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High-throughput, Fluorescent Analysis of Reactive Oxygen Species in C. elegans after Knockdown of mrck-1.

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Abstract

Reactive oxygen species (ROS) produced in living organisms can generate oxidative stress, which contributes to cell damage associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's. Studying responses to oxidative stress in model organisms, like the nematode worm *Caenorhabditis elegans*, may provide insight into basic underlying cellular mechanisms shared with humans. MRCK-1 (myotonic dystrophy kinase-related Cdc42 binding kinase), a highly conserved intracellular kinase, is activated by the G-protein, Cdc42, which contains a redox sensitive motif. Previous studies have shown that MRCK-1 regulates cell polarity and cytoskeleton reorganization, particularly during cell division and in response to cellular damage. To determine if MRCK-1 plays a role in other cellular defense responses, we are testing if reduced *mrck-1* levels through RNA interference (RNAi) results in the production of increased ROS under stressed conditions. The fluorescent, cell permeable ROS indicator, carboxymethyl dichlorodihydrofluorescein derivative, CM-H_2DCFDA, shows significantly increased fluorescence in hydrogen peroxide-treated worms when quantified over a period of 20 hours using the SpectraMax microplate reader. Measuring the fluorescence of worms exposed to dsRNA from two independent *mrck-1* RNAi constructs, *mrck-m* and *mrck-s*, enabled the quantification of ROS levels when MRCK-1 function is decreased. Results suggest that there is no significant difference between *mrck-1* constructs and the empty vector control under stressed conditions over a 5 to 14 hour read (Kruskal-Wallis rank sum test p-value=0.055). Exploring the levels of ROS in RNAi-treated *C. elegans* may give further insight into the cellular role of MRCK-1 both in worms and in humans.

Introduction

Intracellular kinases are proteins involved in many cellular signaling pathways, and are responsible for exerting downstream effects through phosphorylation. Signaling kinases can also be activated, inactivated, and regulated by many other proteins, including guanosine-triphosphates (GTPases), or other kinases. Many kinases have multiple downstream targets, allowing them to regulate different pathways responses. When the myotonic dystrophy-related Cdc42-binding kinase (MRCK) was first discovered, it was named for its role in Cell Division Cycle Protein 42 (Cdc42) binding, and its significant homology to the DMPK (Dystrophia Myotonica Protein Kinase) kinase domain (Leung et al., 1998). The targets of MRCK kinases have not been well characterized (Unbekandt et al., 2014). The lack of information on the role of MRCK in cellular activities can be partly attributed to absence of potent selective inhibitors and other similar chemical biology tools to study MRCK activity and functions (Unbekandt et al., 2014). Compared to other closely related kinases, especially little is known about Cdc42-regulated MRCKα, MRCKβ and MRCKγ in humans (Unbekandt et al., 2014). Fortunately MRCK kinases share conserved domains with other homologs across many organisms including, *M. musculus* (mouse), *D. reiro* (zebra fish), *D. melanogaster* (fruit

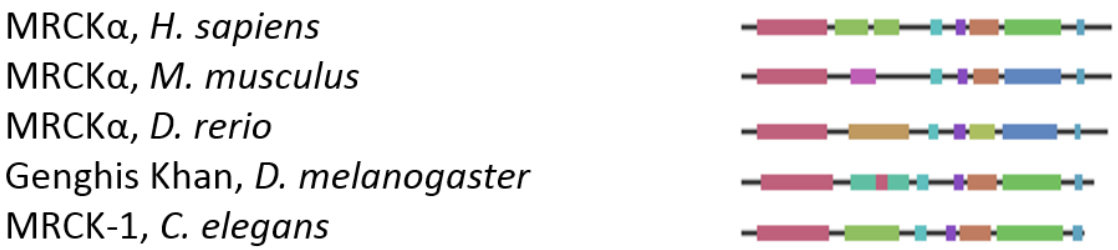


Figure 1. MRCK Conserved Protein Domains in Many Model Organisms. Protein alignment for MRCKα (alpha) in *Homo sapiens* against *mrck-1* in *C. elegans* and other homologs. Conserved protein domains, predicted using rpsblast, are shown in color: Protein Kinases, catalytic domain. DMPK coiled coil domain like. Protein kinase C conserved region 1 (C1). PAK (p21 activated kinase) Binding Domain (PBD), binds Cdc42p- and/or Rho-like small GTPases; also known as the Cdc42/Rac interactive binding (CRIB) motif. (NCBI - Homologene, 2017).

fly), and the nematode worm *Caenorhabditis elegans* (*C. elegans*) (Fig. 1). Studying cellular processes in model organisms such as these can help us better understand similar underlying mechanisms in humans.

The most well established roles of MRCK kinases involve contribution to phosphorylation of the myosin light chain (MLC) and moesin, which functions as a cross-linker of membrane proteins to filamentous actin (Unbekandt et al., 2014). These interactions help to promote enhanced coupling of the cytoskeleton to the membrane (Unbekandt et al., 2014). Regulation, including differential timing and localization of MLC phosphorylation, is the primary function of the MRCK orthologue in *C. elegans* (referred to as MRCK-1), and has been shown through genetic analysis to occur during asymmetric division (Unbekandt et al., 2014, Lant et al., 2015) (Fig. 2). MRCK regulation of such actin-myosin dynamics has been implicated in downstream cytoskeletal effects including actin filament stabilization, increased actin-myosin contraction, and actin-myosin filament coupling in human MRCKα, MRCKβ and MRCKγ (Unbekandt et al., 2014, Wilkinson et al., 2005). Early gastrulation events in *C. elegans* are also regulated by MRCK-1 mediated myosin activation and are essential for normal development (Marston et al., 2016). Additional evidence for this is described by Lant et al., where *mrck-1* ablation by mutation or RNA interference (RNAi) caused canal truncations and cyst formation (2015). In a similar way, the Gek homolog for MRCK in *Drosophila* has been described as essential for proper oogenesis, early development, and establishing and maintaining cell polarity, with Gek mutants also proving lethal (Gomes et al., 2005, Arias-Romero et al., 2013). Additional evidence has been described for

mrck-1 knockout lethality in the ok586 worm strain that is stably maintained by outcrossing and balanced with the nTl[qIs51] chromosome (Kumfer et al., 2003).

MRCK has also been implicated in polarized cell migration, through its upstream GTPase, Cdc42 (Unbekandt et al., 2014, Wilkinson et al., 2005). Cdc42 is a ubiquitously expressed small GTPase belonging to the subfamily of Rho GTPases, including the most well-characterized members RhoA, Rac1, and Cdc42 (Arias-Romero et al., 2013, Aghajanian et al., 2009, Heo et al., 2011). Rho family GTPases act as critical regulators of the organization of the cytoskeleton and membrane trafficking for physiological processes such as cell migration, proliferation, motility, polarity, cytokinesis, cell-cell adhesion, cell matrix adhesion, and cell growth (Arias-Romero et al., 2013, Aghajanian et

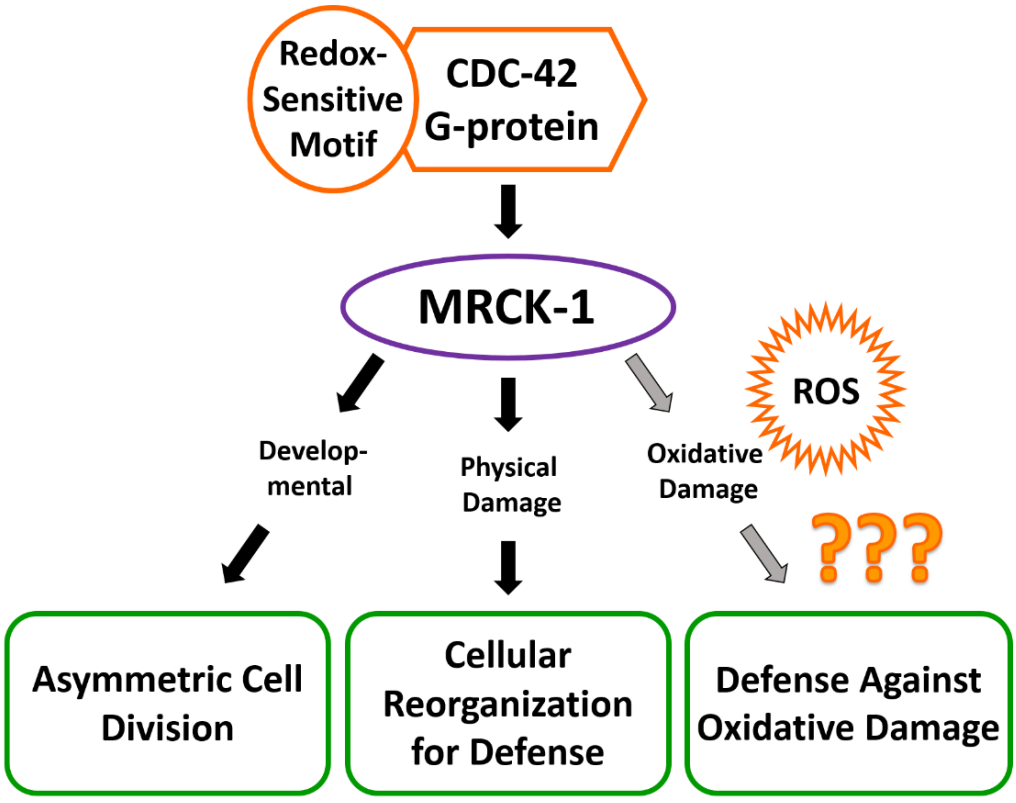


Figure 2. Cdc42 Molecular Signaling Pathway Involving MRCK-1. MRCK-1 is a downstream effector of Cdc42 (Cell Division Cycle Protein 42) shown to protect the nucleus through the process of cellular reorganization (Aghajanian et al., 2009, Gomes et al., 2005, Lant et al., 2015). Cdc42 can be activated by outside forces that may damage critical cellular organelles and membranes (Aghajanian et al., 2009, Kumfer et al., 2010).

al., 2009, Wilkinson et al., 2005). These small GTPases function as molecular switches, fluctuating between an active guanosine triphosphate (GTP)-bound state to an inactive guanosine diphosphate (GDP)-bound state (Arias-Romero et al., 2013, Aghajanian et al., 2009, Kumfer et al., 2010). Activation is mediated by guanine nucleotide exchange factors (GEFs) that catalyze the release of GDP, allowing cellular GTP to be loaded (Arias-Romero et al., 2013, Aghajanian et al., 2009, Kumfer et al., 2010). This causes a conformational change that greatly increases affinity for downstream effector proteins (Arias-Romero et al., 2013, Aghajanian et al., 2009, Kumfer et al., 2010). Cdc42 can activate more than twenty downstream effectors, including MRCK-1 in *C. elegans*, through a signaling cascade which can result in changes to cell polarity, adhesion, migration, proliferation, actin cytoskeletal remodeling, membrane trafficking, and transcription (Arias-Romero et al., 2013, Wilkinson et al., 2005). As a downstream Cdc42 effector, MRCK has been shown to activate myosin II of the MLC through phosphorylation of Ser^{19}, and to play a key role in reorientation of the microtubule-organizing center (MTOC) and rearward nuclear movement in response to physical damage (Gomes et al., 2005) (Fig. 2). Through Cdc42, MRCK has been found to induce actin-myosin retrograde flow that aids in re-orientation of cell nuclei to establish polarity and directionality of migrating cells, which can be viewed as a defense mechanism against physical cellular damage (Unbekandt et al., 2014, Gomes, 2005) (Fig. 2).

In the same way that GTPase activity is classically regulated by GEFs and GTPase activating proteins (GAPs), another mechanism has been indicated where redox agents can target small GTPases through nucleotide binding interactions of redox-sensitive motifs (Aghajanian et al., 2009, Heo et al., 2011). This redox mediated

regulation can often determine the course of downstream cellular signaling cascades and is referred to as redox signaling (Aghajanian et al., 2009, Heo et al., 2011, Ray et al., 2012, Wu et al., 2016). Reactive oxygen species (ROS), in the form of superoxide radials (O_2·-) and hydrogen peroxide (H_2O_2), are generated *in vivo* by enzymatic complexes and as natural byproducts of cellular metabolism. (Aghajanian et al., 2009, Kumsta et al., 2011). Ninety percent of cellular ROS can arise during mitochondrial oxidative phosphorylation, often because of incomplete electron transfer within the electron transport chain (Ewald et al., 2017, Kumsta et al., 2011, Wu et al., 2016, Aghajanian et al., 2009, Heo et al., 2011, Possik et al., 2011).Cellular enzymes such as nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidases) are also major sources of intracellular ROS through electron donation (Ewald et al., 2017, Kumsta et al., 2011, Wu et al., 2016, Aghajanian et al., 2009, Heo et al., 2011, Possik et al., 2011). To cope with these oxidants, cells have evolved ROS-detoxifying enzymes and redox balancing systems, without which an imbalance between pro-oxidants and antioxidants could occur, leading to cellular damage (Wu et al., 2016, Kumsta et al., 2011, Aghajanian et al., 2009, Ray et al., 2012). Excessive ROS molecules that escape the antioxidant defense mechanisms can lead to severe DNA, lipid, and protein damage, a condition termed oxidative stress (Kumsta et al., 2011, Aghajanian et al., 2009, Ray et al., 2012, Possik et al., 2015, Wu et al., 2016). Although ROS serve as critical signaling molecules in cell proliferation and survival, dysregulation of redox signaling cascades and accumulation of oxidative damage has been linked to various diseases including cancers, diabetes, neuronal disorders such as Alzheimer's and Parkinson's, cardiovascular diseases, and

aging (Ray et al., 2012, Kumsta et al., 2011, Heo et al., 2011, Wu et al., 2016, Possik et al., 2015, Ewald et al., 2017).

Recent evidence suggests that GTPases in the Rho family, including RhoA and Cdc42, can be activated by ROS through specific redox sensitive motifs, and therefore may be involved in these highly conserved responses to cellular redox status (Possik et al., 2015, Kumsta et al., 2011, Heo et al., 2011, Aghajanian et al., 2009, Ewald et al., 2017, Wu et al., 2016). In 2009, Aghajanian et al. described two critical cysteine residues located in a redox sensitive motif within the phosphoryl binding loop required for activation and redox-regulation of RhoA. Then in 2011, Heo et al. identified a new class of distinct redox-active motif conserved in half of the Rho family of GTPases including Cdc42, the GXXXXGK(S/T)C motif. This motif can be found for Cdc42 in *C. elegans* as GDGAVGKTC (Wormbase). The sulfur atom of the side chain of the redox sensitive cysteine in the GXXXXGK(S/T)C motif, where X represents any amino acid, is susceptible to redox agents such as O_2·- (Heo et al., 2011). This specific and reversible reaction between ROS and thiol groups on cysteine residues is the mechanism by with ROS can affect cellular signaling and regulation of Cdc42 (Heo et al., 2011, Ewald et al., 2017).

Cdc42 can activate MRCK-1, and MRCK-1 has been shown to protect the nucleus through the process of cellular reorganization (Gomes et al., 2005). We would like to test whether the MRCK-1 kinase is involved in cellular defense mechanisms involving ROS. Suppressing *mrck-1* through RNA interference will prevent the protein from being expressed at normal levels. With reduced MRCK-1 function, we will be able

determine if cells accumulate more potentially damaging ROS, using *C. elegans*, an ideal model organism in which this hypothesis can be tested.

C. elegans is an attractive model to study oxidative stress because it is easy to culture, can quickly propagate large numbers of identical offspring, and allow study in whole organisms versus only in cell systems (Possik et al., 2015, Ewald et al., 2017). The animals transparency also allow for *in vivo* use of fluorescent probes that can measure ROS levels (Possik et al., 2015, Ewald et al., 2017). *C. elegans* are small, soil-dwelling, free-living, nematode worms (Gruber et al., 2009, Wu et al., 2016). The *C. elegans* genome has been fully sequenced, and contains approximately 18,000 genes, over half of which have human homologs (Gruber et al., 2009, Wu et al., 2016, Possik et al., 2015). The ability to reproduce by self-fertilization, synchronize developmental stages, transparency, short lifespan and lifecycle, ease of maintenance, genetic manipulation, and low overall cost make them an ideal model organism to study using reverse genetics

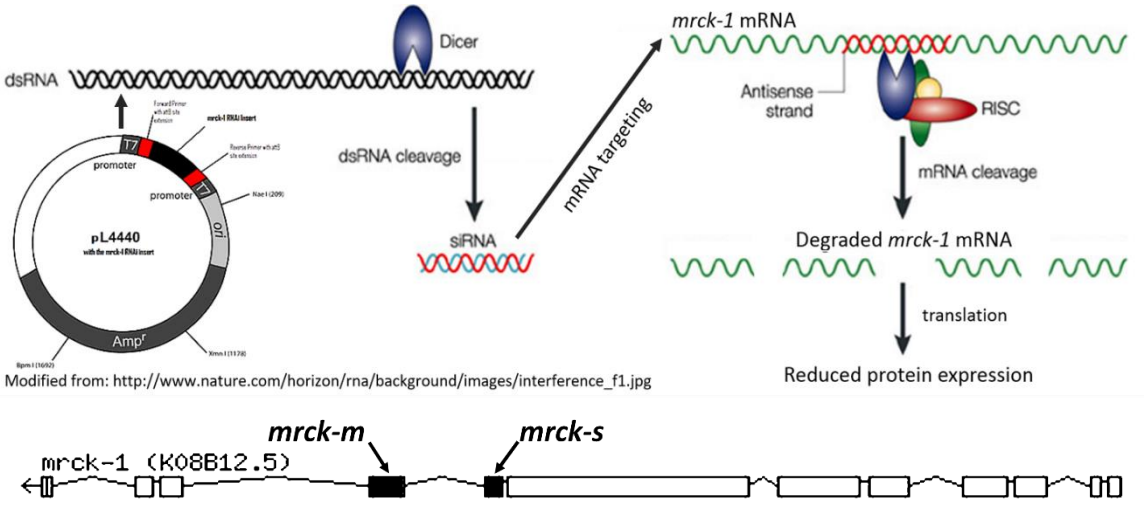


Figure 4. Cellular Mechanism for RNAi Involving *mrck-1*. dsRNA is cleaved by Dicer. The resulting siRNA molecules pair with RISC to form a complex that binds to and degrades complementary mRNA. RNAi can be used to alter an organism's phenotype by targeting specific genes and reducing their expression (Hartwig et al., 2009). *mrck-1* mRNA target sequences for RNAi knockdown, *mrck-m* and *mrck-s*, colored in black.

approaches such as RNAi (Kamath, et al., 2003, Gruber et al., 2009, Gruber et al., 2011, Kumsta et al., 2003).

We are using RNAi to knockdown gene expression of *mrck-1*. RNAi can be used to alter an organism's phenotype by targeting specific genes the accumulation of mRNA (Fig. 4) In *C. elegans,* gene down regulation can be achieved by feeding worms *E. coli* bacteria that harbor a plasmid expressing double stranded RNA (dsRNA) (Possik et al., 2015, Gruber et al., 2009). Once ingested, this dsRNA targets the RNA interference machinery to destroy the mRNA of interest, ultimately leading to decreased levels of protein (Fire et al., 1998). To confirm our RNAi reagents were functioning properly, we targeted the *dpy-13* gene, which affects the formation of collagen in the worm cuticle. This renders an easily visible short and fat phenotype compared to the wild type (Fig. 5).

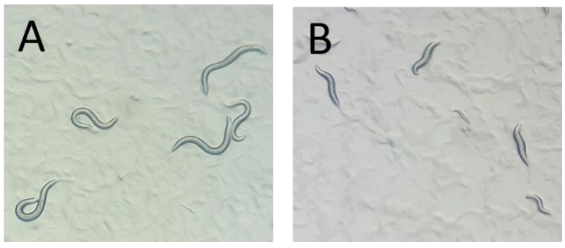


Figure 5. RNAi Induced *C. elegans*. A) N2 wild-type control strain. B) *dpy-13* RNAi worms target and knockdown a collagen gene leading to a short and fat phenotype positive for RNAi.

In this experiment, two previously generated RNAi constructs, *mrck-m* and *mrck-s* (Rigoulot et al., 2012) were used to suppress, or knockdown, the expression of MRCK-1. These two constructs target different parts of the *mrck-1* mRNA, but should both result in the same reduction of protein accumulation (Fig. 4). These constructs were administered to the worms through an HT115 (DE3) *E. coli* bacterial strain that expresse the double-stranded *mrck-1* RNA. The empty vector, plasmid L4440 (pL4440), was used for the experimental negative control. In addition, as a positive control response in our

assay, we used a published RNAi construct that targets the gene *memo-1* (mediator of ErbB2 driven cell motility) in worms (Wormbase). It has been shown that loss of *memo-1* in *C. elegans* leads to an increase in levels of ROS (Ewald et al., 2017).

Previous experiments have tested the effect of oxidative stress on *C. elegans* after knockdown of *mrck-1* (Robben et al., 2014). This was accomplished using strains that were altered to express Green Fluorescent Protein (GFP) in place of the proteins normally

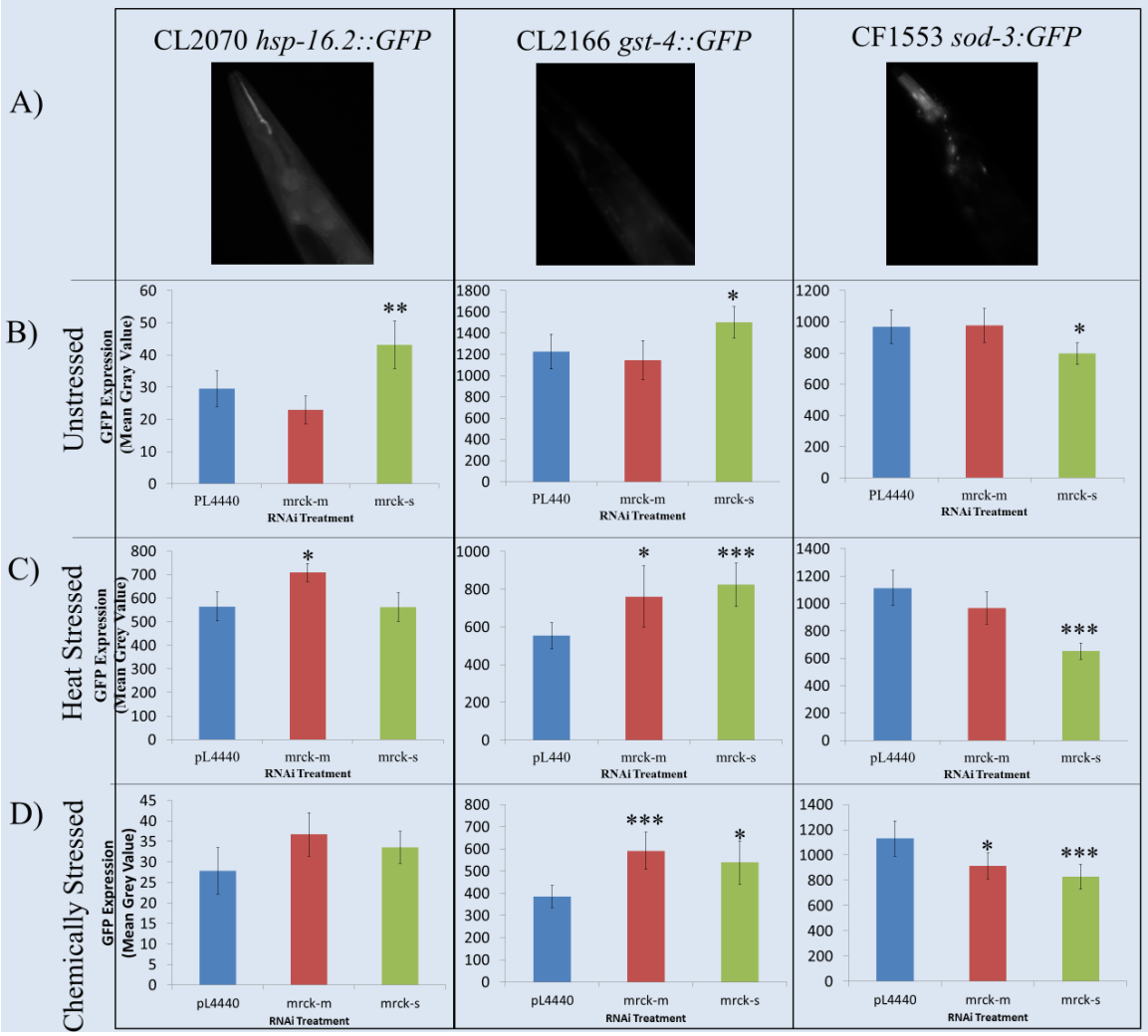
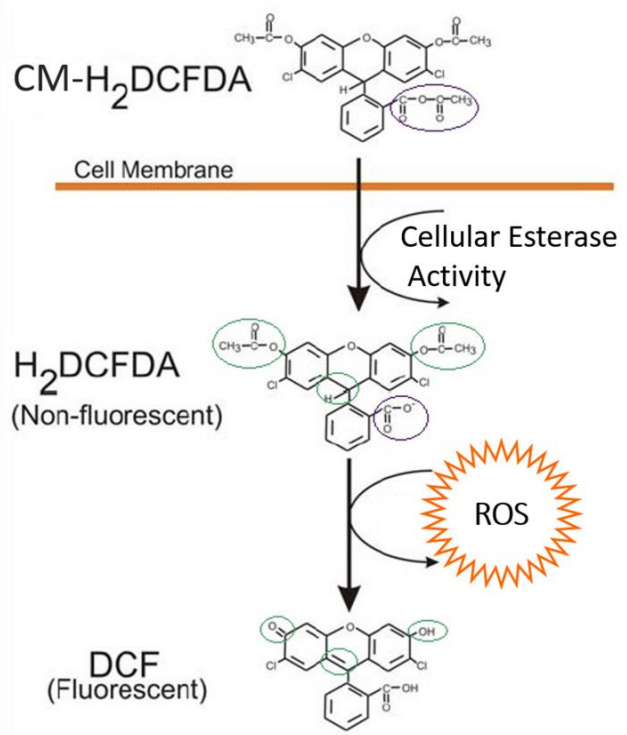


Figure 3. GFP expression driven by redox sensitive promoters in the absence or presence of oxidative stress after treatment with mrck-1 RNAi or the empty vector (pL4440). (A) Anatomical expression of GFP in the anterior region of C. elegans reporter strains under 400X magnification. GFP quantification within pharyngeal region of RNAi-treated strains under normal (22˚C) temperature (B), after 32˚C heat stress (C), and after H_2O_2-induced oxidative stress (D). Deviations in SEM. (*p<0.05) (**p<0.01) (***p<0.005) relative to vector control. (From Robben and Erickson, 2014).

produced in response to oxidative stress. The amount of GFP was measured using fluorescent microscopy and was used to quantify the strength of the response (Fig. 3). Unfortunately, issues with signal intensity and large standard deviations made it difficult to generate reproducible and consistent data. To counter these issues, we have altered our methods to include a highly sensitive, specific fluorescent dye that reacts directly with ROS.

To improve our sensitivity and reproducibility, we have increased the sample size of the worms, and have changed the indicator for ROS, and have altered the method for quantifying ROS levels. To facilitate larger sample sizes, this experiment is high-throughput, using large numbers of *C. elegans* in the SpectraMax fluorescent microplate reader. To obtain a direct, sensitive measurement of ROS, we utilized the membrane soluble, fluorescent ROS indicator 2′,7′-dichlorodihydro-fluoroscein diacetate (CM-H_2DCFDA). This dye acts by passive diffusion into cells where intracellular esterases cleave its hydrophobic acetate groups, effectively trapping the ionic form inside the cell. Here, the thiol-reactive



Modified from: http://www.biotek.com/resources/articles/reactive-oxygen-species-generation.html

Figure 6. CM-H_2DCFDA Mechanism. CM-H_2DCFDA passes through the cell membrane. Once inside, cellular esterases act on the molecule, creating an ionic form that becomes trapped in the cell. Oxidation by Reactive oxygen species converts the molecule to DCF, a form that fluoresces green when stimulated.

chloromethyl groups are oxidized by intracellular reactive oxygen species that convert the dye to a form (dichrolofluoroscein) which fluoresces green (Thermo Fisher Scientific, Pittsburg, PA, catalog # C6827).

Using these methods, we are testing the hypothesis that worms with reduced *mrck-1* expression are less able to defend against chemically-induced oxidative stress. Our prediction is that worms with reduced *mrck-1* expression will result in increased CM-H_2DCFA-detected ROS.

Methods and Materials

Nematode Culture: C. *elegans* were kept on nematode growth medium-lite (NGM-lite) agar plates at 22° C and fed *Escherichia coli* (*E. coli*) of the OP50 strain. Worm strains were maintained by chunking bits of agar from populated plates onto new OP50-seeded NGM-lite plates.

For age synchronization, freshly chunked nematodes were left to grow for three days and then bleached to kill adults and leave only embryos. Chunked worms were allowed to develop for 3 days until a large number of gravid worms were present on NGM-lite plates. Adults were then removed from the plates by washing with M9 salt buffer, and concentrated using gentle centrifugation. Worms were re-suspended in an alkaline sodium hypochlorite solution and incubated until intact worms were destroyed, and embryos were released. After multiple M9 buffer washes, embryos were transferred to NGM-lite plates for further development. The resulting age-synchronized larva grew to adulthood in three days at 22° C.

RNAi knockdown: The gene *mrck-1* was targeted by the two previously generated RNAi constructs *mrck-m* and *mrck-s* (Rigoulot et al., 2012), using the empty vector pL4440 as a control. In addition, the RNAi construct for *memo-1* was used as a positive control for ROS production as described by Ewald et al., 2017. (Dharmacon, Lafayette, CO, catalog # C37C3.8) Bacterial strain HT115(DE3), transformed with the appropriate plasmid, was grown on RNAi-induction plates containing NGM-lite media supplemented with 100 µg/µl ampicillin to select for the plasmid, 0.4 µM IPTG to induce dsRNA expression, and fed to the wild type (N2) *C. elegans* strain (Fire et al., 1998).

Oxidative Stress Treatments: To generate oxidative stress in *C. elegans*, age-synchronized young adults were incubated in a liquid medium containing 0.01% (3 mM) H_2O_2, for 45 minutes, followed by six subsequent wash steps to remove any residual H_2O_2 prior to ROS detection (Kumsta, 2011). The negative control samples included M9 buffer and DMSO only.

ROS Quantification: The oxidative stress-induced nematodes were placed in microplate wells with the ROS-sensitive dye, CM-H_2DCFDA (ThermoFischer, C6827), dissolved in DMSO and 0.1% Tween 20. Fifty nematode worms per well were incubated with 20 µM CM-H_2DCFDA, and fluorescence was quantified every thirty minutes for 14 hours using a SpectraMax microplate reader with an excitation wavelength of 486 nm and an emission wavelength of 520 nm. The sensitivity of the dye was determined using N2 *C. elegans* populations. Six biological replicates under each condition were run with four statistical replicates per experiment. Only three biological replicates were run including the positive control for ROS production, *memo-1*.

Fluorescence data was analyzed to determine the net change in fluorescence over time. The actual fluorescence readings were variable from one time point to the next, but showed a clear linear trend during the time between 5 and 14 hours. It was during this time period that the first differences in fluorescent readout between H_2O_2 treated worms versus untreated was seen. The best-fit line for the slope of fluorescence increase was used to calculate fluorescence change during that time period.

Results

Initial observations after nematodes were grown up on RNAi induction plates indicated no difference in morphological phenotype compared to the empty vector control (Fig. 7).

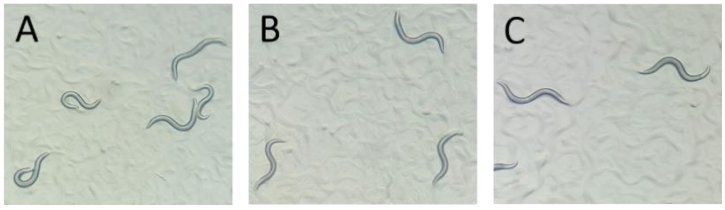


Figure 7. No Obvious Morphological Phenotype Detected in *mrck-1* RNAi *C. elegans*. L4 worms were picked on RNAi induction plates seeded with *E. coli* containing the following plasmids and left to feed for 4 days on transformed bacteria to uptake dsRNA. A) Empty pL4440 vector negative control. B) *mrck-m* RNAi vector. C) *mrck-s* RNAi vector.

ROS quantification results showed a significant increase in ROS levels between hydrogen-peroxide treated worms versus untreated worms in each RNAi knockdown using the Mann-Whitney U test (Fig 8). This demonstrates that a 3mM hydrogen peroxide treatment for 45 minutes is sufficient to induce oxidative stress in adult *C. elegans.* It also indicates that the ROS indicator CM-H_2DCFDA is capable of producing a readout of ROS levels based on fluorescence similar to what is seen in the literature. However, results also indicated that there was no significant difference in accumulated

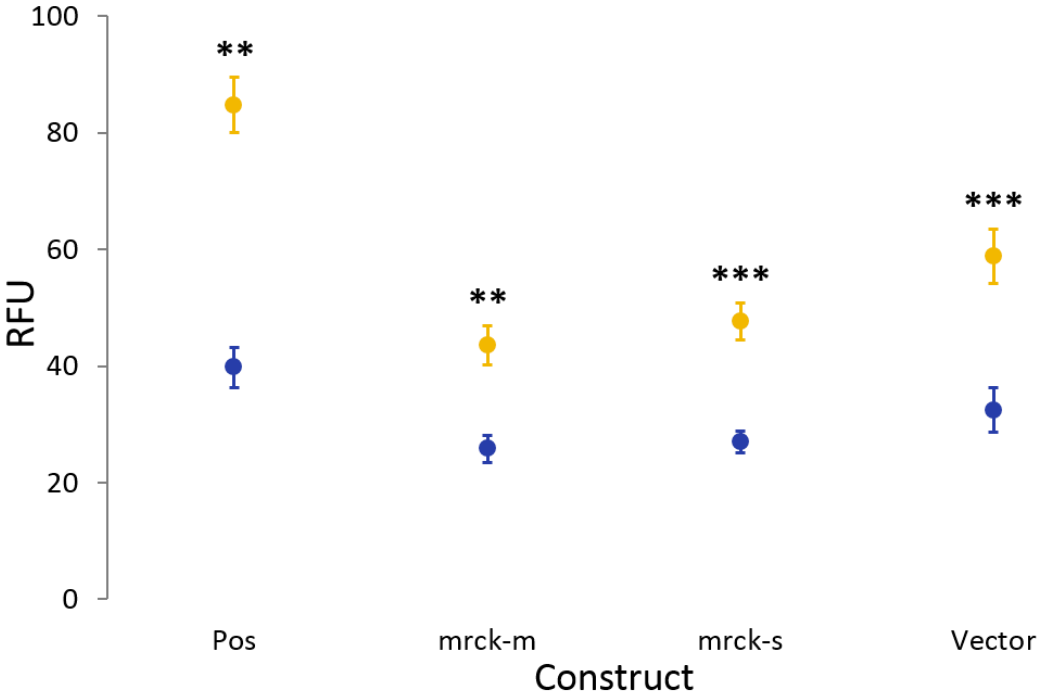


Figure 8. Difference in ROS Levels between Hydrogen Peroxide Treated and Untreated Worms. Worms treated with 0.01% hydrogen peroxide were washed and incubated with 50 µM CM-H_2DCFDA for 20 min in a black 384 well microplate. Wells containing equivalent solvent (DMSO) concentrations, but lacking worms were used as blank, and plate was read at optimal excitation (486nm) and emission (520nm) wavelengths at ambient temperature. Relative fluorescence units (RFU) were quantified over a 5 to 14 hour read period. Positive control for ROS indicated "Pos" is *memo-1* RNAi. Significant difference in ROS levels between hydrogen peroxide (HP) treated (yellow) and untreated (UT) worms (blue) (Mann-Whitney U test: *memo-1* p-value=0.0024, n=12 for HP and UT, *mrck-m* p-value=0.0075, n=24 for HP and UT, *mrck-s* p-value=0.0005, n=24 for HP and UT, vector p-value=0.0008, n=24 for HP and UT). The data represents the mean ± SEM,*p<0.05,**p<0.01,***p<0.001 versus control. Six biological replicates were completed for both *mrck-1* constructs and vector control. Three biological replicates were completed for *memo-1*.

ROS levels between *mrck-1* constructs, the empty vector, and the positive control when deducting untreated worms from treated over a 5 to 14 hour read period using the Kruskal-Wallis rank sum test (Fig 9). These data show no change in ROS levels in worms with decreased MRCK-1 protein production relative to the control (Fig. 9). Both *mrck-m* and *mrck-s* worms gave similar levels of fluorescent readout, with no significant difference between constructs, as expected, if they were working as designed (Fig. 9).

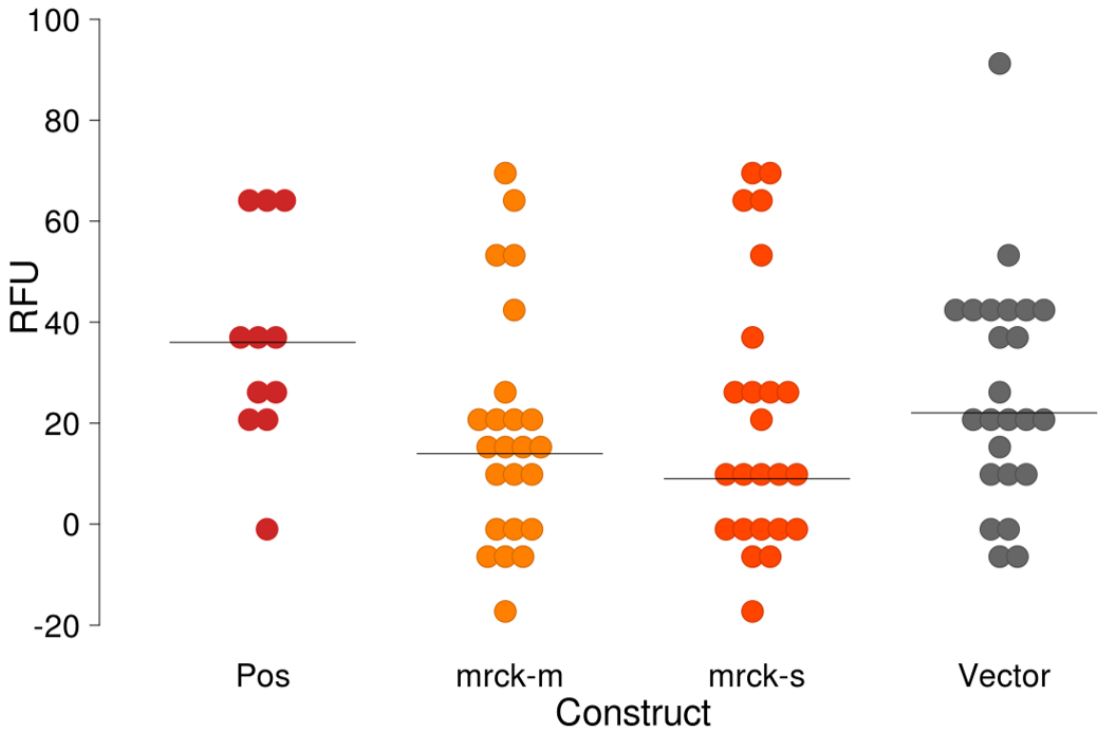


Figure 9. No Difference in ROS Levels between *mrck-1* Constructs and Controls under Stressed Conditions. Positive control for ROS indicated "Pos" is *memo-1* RNAi. No difference in ROS levels between RNAi constructs for *mrck-1* or *memo-1* compared to vector control under stressed conditions (Kruskal-Wallis rank sum test p-value=0.0550). Each dot represents an experimental replicate with ~50 worms. Black line represents the median value, or 0.5 quantile. Six biological replicates were completed for both *mrck-1* constructs and vector control. Three biological replicates were completed for *memo-1*.

Discussion

Our results do not support our hypothesis that knocking down *mrck-1* will lead to increased ROS in the cell. Complicating our analysis, results also did not show a significant difference in ROS production between *memo-1* worms and the empty vector control. The positive control, *memo-1*, was previously shown to have a two-fold increase in endogenous ROS levels after knockdown by RNAi compared to control worms, when quantified using the same fluorescent dye, CM- H_2DCFDA, (Ewald et al., 2017). Therefore, we would like to run further biological replicates including *memo-1,* as only three are included in the data.

We have reason to believe it is possible that fluorescent readout for *memo-1* RNAi construct was lower than expected due to past issues with identity confirmation when sequencing previous positive controls. After the original positive control response in our assay, a published RNAi construct that targets an NADPH oxidase (DUOX) gene (*bli-3*), shown to decrease levels of ROS, was sequenced, it was found that the pL4440-dest-RNAi vector did not contain the sequence for the *bli-3* gene (Cha´vez et al., 2009) Therefore, it is crucial to confirm the identity of the *memo-1* RNAi construct before any definite conclusions can be drawn. In addition, to confirm that the two mrck-RNAi constructs effectively reduced *mrck-1* expression, we would like to perform mRNA expression analysis on worms treated with both *mrck-1* constructs using quantitative PCR to quantify concrete differences in mRNA levels after RNAi is performed. If these possibilities are tested and results point to the same conclusions, another solution could be to increase the sensitivity of our assay or use a different assay altogether. A lot of variation and noise is currently present in the readings, which could be due to the living adult worms moving around in the well. Therefore, we have proposed another experiment where ROS stress responses are not measured in liquid medium.

Methods used for data analysis may also be improved. Assumptions about the linear regression being the best fit for the data may be masking subtle differences based on values for the coefficient of determination (R^2). Specifically, preliminary analysis of ROS increase over the time (5 to 14 hours) was carried out in Excel, where regression lines taken for the data may have had slight rounding errors due to the large fluorescent output from the SpectraMax plate reader. Raw data was run through the statistical programming software, R, to test for better fit to a linear versus parabolic curve, and it

was found that a linear relationship better fit the raw data. Some fluorescent reads had slightly lower R^2 values, suggesting that a different method for analysis may be required. The lowest R^2 value recorded was 0.94, with most values being 0.98 and above. To get a better measurement of the overall change in ROS levels from time points 5 to 14 hours, slope values were multiplied by a factor of nine, which may have inflated any rounding error present. To avoid these issues, we suggest future analysis comparing points of maximal likelihood of sigmoid or S-shaped curves over the course of the full 14 hour read. This would require taking the log of the raw data and may better represent all data points as a growth parameter over time. This may more accurately describe if there was a difference between H_2O_2 treated worms versus untreated, and ultimately, if there was a difference in ROS levels between our *mrck-1* worms and the vector control under stressed conditions.

To test the same hypothesis that MRCK-1 plays a role in defense against oxidative damage, we would like to run a survivorship assay. After gene knockdown by RNAi, will be transferred onto pre-warmed RNAi plates that have been incubated at 35°C for 3 hours (Zevian et al., 2014). Nematodes will then be heat shocked to induce oxidative stress, recover at room temperature for 48 hours, and then be assessed for viability via nose-touch response assays (Kumsta et al., 2011). The nonparametric log-rank test will be used to determine statistical significance of survival after oxidative stress treatment on censored data (Kumsta et al., 2011). Testing acute survivorship in RNAi treated worms would be an additional way to assess survival under oxidative conditions after knockdown. If MRCK-1 plays a role in oxidative stress defense, we hypothesize that *mrck-1* RNAi worms would survive longer compared to the control after short term

exposure to ROS. A survivorship curve may also give better insight into what is occurring biologically.

Another possible explanation for the lack of difference seen in *mrck-1* constructs compared to the vector may be that the redox sensitive motif described for Cdc42 may not be sufficient for ROS binding. The motif described by Aghajanian et al. in RhoA contains two critical cysteine residues, whereas the motif sequence described by Heo et al. for Cdc42, as GXXXXGK(S/T)C, only contains one (2009, 2011). Therefore, it may be possible that ROS-mediated regulation of the Cdc42 GTPase in *C. elegans* requires two cysteine residues. To test if this is the case, we could run an experiment to see if Cdc42 can bind GTP and switch to an active state when exposed to ROS. We could then edit Cdc42, making a base pair change to include a second cysteine residue, and run the same experiment. A last, most general explanation could be that MRCK-1 does not play a role in defensive response against ROS and oxidative damage, but could be regulated upstream by ROS and Cdc42 for other purposes, or ROS may play no role in its function at all.

It is still valuable to study possible roles ROS may play, as experimental research helps us learn more about these cellular signaling mechanisms that are not well characterized, and could lead to unanticipated discoveries. For instance, the mitochondrial-free radical theory of aging (mFRTA), which proposes that aging is due to the accumulation of unrepaired oxidative damage by mitochondrial ROS, is still debated throughout the science community (Heo et al., 2011, Wu et al., 2016, Gruber et al., 2011, Gruber et al., 2009, Ewald et al., 2017, Lee et al., 2010, Cabreiro et al., 2011). Increasing evidence for and against mFRTA, and the associated "vicious cycle" theory which

proposes that ROS promote mitochondrial DNA mutations leading to further increase in ROS production, continues to emerge through research into these signaling pathways (Heo et al., 2011, Wu et al., 2016, Gruber et al., 2011, Gruber et al., 2009, Ewald et al., 2017, Lee et al., 2010, Cabreiro et al., 2011). Certain studies in yeast, fruit fly, and mice have shown that reducing mitochondrial ROS production and oxidative damage can extend lifespan (Wu et al., 2016). Inactivating, as well as overexpressing, antioxidant activities in many other genetically modified organisms, fail to produce outcomes that support the mFRTA (Wu et al., 2016). Conversely, in a study by Gruber et al., *C. elegans* carrying a mitochondrial complex II mutation, which increases ROS production, showed an increase in oxidative and mitochondrial damage and were short-lived (2009). Reduction in mitochondrial superoxide dismutase (SOD) enzymes, which work to convert $O_2^{\cdot-}$ to H_2O_2, that is in turn converted to water and O_2 by catalase and peroxidases, should also lead to shorter lifespan; however, studies using *C. elegans* SOD mutants, deficient in up to five *C. elegans sod* genes, have shown little to no effect on aging. (Possik et al., 2015, Heo et al., 2011, Gruber et al., 2011, Gruber et al., 2009, Cabreiro et al., 2011, Doonan et al., 2008, Wu et al., 2016). An increase in SOD should protect against oxidative damage and aging by converting harmful ROS to less destructive forms, but further studies in *C. elegans* have shown administration of SOD compounds fails to increase lifespan (Cabreiro et al., 2011). Differing results have been obtained based on low or high dose exposure to stressful agents such as paraquat and other superoxide generators (Ewald et al., 2017, Lee et al., 2010). Induction of protective mechanisms at low dose, but harmful effects at high dose, is referred to as hormesis (Ewald el al., 2016) Mild inhibition of respiration and modest increases in ROS have

activated cell-protective pathways in many organisms, promoting longevity and giving evidence to this phenomenon (Lee et al., 2010, Ewald el al., 2016). Therefore, through careful reevaluation and testing, if we are able to determine if MRCK-1 does plays a role in oxidative damage response, data obtained could help provide more evidence for or against the mFRTA.

The extreme complexity of the studies attempted, involving electron transfer in cell signaling, is one of the main reasons why protein systems in which electron transfer plays a role are not well described (Heo et al., 2011). Difficulty in finding specific chemicals and tools with which to measure rates of ROS production and electron transfer remains a challenge (Arias-Romero et al., 2013). In addition, the possibility of other complex interactions among multiple upstream regulators and downstream effectors of Cdc42 contribute to these issues (Arias-Romero et al., 2013). This further stresses the importance and necessity for additional research into these intricate signaling pathways. The more we can piece together the mechanistic elements at play in these complex systems involving redox regulation, the better understanding we will gain into the future development of methods for treatment of diseases involving dysregulation of these redox-mediated GTPase signaling pathways.

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