes. Bottom: Number of shared, under-expressed genes.

transport (Table 5.1). Furthermore, RNA-Seq also validated that the oxidative stress transcriptional response in *trx-1* animals does not greatly differ from that of wild type animals (Figure 5.1B). While *trx-1* animals have overall lower gene expression than wild type animals, regardless of stress, the enrichment levels of glutathione transferase activity do not differ between stressed *trx-1* and wild type animals (Tables 5.2-5.4). While thioredoxins are commonly regarded as general cellular antioxidants, loss of *trx-1* does not elicit a compensatory increase in the expression of alternative antioxidants, suggesting that this is not the major role of TRX-1. The RNA-Seq results were validated using qRT-PCR to verify the changes in gene expression of six down regulated and three up regulated genes (Figure 5.2A). Overall, we conclude that loss of *trx-1* causes a general down regulation of the expression of cuticle components and lipid localization and transport genes. Furthermore, the transcriptional oxidative stress response does not seem to be impaired in *trx-1* animals, as compared to wild type animals.

Three genes, *lips-6*, *lips-11*, and *lbp-8*, are up regulated upon loss of *trx-1*. Interestingly, these three genes are related to lipid metabolism and transport as they encode two uncharacterized lipase-like proteins and one lipid binding protein, respectively. We were interested in addressing whether SKN-1 was important for the up regulation of these genes. Using qRT-PCR, we examined the fold change of these genes in *trx-1* animals, as compared to wild type, after knockdown of *skn-1* via RNAi. Only *lips-6* expression is significantly reduced after knockdown of *skn-1* (Figure 5.2B), though it is not a complete reduction, suggesting that, while SKN-1 has an effect, other factors may be involved in *lips-6* regulation.

#### **Total lipid levels are unaltered in *trx-1* null mutants**

Several genes associated with lipid metabolism were identified in our GO Term analysis of the genes enriched in *trx-1* null mutants (Table 5.1). Interestingly, while four vitellogenins are downregulated upon loss of *trx-1* (*vit-2*, *vit-4*, *vit-6*, *vit-5*), two lipases (*lips-6*

<b>GO Term analysis of genes that changed upon loss of <i>trx-1</i>, as compared to wild type, in unstressed conditions</b>	
<b>GO Term</b>	<b>Fold Enrichment of GO Term*</b>
lipid transport	66.78
lipid localization	40.079
extracellular region	7.77
intrinsic to membrane	1.38
membrane part	1.35
membrane	1.32
integral to membrane	1.29
structural constituent of cuticle	32.37
structural molecule activity	10.72
nutrient reservoir activity	159.27
lipid transporter activity	114.97

\*Includes both positive and negative expression changes in the genes listed in each GO Term category. In this data set, all but three lipid-related genes were downregulated.

**Table 5.1: GO Term analysis of genes that changed upon loss of *trx-1*, as compared to wild type, in unstressed conditions**

<b><i>trx-1</i> (ok1449) stressed vs. unstressed</b>			
<b>Category</b>	<b>Term</b>	<b>Number of Genes</b>	<b>Fold Enrichment</b>
BP	Response to heat	4	35.4
BP	Determination of adult lifespan	7	7.1
BP	Aging	7	6.8
BP	Multicellular organismal aging	7	6.8
BP	Response to abiotic stimulus	5	13.5
BP	Oxidation reduction	8	5.0
MF	Catalytic activity	36	1.92
MF	Glutathione transferase activity	5	58.0
MF	Oxidoreductase activity	13	4.5
MF	Transferase activity, transferring alkyl or aryl (other than methyl) groups	5	28.1

**Table 5.2: GO Term analysis of *trx-1* mutants with and without stress.**

Oxidative stress response genes are enriched upon 5 hours of 10mM sodium arsenite oxidative stress. BP: biological processes; MF: molecular functions.

<i>trx-1</i> (ok1449) vs. WT (stressed)			
Category	Term	Number of Genes	Fold Enrichment
BP	Developmental process	21	1.8
CC	Membrane	35	1.3
CC	Membrane part	34	1.3
MF	Structural constituent of cuticle	24	36.0
MF	Structural molecule activity	25	13.0

**Table 5.3: GO Term analysis of stressed *trx-1* mutants, as compared to wild type.**

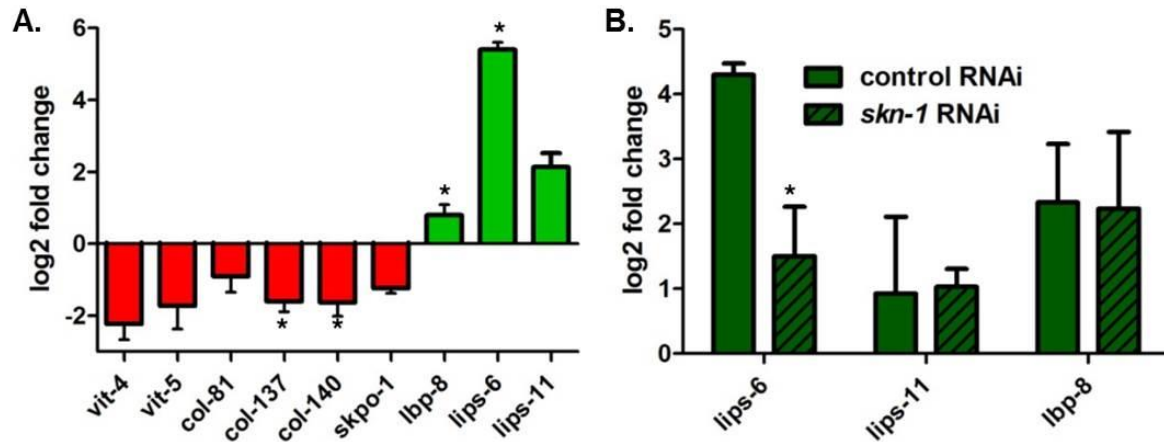
Genes that encode components of the cuticle are also enriched upon loss of *trx-1* during stress. BP: biological processes; CC: cellular component; MF: molecular functions.

WT stressed vs. unstressed			
Category	Term	Number of Genes	Fold Enrichment
BP	Aging	13	8.7
BP	Determination of adult lifespan	13	8.7
BP	Multicellular organismal aging	13	8.7
BP	Response to temperature stimulus	6	22.0
BP	Lipid transport	5	34.0
BP	ER-nuclear signaling pathway	4	71.0
BP	ER unfolded protein response	4	71.0
BP	Response to heat	5	30.0
BP	Response to ER stress	4	65.0
BP	Cellular response to unfolded protein	4	59.0
BP	Response to stress	10	5.4
BP	Response to unfolded protein	4	50.0
BP	Response to protein stimulus	4	50.0
BP	Lipid localization	5	20.0
BP	Response to abiotic stimulus	6	11.0
BP	Response to biotic stimulus	4	30.0
BP	Cellular response to stress	6	9.2
BP	Response to organic substance	4	23.0
BP	Cellular response to stimulus	6	7.6
MF	Nutrient reservoir activity	5	115.0
MF	Glutathione transferase activity	5	41.0
MF	Lipid transport activity	5	39.0
MF	Catalytic activity	45	1.7
MF	Transferase activity. Transferring alkyl or aryl (other than methyl) groups	5	20.0
MF	Oxidoreductase activity	14	3.3

**Table 5.4: GO Term analysis of wild type animals with and without stress.**

Oxidative stress response genes are enriched upon 5 hours of 10mM sodium arsenite oxidative stress. BP: biological processes; MF: molecular functions.

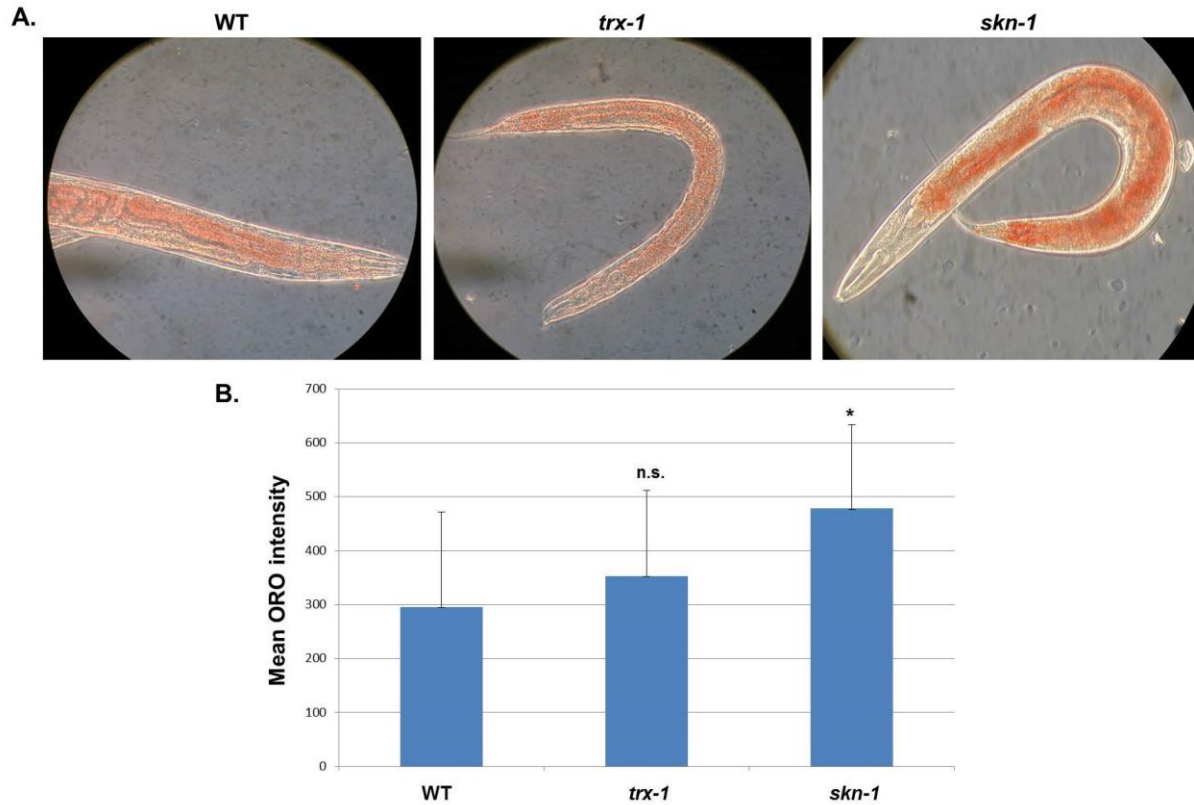




**Figure 5.2: qRT-PCR analysis of changes in gene expression in *trx-1* null mutants.**

**A)** qRT-PCR validation of select genes found to be differentially expressed using RNA Seq upon loss of *trx-1*, as compared to wild type. The average gene expression of biological triplicates was graphed. Error bars represent the standard error of the mean (SEM) and an asterisk indicates a *P*-value < 0.05. **B)** qRT-PCR was used to measure the log<sub>2</sub>fold change in expression of the three up regulated genes (*lips-6*, *lips-11*, *lbp-8*) upon loss of *trx-1*, as compared to wild type, after exposure to either *skn-1* RNAi or control RNAi. Expression of *lips-6* is partially dependent on *skn-1* (*P*-value = 0.032), while *lips-11* and *lbp-8* expression appears independent (*P*-value = 0.446 and *P*-value = 0.453, respectively). The average gene expression of biological triplicates was graphed and error bars represent the standard error of the mean (SEM).

and *lips-11*) and a lipid-binding protein (*lbp-8*) were upregulated. Remarkably, *lips-6* is upregulated 25-fold upon loss of *trx-1*. Taken together, the differential expression of lipid-related genes suggests that lipid homeostasis may be altered in *trx-1* null mutants. To assess this, we analyzed overall lipid levels upon loss of *trx-1*, as compared to wild type animals, using the lipophilic dye Oil-Red-O. Oil-Red-O is a fixative-dependent lipid stain used to visualize the distribution and levels of fat accumulation in the worm. This specificity of this method for solely staining lipid droplets has been stringently evaluated and quantification of lipid levels correlates with biochemical techniques of analyzing total lipid content, including gas chromatography followed by mass spectrometry (152). In *C. elegans*, the intestine is the primary tissue for fat storage (136). To standardize our results, we directed our visualization and quantification of lipid levels to the anterior intestinal region, just below the pharynx, as previously described (see Materials and Methods) (152, 196). Recently, *skn-1* mutants have been demonstrated to exhibit increased levels of Oil-Red-O staining, which is indicative of an general increase in the level of lipids, and were used as a relevant positive control for efficient staining (196). As determined by both visualization and quantification of Oil-Red-O staining, lipid levels remain unaltered in *trx-1* null mutants, as compared to wild type animals ( $P$ -value = 0.18, n.s.), while *skn-1* mutants had increased lipid levels ( $P$ -value = 0.004) (Figure 5.3). Therefore, although genes involved in lipid localization and transport were largely overrepresented in the RNA Seq analysis of genes changed in *trx-1* null mutants, this does not seem to affect the overall level of lipids, as detected by the Oil-Red-O stain.



**Figure 5.3: Total lipid levels are unaltered in *trx-1* null mutants.**

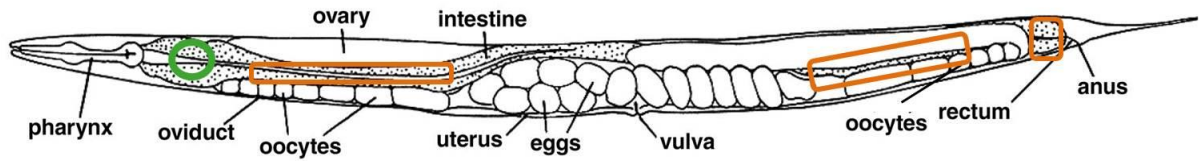
**A)** Brightfield microscopy was utilized to visualize Oil-Red-O staining of WT animals, *trx-1* mutants, and *skn-1* mutants. Worms were visualized using a 20X objective. **B)** Oil-Red-O staining at the anterior intestine was quantified as described in Materials and Methods. *trx-1* mutants did not exhibit quantifiable changes in lipid levels ( $P$ -value = 0.18, n.s.), whereas *skn-1* mutants exhibited a statistically significant increase in lipid content ( $P$ -value = 0.004), as compared to wild type. The mean Oil-Red-O (ORO) intensity of biological triplicates was graphed (N=20 worms per replicate). Error bars represent the standard error of the mean (SEM).

## Discussion

In this chapter, RNA Seq revealed that loss of *trx-1* impacts the expression of both cuticle components and lipid localization and metabolism genes, indicating that TRX-1 may influence these processes (Table 5.1). However, total lipid levels remain unaltered in *trx-1* null mutants (Figure 5.3) and further studies will be required to elucidate the physiological relevance of these transcriptional changes.

Using RNA Seq, we were able to globally evaluate the transcriptional impact of the loss of *trx-1*. This method was powerful for several reasons. First, it allowed us to verify that the SKN-1-dependent oxidative stress transcriptional response remains unchanged in *trx-1* animals, as compared to wild type. Additionally, the response to oxidative stress in *trx-1* null mutants is unimpaired; it was activated in a relatively normal manner under oxidative stress conditions, as compared to wild type animals (Table 5.2, 5.3). Second, this approach allowed us to evaluate in an unbiased manner the classes of genes affected by the loss of *trx-1*. One prominent class of genes down regulated upon loss of *trx-1* was the collagen gene family. Collagen is a critical structural component of the cuticle and the extracellular matrix (ECM) (159). While no obvious cuticle defects are apparent in the *trx-1* mutant, it is possible that the structural integrity of the animals is affected. Further examination of the cuticle of *trx-1* null mutants at high resolution using electron microscopy may allow for detection of aberrant cuticle integrity. Additionally, SKN-1-dependent collagen production is a key factor in maintaining a healthy extracellular matrix, ultimately promoting longevity (60). This is of particular interest given that loss of *trx-1* results in a significant longevity defect (101, 145), which could potentially be explained by the down regulation of collagen gene expression seen in this background. Lastly, this approach allowed us to identify potential modulators of the TRX-1-dependent mechanism of intestinal SKN-1 nuclear localization. For example, the most enriched class of genes with expression changes upon loss of *trx-1* are genes involved in lipid metabolism and transport. Interestingly, lipid gene regulators play a

role in cell non-autonomous signaling in *C. elegans* (236). Therefore, it is possible that TRX-1 expressed in the ASJ neurons modulates lipid metabolism and transport genes as a mechanism to affect intestinal SKN-1 nuclear localization cell non-autonomously. While the expression of genes involved in lipid metabolism and transport is changed upon loss of *trx-1*, this does not result in altered lipid levels in these mutants (Figure 5.3). However, there are two caveats to employing Oil-Red-O staining to discern the difference in total lipid levels in *trx-1* null mutants. First, we restricted our analysis of lipid levels to a limited portion of the anterior intestine. It may be the case that *trx-1* null mutants exhibit altered lipid levels in differing regions of the intestine, which would have been missed in our quantification. Given that vitellogenins, which are yolk proteins that are produced in the intestine but ultimately migrate to the developing oocytes, are downregulated upon loss of *trx-1*, visualization and quantification of the intestinal cells directly surrounding developing oocytes may uncover localized changes in intestinal lipid levels (195). A schematic depicting the region analyzed in our studies and the proposed regions of further study is shown in Figure 5.4. Ultimately, analyzing the entirety of the intestine may serve to uncover localized changes in lipid levels and distribution in *trx-1* null mutants. The second caveat to Oil-Red-O staining is the inherent limitations associated with this method of analysis. Although Oil-Red-O is currently the most facile and cost-effective method to assess lipid levels, this dye stains only a subset of lipids, requires fixing of animals, and relies on a suboptimal quantification strategy, all of which result in the reduced sensitivity of this method (152). Recently, stimulated raman scattering (SRS) microscopy has been developed as a method of sensitively detecting lipid content in *C. elegans*. Briefly, SRS Microscopy is a label-free imaging technique capable of detecting the specific vibrational frequency characteristic of the chemical bonds comprising lipid species. Moreover, this imaging does not require sample preparation and can be done on live worms, as it does not require fixing.



**Figure 5.4: Schematic depiction of the proposed region of Oil-Red-O quantification.**

The *C. elegans* anatomy, as it relates to the intestine and reproductive system, is depicted.

The region quantified upon Oil-Red-O staining is denoted by the green circle and represents the most anterior portion of the intestine. The alternative regions of the intestine proposed to be quantified are boxed in orange and represent the intestinal areas surrounding the developing oocytes. This schematic was obtained and modified from: Strange, K. 2003.

From genes to integrative physiology: ion channel and transporter biology in *Caenorhabditis elegans*. *Physiological Reviews* 83:377-415 (197). Permission was not required for this type of use, as per Copyright Clearance Center.

However, the instrumental setup required to perform SRS microscopy is costly and not feasible to maintain in the general biological laboratory setting. Fortunately, this instrumentation is available to the Garsin lab, as the Wang lab at Baylor College of Medicine utilizes SRS microscopy regularly to analyze the lipid content of worms. Therefore, analyzing the lipid content of *trx-1* null mutants using this sensitive, quantitative technique may uncover subtle differences in lipid distribution that are not discernable via Oil-Red-O staining. Furthermore, the subcellular spatial resolution that is achieved via SRS microscopy is far superior to that of Oil-Red-O staining, which will allow for a more detailed analysis of lipid distribution and may uncover differences in tissue distribution and lipid droplet size (174). Technical caveats aside, and despite exhibiting changes in expression of genes important for lipid metabolism and transport, *trx-1* null mutants may not alter lipid levels or distribution. In this case, the physiological outcome of the changes in expression of genes encoding for lipases, vitellogenins, and fatty acid-binding transport proteins requires further study.

In conclusion, the large number of collagen genes repressed in the *trx-1* mutant, based on our RNA Seq data, may provide an explanation for why these animals are short-lived. Moreover, the increase in expression of genes involved in lipid transport and metabolism may uncover a novel role for lipid-based signaling in SKN-1 activation.

## **Chapter 6: Discussion and Perspectives**

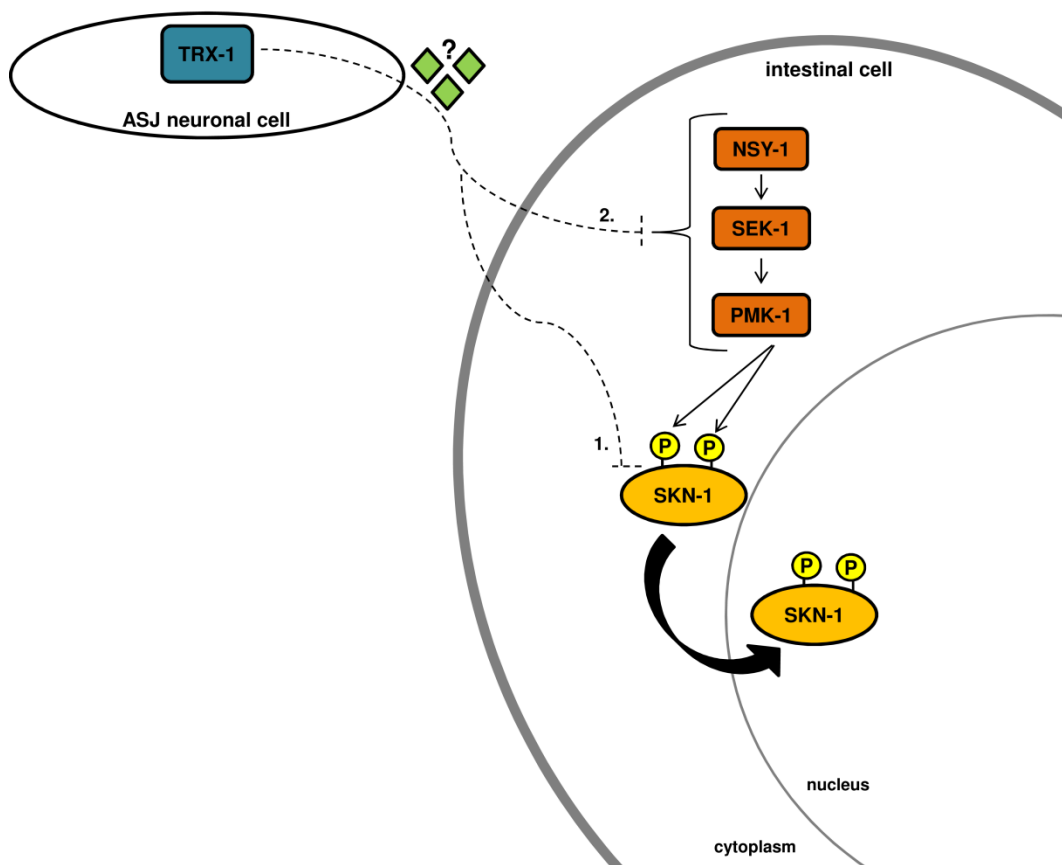


## Summary

In this work, a novel role was uncovered for the thioredoxin TRX-1 as a negative regulator of the major oxidative stress response transcription factor, SKN-1 (Figure 6.1). Specifically, *trx-1* null mutants exhibit increased nuclear accumulation of intestinal SKN-1 in unstressed conditions. This mechanism of regulation is redox-independent, as the conserved, reactive cysteines of TRX-1 are not required to impact SKN-1. Moreover, TRX-1 expressed from the ASJ chemosensory neurons impacts intestinal SKN-1 specifically, indicating that this mechanism of regulation is governed cell non-autonomously via an unknown signaling molecule. Importantly, artificial expression of TRX-1 in the intestine could not rescue proper intestinal SKN-1 function. TRX-1-dependent regulation of intestinal SKN-1 is specific and not a general function of thioredoxins in *C. elegans*.

All known biological stimuli that activate SKN-1 do so through the p38 MAPK signaling pathway (19). TRX-1-dependent regulation of SKN-1 requires the expression of the three p38 MAPK pathway kinases (*nsy-1*, *sek-1*, and *pmk-1*) and the specific SKN-1 serine residues that are phosphorylated by PMK-1. Whether TRX-1 coordinates SKN-1 regulation through activation of the p38 MAPK pathway or acts in a parallel, but dependent, pathway remains unclear. However, TRX-1 does not modulate the phosphorylation status of NSY-1, which is assumed to be the 'active' form of this kinase, or PMK-1, which must be phosphorylated to activate SKN-1. Given this, we favor a model in which TRX-1 acts in a pathway parallel to the p38 MAPK pathway, with TRX-1 ultimately acting on, and requiring, PMK-1-phosphorylated SKN-1. Further experiments will be required to elucidate the relationship between TRX-1 and the p38 MAPK pathway, as it relates to SKN-1 regulation.

Unexpectedly, while TRX-1 impacts intestinal SKN-1 nuclear accumulation, transcriptional activation remains unchanged, as measured by previously characterized



**Figure 6.1: TRX-1 regulates SKN-1 nuclear localization cell non-autonomously.**

See summary text for detailed description of this mechanism. Permission to reproduce this summarizing figure and legend from this article was granted through license number 3830380085023 as assigned by Copyright Clearance Center (CCC), as per Genetics Society of America (GSA) journal of Genetics instructions. I have contributed significantly to this publication. The article is listed for reference: Katie C. McCallum, Bin Liu, Juan Carlos Fierro-González, Peter Swoboda, Swathi Arur, Antonio Miranda-Vizuete, Danielle A. Garsin, 2016. TRX-1 Regulates SKN-1 Nuclear Localization Cell Non-Autonomously in *Caenorhabditis elegans*. *Genetics* (143).

SKN-1-dependent protective responses and gene expression of ten SKN-1-dependent antioxidant genes. Nuclear accumulation of intestinal SKN-1 typically coincides with activation of this transcription factor, which suggests that TRX-1-dependent SKN-1 regulation occurs in a manner that uncouples nuclear accumulation and transcriptional activation of SKN-1 (5, 39, 97, 214). The lack of SKN-1 transcriptional activation was further confirmed using an unbiased RNA Seq approach, which demonstrates that SKN-1-dependent antioxidant transcripts are not enriched in *trx-1* null mutants.

Finally, this study utilized RNA Seq to identify genes with significantly altered expression levels in *trx-1* null mutants. This information served as an unbiased approach to validate the lack of SKN-1-dependent transcriptional activation in *trx-1* null mutants. As discussed in the Conclusion section of Chapter 5, identifying gene expression differences in *trx-1* null mutants provides potential target genes to examine for a potential role in the coordination of the mechanism by which cell non-autonomous regulation of SKN-1 is achieved in a TRX-1-dependent manner. Although not a direct aim of this study, this analysis also provides hints as to the major physiological function of TRX-1, which will be discussed below. Overall, the characterization and analysis of the genes with altered expression levels in *trx-1* null mutants may serve to provide explanations to several unanswered questions.

### **Mechanisms of post-translational regulation of transcription factors and the dissection of subcellular localization and transcriptional activation**

It is not uncommon for transcription factors to be subjected to several layers of post-translational regulation including control of subcellular localization, ability to bind DNA, interaction with cofactors, and protein stability. This is particularly true for stress-activated

transcription factors, which must quickly sense and respond to adverse conditions to confer a cellular advantage. Biological systems do not often rely on transcriptional and/or translational expression to control factors that are required to rapidly respond to damage and acute stress due to the lengthy time both processes require. Rather, their overall cellular abundance is often maintained at a sufficient level, and these transcription factors are post-translationally controlled until stress-dependent induction or relief of repressive regulation is achieved (83).

Post-translational regulation can be achieved through numerous types of modifications including phosphorylation, methylation, ubiquitination, SUMOylation, and glycosylation. Importantly, several types of post-translational modifications may be utilized simultaneously to achieve a sophisticated level of regulation that integrates information from several signaling pathways. Phosphorylation is the most well-defined mechanism of post-translational modification and is characterized by the addition of a phosphate group to serine or threonine residues of a protein, including transcription factors. Serine/threonine kinases, such as p38 MAPK, typically carry out the addition of these phosphate groups. The downstream biological consequences of transcription factor phosphorylation vary, ranging from activation to repression through changes in stability, DNA-binding efficiency, cofactor interaction, and subcellular localization. Furthermore, the downstream physiological consequences of transcription factor phosphorylation span from apoptosis to differentiation (209). For instance, ERK-dependent phosphorylation of Net, a mammalian transcriptional repressor, facilitates its conversion from a repressor to an activator (54). Conversely, JNK-dependent phosphorylation of Net facilitates its export from the nucleus, thereby relieving its ability to repress transcription (53). In the case of Net, regulation of transcriptional activation and subcellular localization are controlled via separate signaling pathways and separate

phosphorylation events. In ways such as this, several layers of post-translational regulation are utilized to tightly control transcription factors.

In a majority of the previous studies investigating *C. elegans* SKN-1 regulation, visualization of SKN-1::GFP nuclear accumulation in intestinal cells typically corresponded with increased expression of SKN-1-dependent transcripts (5, 39, 97, 107, 153, 179, 214). However, SKN-1-dependent gene transcription does not seem to be inextricably linked to the visualization of SKN-1::GFP subcellular accumulation in intestinal nuclei. Specifically, levels of SKN-1::GFP nuclear accumulation that are beyond detection via fluorescence microscopy seem to be sufficient to allow for increased SKN-1-dependent gene expression. For instance, jugalone exposure does not result in visible intestinal SKN-1::GFP nuclear accumulation, yet results in a SKN-1-dependent increase in antioxidant gene expression (107). Conversely, in this study, we demonstrate a genetic situation in which SKN-1::GFP nuclear accumulation does not result in increased transcriptional activation of SKN-1. This is not specific to TRX-1-dependent SKN-1 regulation, as loss of select proteasomal subunits, the chaperonin complex, and a subset of ubiquitin hydrolases also impact SKN-1 nuclear accumulation without impacting transcriptional activation (107). These data suggest that conditions exist, examples being TRX-1-dependent regulation and proteasomal stress, in which SKN-1 subcellular localization can be uncoupled from transcriptional activation. These results suggest that SKN-1 regulation is complex and may require separate modifications to impact its accumulation in intestinal nuclei and activation of antioxidant transcription. However, it remains unclear how these two layers of post-translational regulation are distinctively achieved in this subset of conditions.

As previously mentioned, SKN-1 is both inhibited and activated by phosphorylation. Recall, *daf-2* and *gsk-3* are required to inhibit, by inhibitory phosphorylation events, both SKN-1 nuclear accumulation and transcriptional activation (5, 214). Conversely, PMK-1-

dependent phosphorylation of SKN-1 is critical for nuclear accumulation and the increased expression of antioxidants (97). In all of these studies, however, genetic or *in vitro* mammalian cell culture analyses were utilized to identify these mechanisms of SKN-1 regulation. It is currently still unclear how SKN-1 phosphorylation specifically impacts nuclear exclusion/inclusion and transcriptional deactivation/activation, but phosphorylation seems to impact these two layers of regulation in concert. TRX-1-dependent inhibition of SKN-1, however, impacts nuclear accumulation but not the transcriptional activation of antioxidants. This suggests that an alternative TRX-1-dependent post-translational modification event controls SKN-1 and dissociates these two layers of regulation. However, the identity of this modification remains elusive.

Myristoylation, prenylation, and palmitoylation are three lipid-based modifications in which fatty acid chains are covalently or reversibly linked to proteins either co-translationally or post-translationally. The RNA Seq analysis presented herein implicates a genetic link between *trx-1* and genes encoding lipid metabolism and lipid-binding proteins. Taken together, it is plausible that TRX-1-dependent control of SKN-1 may be achieved via a lipid-based modification, impacting its ability to accumulate in intestinal nuclei, but not activate the expression of antioxidants. Given that lipid-based post-translational modification of SKN-1 should be reversible, myristoylation and prenylation are irreversible modifications, and that SKN-1 lacks the N-terminal glycine required for myristoylation, reversible palmitoylation seems to be the most likely lipid-based modification of SKN-1. Moreover, intriguing parallels exist to suggest that Nrf/SKN-1 could be regulated via palmitoylation in mammals and worms. First palmitoylation, the most common lipid-based modification, occurs at cysteine residues of a variety of proteins, and regulates the subcellular localization of proteins (2). Second, while palmitoylation modification is severely understudied in *C. elegans*, 12 palmitoyl-acyl transferases (PATs) were recently identified in the worm, increasing the

likelihood that lipid-based modifications may be relevant in this context (59). Third, dysfunction of PATs is linked to the pathology of neurodegenerative diseases such as Huntington's disease (67). In summary, TRX-1 impacts SKN-1 subcellular localization without altering its transcriptional activation, suggesting that an alternative form of post-translational modification may control SKN-1. Given the changes in expression of genes coding for lipid metabolism and transport in *trx-1* null mutants, it seems plausible, and warranting of further analysis, that a lipid-based modification may control SKN-1 nuclear accumulation.

### **Redox-independent regulation of a major oxidative stress transcription factor**

In this study, we uncovered a novel role for a *C. elegans* thioredoxin, TRX-1, in the regulation of the major oxidative stress transcription factor, SKN-1. As previously mentioned, thioredoxins utilize a pair of conserved, reactive cysteine residues to facilitate their roles as both a protein disulfide reductase and redox regulator. Intuitively, given that it is both a redox sensitive protein and involved in the regulation of the oxidative stress response, one would predict that TRX-1 regulates SKN-1 in a redox-dependent manner. Interestingly, TRX-1-dependent regulation of SKN-1 occurs independently of its catalytic cysteine residues, suggesting that SKN-1 regulation is a redox-independent function of TRX-1. Precedence for redox-independent roles of thioredoxins exist and induce the alternative mechanisms by which mammalian TRX1 regulates ASK1 and *C. elegans* TRX-1 impacts lifespan extension during dietary restriction. However, neither of these processes directly relate to the oxidative stress response, making redox-independent mechanisms of regulation less puzzling. The question then remains why SKN-1, the major oxidative stress response transcription factor, is regulated redox-independently. Although it is often claimed that “the devil is in the details,” the answer to this question most likely lies in the larger picture of SKN-1 functions, of which many are redox-independent. In scientific literature, Nrf2 and SKN-1 are typically referred to

as major oxidative stress transcription factors, because to date this is the most well-defined functional role for these two proteins. However, as mentioned previously, *skn-1* was originally identified as a critical modulator of development (21). Additionally, SKN-1 exhibits intestinal functions beyond that of regulating the oxidative stress response, such as its role in proteostasis, the unfolded protein response (UPR), and immunity (73, 85, 126). Interestingly, recent findings suggest that that SKN-1 plays important roles in diverse physiological processes such as lipid metabolism, mitochondrial function, and maintenance of the extracellular matrix (60, 158, 161, 163, 196). TRX-1 is understudied and has not been determined to impact any of these processes. Importantly uncovered in this study, *trx-1* impacts the expression of genes encoding proteins involved in lipid transport and collagen biosynthesis, drawing a potential connection between SKN-1 and TRX-1 functions.

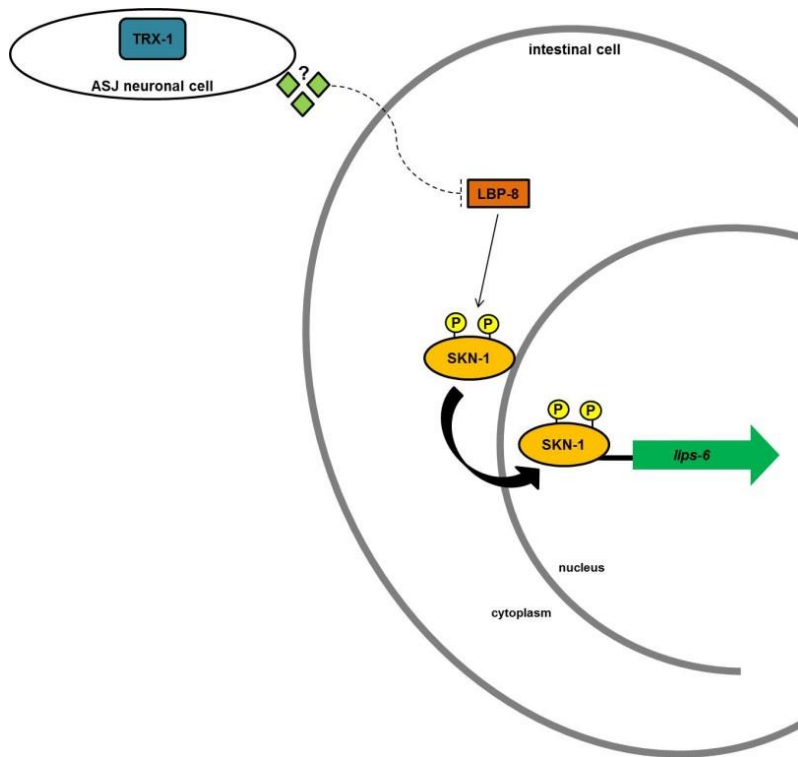
The Blackwell lab recently identified a role for SKN-1 in the maintenance of lipid homeostasis, in which both intestinal SKN-1 nuclear localization and SKN-1 transcriptional activation are prompted by lipid accumulation. However, rather than the antioxidant-rich increase in gene expression seen upon oxidative stress, lipid-dependent activation of SKN-1 increases the expression of genes involved in lipid metabolism, such as lipases, lipid binding and transport, fatty acid desaturation and elongation, and lipophagy. Moreover, lipid-dependent transcriptional activation is dependent on *lbp-8*, a lipid binding protein expressed exclusively in the intestine. Loss of *lbp-8* reduces both intestinal SKN-1 nuclear accumulation and SKN-1 transcriptional activation, as determined by SKN-1-dependent increase in expression of genes involved in lipid metabolism. Concordantly, loss of *skn-1* results in a dramatic accumulation of lipids in the intestine and is required for the increased longevity of animals lacking germline stem cells, indirectly resulting in the accumulation of yolk lipoproteins destined for developing oocytes (196). In summary, the Blackwell lab uncovered a role for SKN-1 in lipid homeostasis, in which lipid accumulation activates SKN-



1, in an *lbp-8*-dependent fashion, to increase the expression of genes involved in lipid metabolism, thereby maintaining homeostatic lipid levels (196).

Analysis of the transcriptome of *trx-1* null mutants revealed changes in the expression levels of genes associated with lipid metabolism and transport. Specifically, four vitellogenins, *vit-2*, *vit-4*, *vit-5*, and *vit-6*, were downregulated more than 4-fold in *trx-1* null mutants. Vitellogenins are intestinally-produced, lipid-binding proteins that are precursors to egg yolk, which upon internalization by oocytes provides the nutrients for development (237). As previously discussed, two lipases, *lips-6* and *lips-11*, were upregulated upon the loss of *trx-1*, of which *lips-6* expression seemed to be partially dependent on *skn-1*. Therefore, while *skn-1* is required for expression of lipases, *trx-1* seems to play an inhibitory role in the expression of these proteins. This corresponds to the increased fat accumulation seen in *skn-1* mutants, whereas *trx-1* mutants display normal lipid distribution (Figure 5.3) (196). Moreover, *lbp-8* expression was increased in *trx-1* null mutants, suggesting that *trx-1* negatively regulates *lbp-8*. Given this, and that *lbp-8* regulates SKN-1 activation in response to lipid accumulation, it is tempting to envision a mechanism by which TRX-1 regulates SKN-1, in an LBP-8-dependent manner, to localize to intestinal nuclei and activate the expression of a specific and limited transcriptional program comprised of genes responsible for lipid metabolism (Figure 6.2).

Precedence exists for the activation of distinct SKN-1-dependent transcriptional programs other than the antioxidant-enriched transcriptional response used to restore redox homeostasis. For instance, in response to ER stress, SKN-1 activates the expression of genes required for the UPR rather than detoxification (73). As mentioned, SKN-1 activates the expression of yet another differing set of genes, those involved in lipid metabolism, in response to lipid accumulation. Currently the only verified overlapping gene that both *skn-1* and *trx-1* impact is *lips-6*. However, this may suggest that TRX-1-dependent regulation of



**Figure 6.2: Putative Model by which TRX-1 regulates SKN-1 nuclear accumulation, and (limited) transcriptional activation, via LBP-8.**

TRX-1 expressed in the ASJ neurons negatively controls nuclear localization of intestinal SKN-1, independently of its redox activity, probably through an unknown signaling intermediate. TRX-1 inhibits intestinal *lbp-8* expression, preventing SKN-1 nuclear accumulation and activation of a limited SKN-1-dependent transcriptional program consisting of genes involved in lipid metabolism.

SKN-1 activates a distinct transcriptional program that alters genes involved in lipid metabolism.

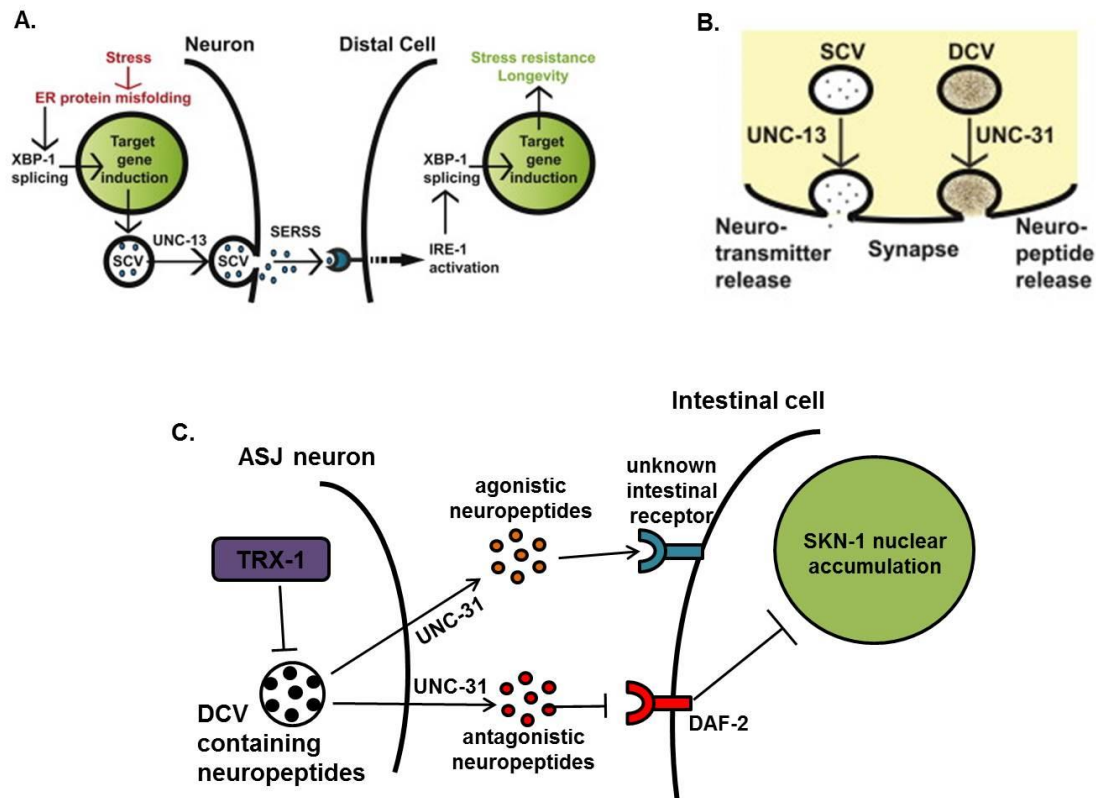
### **Potential Mechanisms of Cell Non-autonomous Regulation of SKN-1**

Most tissues have established cell autonomous mechanisms to respond to states of internal stress. However, the organismal damage inflicted by environmental stressors is typically not confined to a single cell or tissue. To account for this, multicellular organisms employ mechanisms of cell non-autonomous signaling to facilitate communication between both cells and tissues. While inter-tissue communication is a familiar biological concept, its importance and complexity in *C. elegans* physiology is only just recently being appreciated (22). Garnering support for the pivotal role of cell non-autonomous regulation is the finding that several stress responses are activated and governed from distant cell types, most commonly the neurons (172, 198, 203, 219, 222, 236). However, a thorough understanding of the signals and receptors involved in stress-activated cell non-autonomous regulation is lacking. Herein, we report that the subcellular localization of SKN-1, the major oxidative stress response transcription factor, is regulated cell non-autonomously via TRX-1 expressed in the ASJ neurons. This finding expands the list of stress responses that can be regulated cell non-autonomously in *C. elegans*. However, the mechanism by which TRX-1 impacts SKN-1 cell non-autonomously remains elusive.

In mammals, neurons secrete neurotransmitters to relay messages to recipient cells, which are primarily other neuronal or muscle cells. *C. elegans* has 302 neurons and produce many of the same neurotransmitters as mammals, including acetylcholine, serotonin, dopamine, gamma-aminobutyric acid (GABA), and glutamate (Worm Atlas)(226). The *C. elegans* heat shock response was found to be regulated cell non-autonomously eight years ago, however it was only just last year that the mechanism by which this occurs was

elucidated. Specifically, while it was known that the AFD thermosensory neurons were required to elicit an organism-wide heat shock response upon exposure to elevated temperatures, it was unclear how this message was communicated to distant tissues, including the germline and intestine (172). Recently, serotonergic neurons and serotonin were shown to be required for the AFD neurons cell non-autonomous control of the heat shock response, demonstrating the use of neurotransmitters as signaling molecules to elicit organism-wide stress responses (201). The utilization of neurotransmitters to elicit cell non-autonomous control of stress signaling pathways was suggested by previous findings, which demonstrated that synaptic vesicles, which transport neurotransmitters, are necessary for control of both organism wide immune and unfolded protein responses (203, 222). The mechanism by which the unfolded protein response is regulated cell non-autonomously is depicted in Figure 6.3A. The requirement for synaptic vesicles was analyzed using a mutant, *unc-13*, defective in small clear vesicle (SCV) release (133). Dense core vesicle (DCV), another neurosecretory vesicle which carries neuropeptides, release can be examined using a mutant defective in this process, *unc-31*; however, DCV release has yet to be associated with cell non-autonomous signaling (194). Figure 6.3B depicts the mechanism by which SCVs and DCVs are released.

Given that the signaling molecule required for cell non-autonomous SKN-1 regulation is unknown, analyzing whether TRX-1-dependent SKN-1 regulation requires *unc-13* or *unc-31* would serve to significantly narrow the potential list of signaling molecules. Interestingly, several neuropeptides, including *daf-28*, *ins-1*, *ins-9*, *flp-21*, and *nlp-3*, are expressed in the ASJ neurons, the site from which TRX-1 regulates intestinal SKN-1 nuclear accumulation (Wormbook). Neuropeptides can act as either agonists or antagonists of receptors on distant tissues, such as intestinal cells. For instance, DAF-28 is an agonist of insulin-like signaling receptor, DAF-2, INS-1 is a DAF-2 antagonist (93). Moreover, TRX-1 inhibits



**Figure 6.3: Neurotransmitter and neuropeptide control of cell non-autonomous regulation.**

**A)** Schematic depicting *unc-13* facilitated release of small clear vesicles promotes Secreted ER Stress Signal (SERSS) secretion from neurons in order to activate the UPR in distant intestinal cells. The identity of the SERSS is unknown. This schematic was reproduced from: Taylor, R. C., and A. Dillin. 2013. XBP-1 is a cell-nonautonomous regulator of stress resistance and longevity. *Cell* 153:1435-1447 (203). Permission to use this figure was granted by Copyright Clearance Center (License #:3831980887657). **B)** Schematic of the roles of UNC-13 and UNC-31 in SCV and DCV neurosecretory release, respectively. SCVs contain small-molecule neurotransmitters, while DCVs primarily contain neuropeptides. This schematic was reproduced from: Taylor, R. C., and A. Dillin. 2013. XBP-1 is a cell-

nonautonomous regulator of stress resistance and longevity. Cell 153:1435-1447 (203).

Permission to use this figure was granted by Copyright Clearance Center (License #:3831980887657). **C)** Potential mechanism by which TRX-1 may modulate intestinal SKN-1 nuclear accumulation. TRX-1 inhibits the expression of DAF-28 or other ASJ-specific neuropeptides (61). Upon relief of TRX-1 repression, neuropeptides are produced and the release of DCVs containing neuropeptides is facilitated by UNC-31 (194). DAF-28 and other agonistic neuropeptides may activate an unknown intestinal receptor, which signals the nuclear accumulation of intestinal SKN-1. Alternatively, ASJ-specific DAF-2 antagonists, such as INS-1, may prevent intestinal DAF-2 activation, allowing intestinal SKN-1 nuclear accumulation.

ASJ-specific *daf-28* expression in manner which affects *daf-2*-dependent lifespan regulation, suggesting a potential mechanism by which cell non-autonomous signaling may be achieved to impact intestinal SKN-1(61). Intestinal DAF-2 is activated by neuronally-produced DAF-28 to elicit control over intestinal DAF-16-dependent dauer gene expression (93). Additionally, intestinal SKN-1 nuclear accumulation is inhibited by DAF-2 (214). Taken altogether, these data suggest that analyzing whether *unc-31* and *daf-28* (or other ASJ-specific neuropeptides) are required for TRX-1-dependent intestinal SKN-1 nuclear accumulation may elucidate a potential mechanism by which this form of cell non-autonomous communication is achieved (Figure 6.3C).

Another mechanism by which cell non-autonomous signaling is achieved in *C. elegans* is via the use of lipid-derived signals, such as ascarosides, branched chain fatty acids, polyunsaturated fatty acids, and sterol-derived hormones (58, 71, 113, 117, 190). These lipid-derived signals are generated via endogenous synthesis or metabolism of pre-existing lipid components via lipases, elongases, and desaturases and are then transported by lipoproteins or free fatty acid binding proteins (22).

There are several connections between lipid signaling, transport, and metabolism and both *trx-1* and *skn-1*, suggesting that lipid signaling may be involved in TRX-1-dependent SKN-1 regulation. Herein, we uncovered a connection between *trx-1* and the fatty acid binding protein, *lbp-8*. Recently, Steinbaugh et al. uncovered a role for *lbp-8* in SKN-1 regulation in response to lipid accumulation (196). Taken together, I have proposed a mechanism by which TRX-1 may regulate SKN-1 in an *lbp-8*-dependent manner (Figure 6.1). Moreover, in response to oxidative stress, MDT-15, a transcriptional regulator of genes involved in lipid metabolism and a subunit of the Mediator complex, binds SKN-1 and acts a transcriptional cofactor in the upregulation of SKN-1-dependent antioxidants (75).

This is yet another example of the connection between SKN-1 and a lipid metabolism regulator. Lastly, mutations in germline stem cell translation machinery stimulates intestinal *gst-4* expression, via a bile-acid-like lipid derivative, in a manner which is dependent on both PMK-1 and SKN-1 (77). This lipid signal-dependent, cell non-autonomous regulation of SKN-1-dependent antioxidant expression is yet another example of how SKN-1 regulation may be impacted by lipid signals and lipid modifiers. Given that TRX-1 impacts both lipid metabolism genes and intestinal SKN-1, and intestinal SKN-1 is associated with several layers of lipid-based regulation, it seems plausible that cell non-autonomous regulation of SKN-1 via ASJ-expressed TRX-1 may be achieved via a lipid-derived signal.

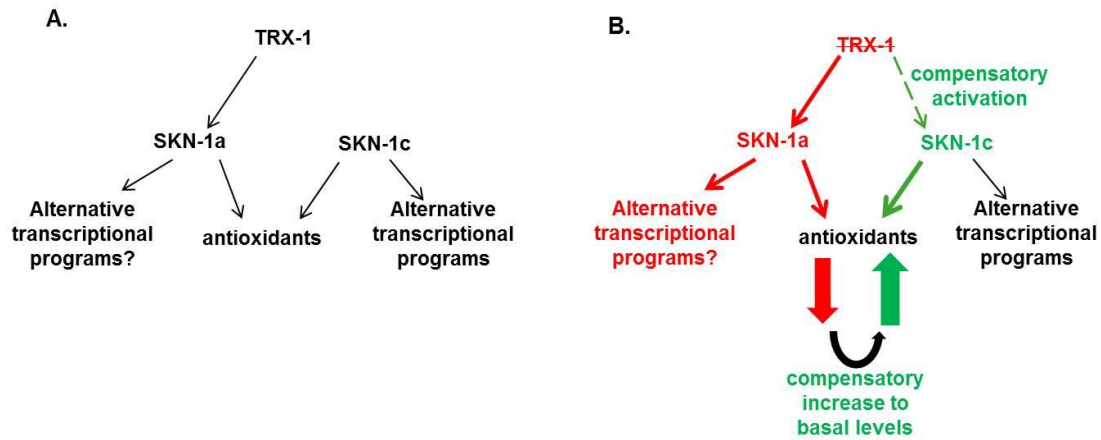
### **Analysis of the *skn-1a* isoform offers an alternative potential model for TRX-1/SKN-1 interaction**

Four *skn-1* isoforms are predicted to be expressed in *C. elegans*, of which three (*skn-1a*, *skn-1b*, and *skn-1c*) have been shown to be expressed *in vivo*. SKN-1b controls dietary restriction-induced longevity in the ASI neurons and SKN-1c controls the oxidative stress response in the intestine (4, 17). Somewhat ironically, given that Expressed Sequence Tagging (EST) suggests SKN-1a to be the most highly expressed, no specific role for this isoform has been uncovered (214). The association between TRX-1 and SKN-1c has been addressed thoroughly throughout this work and, moreover, SKN-1b function is not dependent on TRX-1. Moreover, the SKN-1::GFP translational fusion strain used throughout this study (*skn-1b/c::gfp*) only encodes for the *skn-1b* and *skn-1c* isoforms. Therefore, in this section, I intend to focus on what is known about SKN-1a and suggest a potential SKN-1a-dependent explanation for the perplexing TRX-1/SKN-1c connection demonstrated throughout this study.



Similar to SKN-1c, SKN-1a is expressed in the intestine and contains the conserved bZIP DNA binding domain. Unlike SKN-1c, SKN-1a contains a 90 amino acid N-terminal transmembrane domain (17). Six separate bioinformatics algorithms, including Phobius, predict amino acids 39-59 as the region responsible for transmembrane insertion of SKN-1a (73). Moreover, *skn-1a* is controlled by an upstream promoter region distinct from the one controlling *skn-1c* (17). Interestingly, SKN-1a associates with both the ER and mitochondrial membranes via interactions with HSP-4 and PGAM-5, respectively (73, 158). Specifically, tunicamycin treatment, which induces ER stress and the UPR, greatly enhances SKN-1a protein levels. Furthermore, coimmunoprecipitation studies demonstrate that SKN-1a strongly binds HSP-4, an ER-resident chaperone, with and without ER stress (73). Similarly, coimmunoprecipitation studies demonstrate that SKN-1a binds PGAM-5, a mitochondrial outer membrane protein. Taken together, these data suggest that SKN-1a may utilize its transmembrane domain to interact with both the ER and mitochondria. In mammals, Nrf1 is inserted in the ER membrane, indicating that this may be a conserved attribute of Nrf/SKN-1a (238). Recall that DAF-2-dependent activation of AKT negatively regulates SKN-1c. Interestingly, both AKT-1 and AKT-2 have been shown to phosphorylate SKN-1a *in vitro*. Moreover, when a predicted AKT phosphorylation site, serine residue 12, is replaced with alanine, AKT-2-dependent phosphorylation decreases dramatically and intestinal SKN-1a nuclear accumulation and SKN-1a-dependent antioxidant expression increases, suggesting that overlapping mechanisms of regulation exist for these two isoforms. (214). Unlike SKN-1c, however, SKN-1a is not negatively regulated by GSK-3 (via phosphorylation of serine residue 393), suggesting that distinct mechanisms of regulation are utilized to control SKN-1a (158). Furthermore, this implies that unique physiological roles, which are divergent from those of SKN-1c, may exist for SKN-1a.

Herein, we have uncovered a perplexing situation in which loss of *trx-1* results in increased intestinal SKN-1c nuclear localization but not increased SKN-1 transcriptional activation. We favor a model where the role of TRX-1 is to uncouple the regulation of subcellular localization and transcriptional activation of SKN-1, resulting in a more tightly controlled oxidative stress response. However, given what is known (and not known) about SKN-1a, another intriguing potential model can be suggested in which TRX-1 promotes intestinal SKN-1a activation and protective responses (Figure 6.4). Loss of *trx-1* reduces SKN-1a activation, thereby decreasing the expression of SKN-1a-dependent antioxidants and unknown alternative transcriptional programs. The reduction in expression of antioxidants triggers a compensatory response in which SKN-1c accumulates in intestinal nuclei to increase the expression of redundantly-controlled antioxidants, thereby restoring basal levels of detoxification enzymes throughout the intestine. In this way, loss of *trx-1* results in increased nuclear accumulation of SKN-1c but does not result in an increase in detoxification enzyme expression beyond that which is seen in wild type animals. Additionally, the alternative transcriptional programs of SKN-1c would not need to be increased in this compensatory response. However, the alternative transcriptional program of SKN-1a, would be decreased, and this may account for the overall reduction in gene expression exhibited upon the loss of *trx-1*. Recall that the loss of *trx-1* reduced the expression of 62 genes and increased the expression of only 3 genes, which is contradictory to the predicted gene expression changes that would be expected upon loss of a negative regulator of a major transcription factor such as SKN-1 (Figure 5.1A). Genes whose expression are dependent on SKN-1 were initially identified via *skn-1a/c* RNAi and microarray analysis. Recently, the Blackwell lab utilized RNA Seq to expand upon this microarray data (196). While the list of *skn-1*-dependent genes has grown tremendously, it still remains elusive which of the identified genes are impacted by SKN-1a and which are impacted SKN-1c, as *skn-1a/c* RNAi was used for this analysis. However, a subset of the



**Figure 6.4: Alternative model by which TRX-1 promotes SKN-1a**

**A)** In wild type worms, TRX-1 promotes both the nuclear accumulation and transcriptional activation of SKN-1a. The nuclear accumulation and transcriptional activation of SKN-1c is regulated via the numerous previously described mechanisms (See Figure 1.6). **B)** Loss of *trx-1* results in reduced SKN-1a nuclear accumulation and gene expression, thus reducing the expression of its alternative transcriptional programs and antioxidant gene expression (red arrows and text). The reduced antioxidant gene expression is sensed and compensated for by activation of SKN-1c. Both the nuclear accumulation and transcriptional activation of SKN-1c are increased, however the basal level of antioxidant expression does not change in this purely compensatory reaction (green arrows and text). Moreover, the expression of the alternative transcriptional programs of SKN-1c is not activated in this compensatory reaction (black arrows and text).

genes with reduced expression upon loss of *trx-1*, of which a majority encode collagens, overlap with the newly identified SKN-1a/c-dependent genes. Taken altogether, I propose an alternative model to be tested, in which TRX-1 may promote the nuclear accumulation and transcriptional activation of SKN-1a.

### **What is the physiological role of TRX-1?**

Recall that *trx-1* mutants are short-lived, suggesting that *trx-1* plays a positive role in mediating lifespan and proper aging. The *skn-1/trx-1* interaction uncovered herein, as well as findings from previous studies, do not discern a physiological role for *trx-1* that serves to explicitly justify this inherent and short-lived phenotype (61, 101, 145). Although not a direct aim of this study, RNA Seq results provide potential clues to illuminate the role TRX-1 plays in aging.

*C. elegans* has been employed to analyze the genetic requirements, physiological outcomes, and mechanisms of regulation of lifespan since the late 1970s and is a considered to be one of the major model organisms for the study of aging (205). The process of aging is attributed to a range of circumstances, including damage from a variety of stressors, such as oxidative and pathogen stress, that accumulates over time (69). This accumulation of cellular damage can be detected by examining changes at the molecular level including increases in protein oxidation/aggregation and lipofuscin, an age-related pigment (72). Interestingly, aging worms do not chronically activate the stress responses required to ameliorate the forms of damage accrued over time, such as those incurred as a result of oxidative stress (131). While this serves to highlight the complexity of the aging process, it suggests that the progression of aging is specifically regulated. Unsurprisingly, an overwhelming number of pathways and processes have been demonstrated to affect lifespan in *C. elegans*, including Insulin/IGF-1-like, TOR, and SKN-1 signaling and dietary

restriction, germline stem cell maintenance, and mitochondrial homeostasis, respectively (228). For instance, loss of germline stem cells results in increased lifespan, as previously mentioned (196). Recall, *trx-1* is important for lifespan and has been shown to be required for lifespan extension affected by Insulin/IGF-1-like signaling, dietary restriction, and mitochondrial homeostasis. Moreover, RNA Seq analysis implicates *trx-1* to be important for expression of genes encoding for collagens, which have recently been shown to be important for longevity. Taken altogether, it is tempting to speculate that *trx-1* may be involved in the regulation of aging in the worm.

Collagen is a structural protein that plays a crucial role in the formation and maintenance of the extracellular matrix of connective tissue and represents one third of the total protein in humans (150). Collagen, like most biological molecules, accumulates damage as an organism ages which can result in tissue damage and diseases such as osteoporosis (150, 191). Moreover, collagen expression declines with age (220). *C. elegans* encodes around 175 collagen proteins to form its cuticle, which serves as the exoskeleton of this invertebrate. Collagens contain many intramolecular disulfide bonds and are crosslinked via intermolecular disulfide bonds to form the cuticle. Mutations in or improper crosslinking of many of these collagens results in body morphology defects in the worm (160). Not all collagens are produced at the same level, at the same time, or in the same location, suggesting that collagen expression is tightly regulated, both temporally and spatially. This is indeed the case, and collagen expression regulation is an area of ripe investigation (106). Twenty longevity-associated genes sets implicate that the pathways and processes impacting longevity link increased collagen levels to lifespan extension (as referenced in Supplemental Table 10 of Ewald et al). As the worm naturally ages, collagen expression decreases. Also, overexpression of certain collagens extends lifespan (60). Taken together, it seems plausible that collagen biogenesis is an underlying factor important for normal

lifespan. Interestingly, numerous *trx-1*-dependent genes encoding collagens overlap with those found to be regulated in a longevity-dependent manner (as referenced in Supplemental Table 10 of Ewald et al).

Given both the lifespan defect and the reduced expression of genes encoding collagens in *trx-1* null mutants, it seems reasonable that *trx-1*-dependent expression of collagens may promote lifespan, potentially through dietary restriction processes or Insulin/IGF-1-like signaling. Since collagens are produced in the epithelial layer directly underneath the hypodermis, and *trx-1* expression is restricted to the ASJ neurons, TRX-1-dependent regulation of collagen expression would occur cell non-autonomously (106). However, a majority of the pathways regulating longevity do so cell non-autonomously. Herein, we demonstrate that TRX-1 regulates SKN-1 cell non-autonomously. Taken together, significant precedence exists for a process by which *trx-1*-dependent regulation of collagen expression may occur cell non-autonomously to promote lifespan.

The redox-independent function of TRX-1 was a surprising finding in this study, as thioredoxins from other organisms are most well-known for their redox-dependent roles as both antioxidants and redox regulators of signaling pathways (7). In *C. elegans*, TRX-1 impacts insulin-like neuropeptide function and SKN-1 subcellular localization redox-independently (61). It remains elusive whether the other characterized roles of TRX-1, in dietary restriction, longevity, and mitochondrial homeostasis, occur in redox-dependent or redox-independent manners. Therefore, no redox-dependent roles of TRX-1 have been characterized in *C. elegans* to date. In future studies it would be intriguing to analyze whether a 'redox dead' version of TRX-1 can rescue the phenotypic deficiencies, such as reduced lifespan, of *trx-1* mutants in an effort to further characterize the physiological role of TRX-1.

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