The Oxidative Stress Response in *Caenorhabditis elegans* Requires the GATA Transcription Factor ELT-3 and SKN-1/Nrf2

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ABSTRACT Cellular damage caused by reactive oxygen species is believed to be a major contributor to age-associated diseases. Previously, we characterized the *Caenorhabditis elegans* Brap2 ortholog (*BRAP-2*) and found that it is required to prevent larval arrest in response to elevated levels of oxidative stress. Here, we report that *C. elegans* *brap-2* mutants display increased expression of *SKN-1*-dependent, phase II detoxification enzymes that is dependent on *PMK-1* (a p38 MAPK *C. elegans* ortholog). An RNA-interference screen was conducted using a transcription factor library to identify genes required for increased expression of the *SKN-1* target *gst-4* in *brap-2* mutants. We identified *ELT-3*, a member of the GATA transcription factor family, as a positive regulator of *gst-4p::gfp* expression. We found that *ELT-3* interacts with *SKN-1* to activate *gst-4* transcription *in vitro* and that *elt-3* is required for enhanced *gst-4* expression in the *brap-2(ok1492)* mutant *in vivo*. Furthermore, nematodes overexpressing *SKN-1* required *ELT-3* for life-span extension. Taken together, these results suggest a model where *BRAP-2* acts as negative regulator of *SKN-1* through inhibition of p38 MAPK activity, and that the GATA transcription factor *ELT-3* is required along with *SKN-1* for the phase II detoxification response in *C. elegans*.

KEYWORDS oxidative stress; SKN-1; GATA transcription factors; BRAP-2; ELT-3

REACTIVE oxygen species (ROS) can cause damage to a variety of cellular components and are believed to contribute to the development of diseases associated with aging such as Alzheimer's or Parkinson's. The overproduction of these oxygen derivatives can lead to extensive tissue and cellular damage that ultimately leads to cell death (Finkel 2011). To cope with changes in ROS levels and to maintain homeostasis and cell survival, organisms possess a sophisticated defense mechanism that induces the expression of detoxification enzymes to remove excessive oxygen radicals. Two key regulators of the mammalian stress response are

FOXO and the NF-E2-related factor (Nrf2) transcription factors, which are responsible for inducing the expression of genes required for detoxifying stress compounds and promoting longevity (Moi *et al.* 1994; Itoh *et al.* 1999; Kops *et al.* 2002).

Nrf2 is evolutionarily conserved and is present in *Caenorhabditis elegans*, where it is known as *SKN-1* (Blackwell *et al.* 2015). *SKN-1* was initially identified as the transcription factor that initiates endoderm specification during embryogenesis (Bowerman *et al.* 1992, 1993). However, like its homolog in humans, *SKN-1* also functions in regulating the expression of the *C. elegans* phase II detoxification response (An and Blackwell 2003). In the intestine, the main detoxification organ in *C. elegans*, *SKN-1* accumulates in nuclei and activates target genes in response to environmental stresses. Genes upregulated by activated *SKN-1* include glutathione-S-transferase 4 (*gst-4*), the γ -glutamine cysteine synthetase heavy chain (*gcs-1*), and a wide range of genes that are involved in membrane, lysosomal, and proteasomal homeostasis (Oliveira *et al.* 2009). However, these detoxification genes are often regulated through complex transcriptional networks,

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and some *SKN-1* target genes are also under the regulatory control of *SKN-1*-independent mechanisms.

Nrf2/*SKN-1* activity is regulated by proteasomal degradation and by phosphorylation-dependent control of subcellular localization. Phosphorylation of Nrf2/*SKN-1* by specific kinases can either promote or prevent translocation from the cytosol to the nucleus, where gene expression can then be initiated. Kinase-signaling pathways that promote Nrf2/*SKN-1* nuclear localization, thus acting as positive regulators, include the p38/Erk MAPK pathways and the JNK stress-activated pathway (Inoue *et al.* 2005; Okuyama *et al.* 2010); while negative regulators (*i.e.*, phosphorylation that promotes cytosolic localization) of Nrf2/*SKN-1* include *GSK-3 β* and *SGK-1* pathways (An *et al.* 2005; Tullet *et al.* 2008). Nrf2 is negatively regulated by the oxidative stress sensor Keap1 that promotes cytoplasmic localization and degradation of Nrf2 (Kobayashi *et al.* 2006). Proteasomal degradation of Nrf2 also occurs in a Keap1-independent manner, demonstrated by mutations in Nrf2 that abolish the Nrf2/Keap1 interaction yet still exhibits targeted degradation (Suzuki and Yamamoto 2015). *C. elegans* lacks a Keap1 ortholog; however, the *C. elegans* protein *WDR-23* (which interacts with the *CUL-4/DDB-1* complex) acts analogously by targeting *SKN-1* for degradation, thus preventing its accumulation (Choe *et al.* 2009).

Our interest lies in further understanding the mechanisms by which an organism responds to oxidative conditions. To this end, we examined the role of Brap2 (BRCA1-binding protein 2 or Brap as listed in the Human Genome Organisation database) in this response. Mammalian Brap2 is an E3 ubiquitin ligase that inhibits Brca1 nuclear localization by binding to the Brca1 nuclear localization signal motifs (Li *et al.* 1998). Brap2 also acts as a cytoplasmic retention protein that prevents nuclear import of the SV40 large T antigen, mitotin (CENP-F), p21^{WAF1/CIP1}, Cdc14, along with a number of proteins isolated by yeast two-hybrid screening using a testis library (Li *et al.* 1998; Chen *et al.* 2009; Davies *et al.* 2013; Fatima *et al.* 2015). Brap2 was found to be autopolyubiquitinated in response to Ras activation. This action then releases the Brap2 inhibition of kinase suppressor of Ras (KSR) and activates the KSR–RAF–MEK complex, resulting in increased stimulus-dependent MEK activation (Matheny *et al.* 2004).

Previously, we identified the *C. elegans* Brap2 ortholog (*BRAP-2*) and found that it is required to prevent larval arrest in response to elevated levels of ROS (Koon and Kubiseski 2010). Because *brap-2* mutants display increased sensitivity to oxidative stress, we measured the expression levels of ROS detoxification enzymes in these animals. The work presented here provides evidence for a new role for *BRAP-2* in response to oxidative stress through *SKN-1*. We report that the *brap-2(ok1492)* mutation triggered a *PMK-1/SKN-1*-dependent induction of the phase II detoxification response. An RNA interference (RNAi) screen found that this enhancement is dependent on the transcription factor *ELT-3*, a member of the GATA transcription factor family. We demonstrate that *ELT-3* physically interacts with *SKN-1* to activate *gst-4* transcription

in vitro and that worms overexpressing *SKN-1* required *ELT-3* for the phase II detoxification response *in vivo*. Together, these observations suggest a model where the removal of *BRAP-2* leads to the *PMK-1*-dependent activation of *SKN-1* and *ELT-3* to promote expression of nematode phase II detoxification enzymes.

Materials and Methods

C. elegans strains

All *C. elegans* strains were maintained as described by Brenner (1974). Experiments were performed at 20° unless stated otherwise. *C. elegans* strains used in this study are listed in Supplemental Material, Table S1. Unless noted, all *C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center.

GFP reporter analysis

To investigate GFP expression in a *brap-2(ok1492)* background, transgenic worms were generated using standard *C. elegans* techniques and confirmed by single-worm PCR. L4-stage animals were picked and anesthetized using 25 mM Levamisole (#L9756, Sigma Chemical, St. Louis, MO) and mounted on 2% agarose pads. Images of fluorescent animals were taken using a Carl Zeiss (Thornwood, NY) LSM 700 confocal laser-scanning microscope with Zen 2010 Software. For *SKN-1B/C::GFP* localization studies, the accumulation of nuclear localization was categorically quantified ($n = 22$). “Low” refers to animals in which *SKN-1B/C::GFP* was barely detectable in either anterior or posterior intestinal nuclei, “medium” refers to detection in both anterior and posterior intestinal nuclei, and “high” refers to detection throughout the intestinal nuclei (Inoue *et al.* 2005).

Cell culture and transfection

Human embryonic kidney (HEK) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. For transfection, 5 μ g (pull-down assay) or 0.5 μ g (luciferase assay) of mammalian expression constructs were cotransfected in HEK-293T cells using the transfection reagent polyethylenimine.

Pull-down assay and Western blot

A GST pull-down assay was performed to investigate *SKN-1/ELT-3* interaction. HEK-293T cells were cotransfected with plasmids coding EGFP::*SKN-1* and GST::*ELT-3*, and cells were lysed in Tris-buffered saline containing 1% NP-40 and Complete Protease Inhibitor Cocktail (#D06159166, Calbiochem, San Diego, LA) 72 hr after transfection. The GST-tagged protein was then pulled down from cell lysates using glutathione sepharose beads (#17-0756-01, Roche), according to the manufacturer's protocol. Samples were analyzed by standard SDS polyacrylamide gel, followed by Western blot analysis. The antibodies used were anti-GST (#2622, NEB), and anti-GFP (1:5000) (#SC-9996, Santa Cruz).

For Western blots of whole-worm lysates, synchronized adult worms were grown for each genotype. Packed worms (100 μ l) were washed with M9 buffer and resuspended in worm lysis buffer [50 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM EDTA, protease inhibitor and phosphatase inhibitor cocktail (#P5726 and #P0044, Sigma Chemical)] followed by sonication (10 sec ON/10 sec OFF, 10% amplitude for 2 min). The lysates were cleared by centrifugation and the total protein lysate concentrations were quantified using a bicinchoninic acid (BCA) assay according to the manufacturer's protocol (#23227, Thermo Scientific). Western blots were performed by loading 50 μ g of total protein lysates and blots were probed with anti-phospho p38 MAPK antibody (1:200) (#4511, NEB) and anti-tubulin (1:1000) (#3873, Cell Signal). The *PMK-1* band densities were measured and normalized to their respective anti-tubulin protein levels using ImageJ software (National Institutes of Health).

Luciferase assay

The *gst-4* promoter region (724 bp) was amplified from wild-type *C. elegans* genomic DNA by PCR, and the DNA product was cloned into a *KpnI* digested pGL4.10 luciferase vector using the In-Fusion HD Cloning Plus, following the manufacturer's instructions (#638909, Clontech).

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

Paraquat/arsenite treatment

N2 and *elt-3* strains were collected with M9 buffer and treated with 100 mM paraquat (#856177, Sigma Chemical) or 5 mM sodium arsenite (#35000-1L-R, Sigma Chemical) for 2 hr to induce stress shock, followed by RNA isolation, and quantitative real-time PCR (qRT-PCR) for *gst-4* messenger RNA (mRNA) quantification. M9 buffer treatment was performed simultaneously as control. The experiment was completed in three independent trials. To assay for arsenite and paraquat toxicity, young adults were placed in M9 buffer as above and the number of surviving worms were counted at the indicated times. Results from the three independent trials were pooled and the average number of surviving animals ($\pm$ SE) per time point was plotted.

Transcription factor RNAi screening and RNAi assays

To identify new regulators and coactivators of *SKN-1*, an RNAi screen was performed using a near-complete transcription factor RNAi library that contains 882 of the 934 predicted transcription factors (Reece-Hoyes *et al.* 2005) and 30 unconventional DNA binding proteins (Table S2), as described

previously (MacNeil *et al.* 2015). The RNAi screen was performed in 96-well plates, with each transgenic strain screened in three independent trials using *dvls19* and *brap-2(ok1492);dvls19* strains which express GFP under the control of the *gst-4* promoter. RNAi was carried out by feeding animals with bacteria containing a plasmid with the ORF of a specific transcription factor, and the change in GFP expression in the whole worm was visually monitored in the RNAi-treated worms. Each plate contained a positive (RNAi for *gfp*) and negative (empty vector pL4440) control. Animals that showed a diminished GFP expression in at least two of the three trials were scored as positives, excluding RNAi samples that produced dead, sick, or slow-growing worms. Further RNAi assays to validate the positive candidates were carried out by feeding worms double-stranded RNA on bacterial NGM plates containing ampicillin (100 μ g/ml), IPTG (0.4 mM), and tetracycline (12.5 μ g/ml).

Chromatin immunoprecipitation

Protein lysates were prepared and chromatin immunoprecipitation (ChIP) was performed as described with some modification (Mukhopadhyay *et al.* 2008; Claycomb *et al.* 2009). Briefly, synchronized L4 worms were collected and frozen worm pellets were prepared using liquid nitrogen prior to sonicating and cross-linking. Lysates were collected by centrifugation and the total protein concentration was determined using a Thermo Fisher BCA Assay Kit (#23227). An amount of 0.5 mg of total protein lysates along with IgG (#ab37415, Abcam) or anti-GFP (#ab290, Abcam) antibody was used for overnight incubation. DNA collected from reverse cross-linking was resuspended in 25 μ l double-distilled H₂O followed by qRT-PCR. The sequences of oligonucleotides used in this study are listed in Table S3.

RNA isolation and qRT-PCR

For each strain, mixed-stage worms were collected and washed three times with M9 buffer. Total RNA was isolated using TRI Reagent (#93289, Sigma Chemical) as described by the manufacturer's protocol. The collected RNA was then treated with DNA-free DNA Removal Kit (#AM1906, Ambion). Total RNA concentration was determined using the Thermo Fisher NanoDrop2000 and 0.5 μ g of RNA was used to carry out reverse-transcription PCR with Applied Biosystems (Foster City, CA) RNA-to-cDNA Kit (#4387406) in a 20- μ l reaction following the manufacturer's protocol. SYBR Green (#98005, FroggaBio) qRT-PCR was conducted using QIAGEN (Valencia, CA) Rotor-Gene Q Detection System and analyzed using the comparative method ($\Delta\Delta$ Ct). qRT-PCR data were derived from three independent replicates. Results were graphed and the mRNA expression level of each strain was compared relative to *N2*. The endogenous control used for normalization was *act-1* (Tullet *et al.* 2008) unless otherwise specified. *act-1* mRNA levels were compared to other reference genes in a number of strains used in this study, and its level of expression was consistent between them (Figure S1A in File S1). A standard curve for each set of primers was constructed using

a serial dilution of complementary DNA to verify equal amplification efficiency.

Statistics

Statistical analysis was performed with Prism 7 software (GraphPad). Statistical significance was determined using a Student's *t*-test when two means were compared and *P*-values < 0.05 were taken to indicated statistical significance. Error bars represent $\pm$ SEM.

Life-span assay

Life-span assays were performed as previously described (Wilkinson *et al.* 2012). The long-lived strain LD1250 (*ldEx7*) and N2 were raised at 20° on NGM plates seeded with *Escherichia coli* HT115. At L1, 40 worms were placed onto *elt-3* RNAi seeded plates. Worms were transferred to fresh plates (away from progeny) and survival was scored every day as response to prodding. Missing nematodes or worms that died from vulval bursting were censored. The online program Oasis2 was used to generate the Kaplan–Meier curves and log-rank analysis was used to compare the significance of the survival curves using median life span (Han *et al.* 2016).

Data availability

Strains are available upon request or through the *Caenorhabditis* Genetics Center. All the data necessary for confirming the conclusions presented in the article are represented fully within the article and Supplemental Material.

Results

brap-2 mutants display increased expression of phase II detoxification genes

Our current interest in studying the mechanisms controlling an organism's response to oxidizing conditions came about from studying the physiological role of *C. elegans* BRAP-2. We observed that *brap-2(ok1492)* worms arrest at the L1 larval stage when exposed to oxidative stress, and we hypothesized that the *brap-2(ok1492)* mutant may have altered expression of ROS detoxification enzymes. To test this hypothesis, we used transgenic animals carrying either the transcriptional oxidative-stress-sensitive reporters *gst-4p::gfp* (a SKN-1 reporter gene) or *sod-3p::gfp* (a DAF-16 responsive transgene) and analyzed GFP expression levels in wild-type and *brap-2(ok1492)* mutant animals. We found that *brap-2(ok1492)* worms expressed elevated levels of GFP in intestinal and hypodermal cells in *gst-4p::GFP* animals compared to wild-type animals, while no change in *sod-3p::gfp* reporter levels was observed (Figure 1A). We also measured whole-worm fluorescence of animals expressing GFP under control of the *gst-4* promoter and found that the *brap-2* mutant worms had a 2.5-fold increase in GFP levels compared to wild type (Figure 1B). We next examined endogenous mRNA levels by qRT-PCR. Consistent with the results obtained from fluorescent microscopy, relative to wild-type animals, *brap-2(ok1492)* mutants displayed an ~10- to 15-fold increase

in *gst-4* expression (Figure 1C and Figure S1B in File S1). To confirm activation of phase II genes, we examined 10 additional phase II genes and 8 out of 10 showed a significant increase in expression in the *brap-2* mutant relative to wild type (Figure 1C). We also quantified mRNA levels of other nonphase II stress response genes, and of the seven tested, we found one, *ctl-2*, increased in the *brap-2* mutant by 1.7 times as compared to wild type (Figure 1D). Therefore, the *brap-2(ok1492)* mutation generates a specific activation of the phase II detoxification genes and not a general stress response.

In response to oxidative stress, the phase II detoxification genes in *C. elegans* are induced by the transcription factor SKN-1. To determine whether the activation of these genes in *brap-2(ok1492)* mutants is mediated by SKN-1, RNAi was used to knockdown *skn-1* in *brap-2(ok1492)* animals. As expected, a reduction of *gst-4* expression was seen with *skn-1* depletion (Figure 2A). In addition, *skn-1(zu67)* (a loss-of-function mutant of *skn-1*) and *brap-2(ok1492); skn-1(zu67)* mutants demonstrated a decrease in *gst-4* levels (Figure 2B). These results indicate that the increased expression of *gst-4* in *brap-2(ok1492)* mutants is dependent on the transcription factor SKN-1.

As a common mechanism of SKN-1 control is the regulation of its transport into the nucleus, we investigated the subcellular localization of SKN-1 in N2 and *brap-2(ok1492)* worms. *C. elegans* *skn-1* encodes three splice variants (SKN-1A, SKN-1B, and SKN-1C), each with its own distinct expression pattern and function. SKN-1A is an ER-associated isoform involved in promoting transcriptional response to proteasomal dysfunction, while SKN-1B and SKN-1C have roles in caloric restriction and stress resistance, respectively (An and Blackwell 2003; Bishop and Guarente 2007; Glover-Cutter *et al.* 2013; Lehrbach and Ruvkun 2016). Using L4 nematodes carrying a translational *skn-1b/c::gfp* reporter, we found that, in the absence of *brap-2*, the incidence of SKN-1B/C localization within intestinal nuclei was 2.6-fold higher compared to wild-type animals (Figure 3, A and B). This suggests that BRAP-2 functions to inhibit the nuclear localization of SKN-1, either directly or indirectly. To determine whether BRAP-2 inhibits SKN-1 nuclear localization through direct interaction with SKN-1, we attempted to co-immunoprecipitate the two proteins. However, we were unable to detect a physical interaction between BRAP-2 and SKN-1.

Interestingly, we saw an overall increase in intestinal *skn-1::gfp* expression in *brap-2* mutant animals, suggesting that *skn-1* is transcriptionally upregulated in *brap-2(ok1492)* mutant worms. To determine if the *brap-2* mutation causes an increase in endogenous *skn-1* expression, qRT-PCR was performed and revealed a 1.5-fold increase in *skn-1b* and a 2.75-fold increase in *skn-1c* mRNA levels in *brap-2(ok1492)*, indicating that an accumulation of SKN-1 probably occurs in *brap-2* mutant worms (Figure 3C). Notably, we also used *skn-1* isoform-specific RNAi constructs to determine the isoform responsible for increased *gst-4* expression in *brap-2* mutant worms and found that when *skn-1c*, but not *skn-1a* or *skn-1b*, was knocked down, it resulted in a decrease in *gst-4* mRNA

Figure 1 *gst-4* expression levels are higher in *brap-2(ok1492)* worms. (A) *brap-2(ok1492)* mutants display increased *gst-4p::GFP* in hypodermis and the intestine, but the *sod-3p::GFP* expression is not affected. (B) GFP levels of *brap-2(ok1492)* worms expressing *gst-4p::GFP* were ~2.5 times that of wild type. Quantification was done using ImageJ ($n = 18$ for each sample). (C) The mRNA levels of 11 phase II detoxification genes (log₂ scale) were compared between wild type and *brap-2(ok1492)* mutants. A significant increase in mRNA levels was found in 9 of 11 phase II genes in the mutant strain. (D) The mRNA levels of nonphase II stress-related genes were measured by qPCR. Of the seven genes tested, only one showed a significant increase in the *brap-2(ok1492)* mutant. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

levels (Figure 3D). Therefore, the *brap-2(ok1492)* mutant increases both the amount of SKN-1C and its subcellular localization to the nucleus.

SKN-1 directly binds to the promoter regions of its target genes to increase their expression. The modENCODE project has mapped SKN-1 transcription factor binding sites at every stage of *C. elegans* development using ChIP. These data reveal an enrichment of SKN-1 binding to a number of sites in the *gst-4* promoter at the L4 stage of development. Using ChIP, we examined two *gst-4* promoter regions containing the canonical SKN-1 binding sites WWTRTCAT or TCTTATCA (An and Blackwell 2003). ChIP–quantitative PCR (qRT-PCR) analysis revealed at least a twofold enrichment in SKN-1 binding at two *gst-4* promoter sites in *brap-2(ok1492)* mutants expressing SKN-1::GFP::3xFLAG compared to wild type at

the L4 stage (Figure 3E). These findings confirm that the activation of *gst-4* is dependent on the direct interaction of SKN-1 on the *gst-4* promoter, and that this interaction is enhanced in the *brap-2(ok1492)* mutant.

Regulation of SKN-1 by BRAP-2 involves the p38 MAPK pathway

Since SKN-1 is mainly localized in the nucleus of *brap-2(ok1492)* mutants and we could not detect a physical interaction between BRAP-2 and SKN-1, we hypothesized that the change in SKN-1 subcellular localization may be due to the phosphorylation state of SKN-1. One candidate kinase that may carry out this phosphorylation is PMK-1 (the *C. elegans* ortholog of mammalian p38 MAPK) whose phosphorylation of SKN-1 promotes its nuclear localization, resulting in

Figure 2 Enhanced *gst-4* expression in *brap-2(ok1492)* requires SKN-1. (A) Synchronized *gst-4p::gfp* and *brap-2(ok1492);gst-4p::gfp* worms were grown in control (L4440) or *skn-1* RNAi. *brap-2(ok1492)* shows decreased *gst-4* expression when *skn-1* is knocked down in L4 worms. (B) RNA was extracted and the *gst-4* mRNA levels (log₂ scale) were quantified using qRT-PCR. The *brap-2(ok1492);skn-1(zu67)* double mutant showed a reduction of *gst-4* mRNA expression. ** $P < 0.01$.

activation of **SKN-1** target genes that promote longevity (Inoue *et al.* 2005).

We asked if the p38/**PMK-1** pathway was required for **BRAP-2** regulation of the **SKN-1** detoxification pathway and we found that the *brap-2(ok1492)* mutation induced a 1.4-fold increase in phospho-PMK levels, as measured by Western

blot (Figure 4A); indicative of a modest increase in activated PMK levels. We also quantified the expression of two genes, *F08G5.6* and *F35E12.5*, previously shown to be targets of *pmk-1* (Troemel *et al.* 2006; Cornejo Castro *et al.* 2010). We found higher levels of mRNA for these two transcripts in *brap-2(ok1492)* animals compared to wild type, and that

Figure 3 Increased SKN-1 intestinal nuclear localization and binding to the *gst-4* promoter in *brap-2(ok1492)*. (A) Localization of *skn-1::gfp* in N2 (top) and *brap-2(ok1492)* (bottom) worms. Synchronized worms were grown on NGM plates and L4 worms were picked to visualize GFP expression using confocal microscopy. The white arrows indicate the locations of intestinal nuclei. Results indicated an accumulation of SKN-1::GFP in intestinal nuclei in *brap-2(ok1492)* animals. (B) Percentage of SKN-1::GFP nuclear localization in *brap-2(ok1492)* and N2 worms were categorically scored and quantified as described in *Materials and Methods*. The percentage of nuclear localization increased 2.6-fold in *brap-2* mutant worms as compared to wild type. (C) RNA was isolated from N2 and *brap-2(ok1492)* worms followed by quantification of *skn-1a*, *skn-1b*, and *skn-1c* mRNA transcript levels using qRT-PCR. Results demonstrated an enhanced *skn-1b* and *skn-1c* expression in *brap-2(ok1492)*. *** $P < 0.001$, ** $P < 0.01$ vs. N2. (D) Isoform-specific RNAi of *skn-1a*, *skn-1b*, and *skn-1c* isoforms of *skn-1* was performed and *gst-4* mRNA levels quantified by qRT-PCR. Knockdown of *skn-1c* showed a significant reduction of *gst-4* expression (log₂ scale) in wild-type and *brap-2* mutant animals. *** $P < 0.001$. (E) ChIP-qPCR analysis of SKN-1 binding at two *gst-4* promoter regions in *skn-1::gfp::3xFlag*- and *brap-2(ok1492); skn-1::gfp::3xFlag*-expressing worms. Relative percentage input is normalized to N2 (absence of GFP antigen). Data represented the mean of four independent trials.

this increase was dependent on functional PMK-1, consistent with PMK-1 being activated in *brap-2(ok1492)* (Figure 4B). Furthermore, we found that PMK-1 was at least partially required for the enhanced expression of phase II detoxification

enzymes in *brap-2* mutants, as the *brap-2;pmk-1* double mutant showed a significant decrease in the mRNA levels of genes tested compared to the *brap-2* mutant (Figure 4C). These findings indicate that the p38 MAPK pathway is at least

partially responsible for regulating the expression of phase II detoxification genes in the *brap-2(ok1492)* animals, although whether this is due to BRAP-2 directly regulating PMK-1 activity or some indirect mechanism of activation in the mutant animals remains to be determined.

The ELT-3 GATA transcription factor is required for *gst-4* expression

Transcription factors are integral components of gene regulatory networks, serving as key regulators of gene activation or repression. The regulation of gene expression could be a product of the interactions between multiple transcription factors, and identifying transcription factors that work in combination with SKN-1 will increase our understanding of the complex regulatory network involved in the phase II detoxification response. Since *brap-2(ok1492)* activated a signaling cascade that enhanced the SKN-1 phase II detoxification pathway, we used the *brap-2(ok1492);gst-4p::gfp* strain as the basis for an RNAi screen to test for transcription factors that, when knocked down, suppress *gst-4p::gfp* expression. Out of 912 tested clones, we identified 18 transcription factors that, when knocked down, resulted in decreased GFP expression in *brap-2(ok1492)* (Table S4). Among the candidates were three GATA transcription factor family members (*elt-1*, *elt-2*, and *elt-3*), suggesting that GATA transcription factors may play an important role in regulating expression of phase II detoxification enzymes in *C. elegans*. *elt-3* was chosen for further study because it is a potential regulator of aging (Budovskaya *et al.* 2008) and because *elt-3* homozygous mutant animals are viable and amenable to genetic studies. We observed that *brap-2(ok1492)* worms treated with *elt-3* RNAi displayed reduced levels of GFP compared to wild type (Figure 5A). To further validate our RNAi screen, we quantified mRNA levels in *brap-2(ok1492);elt-3(vp1)* double mutants and found a >60% reduction in *gst-4* expression (Figure 5B). These results indicate that ELT-3 is required for the induction of *gst-4* expression in the absence of BRAP-2.

After defining the importance of ELT-3 in the expression of *gst-4*, we asked whether ELT-3 could coactivate and thus enhance SKN-1 activity. To determine if ELT-3 is required for the activation of *gst-4* during oxidative stress, we measured *gst-4* mRNA levels after arsenite or paraquat treatment in *elt-3* mutants. In response to either stressor, *gst-4* mRNA levels increased in wild-type animals. However, induction of *gst-4* mRNA was significantly decreased in *elt-3(vp1)* mutants relative to wild-type animals, indicating that ELT-3 is required to induce SKN-1 targets upon oxidative stress (Figure 6A). We also measured the ability of *elt-3* mutant worms to survive exposure to oxidative stress. We found that *elt-3(vp1)* animals were hypersensitive to arsenite, but responded similarly to paraquat as the wild type (Figure 6B and Table S5). The reason for the difference between arsenite and paraquat treatments is not known, but may be due to differences of mechanisms of action in promoting oxidative stress (Lee *et al.* 2003), or differences in the tissues these compounds may target *in vivo*.

We then tested whether SKN-1 and ELT-3 physically interact to promote gene expression. ELT-3 activation of *gst-4* appears to be direct and, furthermore, ELT-3 binds to the promoters of phase II detoxification enzymes at regions that overlap those of SKN-1, as indicated by modENCODE genome-wide ChIP surveys (Figure S2 in File S1). We generated a luciferase reporter construct using the *gst-4* promoter that we transfected into HEK293T cells along with SKN-1 and/or ELT-3. Although a moderate increase in *gst-4* promoter expression was seen when either SKN-1 or ELT-3 alone was transfected, a synergistic increase in luciferase expression was detected in the presence of both SKN-1 and ELT-3 (Figure 6C). Furthermore, we cotransfected HEK293T cells with tagged SKN-1 and/or ELT-3 and detected an interaction between SKN-1 and ELT-3 *in vitro*, suggesting that these two transcription factors heterodimerize (Figure 6D). Therefore, the data presented here show that the GATA transcription factor ELT-3 has an essential role in regulating *gst-4* and the oxidative stress response with SKN-1.

Since *skn-1b* and *skn-1c* mRNA levels are increased in *brap-2(ok1492)* mutants, we asked whether ELT-3 was required for this increase. We found that the induction of *skn-1b* and *skn-1c* transcripts observed in the *brap-2* mutant was completely lost in *brap-2(ok1492);elt-3(vp1)* double mutants (Figure S3A in File S1). Since SKN-1 has also been found to interact with its own promoter (Gerstein *et al.* 2010; Figure S3B in File S1), we also tested if SKN-1 could function with ELT-3 in inducing *skn-1c* expression. We generated a luciferase reporter construct containing the *skn-1c* promoter, used it to transfect HEK293T cells along with expression vectors for SKN-1 and ELT-3, and found that ELT-3 and SKN-1 synergistically induced the *skn-1c* promoter (Figure S3C in File S1). These results show that ELT-3 was required for the induction of SKN-1C in the *brap-2* mutant, and that these inductions likely required SKN-1.

We also determined the expression pattern and cellular localization of ELT-3 *in vivo*. The translational reporter strain *elt-3::gfp* was used and we were able to detect ELT-3 in hypodermal nuclei in both the wild type and *brap-2(ok1492)* (Figure S4 in File S1). Thus, unlike SKN-1, ELT-3 subcellular localization appears to be independent of BRAP-2 activity. Furthermore, no change in ELT-3::GFP levels was seen when wild-type and *brap-2(ok1492)* worms were compared, indicating that the *brap-2* mutation does not influence ELT-3 expression.

SKN-1 has been shown to promote longevity under certain conditions (Blackwell *et al.* 2015). For example, while transgenes that produce high levels of SKN-1 are harmful, moderate levels of SKN-1 overexpression lead to life-span extension (Tullet *et al.* 2008; Blackwell *et al.* 2015). A number of *skn-1* gain-of-function mutations have been identified. A mutation in SKN-1 that disrupts its interaction with the inhibitor WDR-23 and promotes SKN-1 stability leads to life-span extension (Choe *et al.* 2009), while two other characterized gain-of-function mutations (*lax120* and *lax188*) have no significant effect on life span (Paek *et al.* 2012); demonstrating the

Figure 4 PMK-1 is activated in *brap-2(ok1492)* mutants and is partially required for enhanced *gst-4* expression. (A) Phosphorylation level of PMK-1 is increased in *brap-2* mutants. (Left) A Western blot of lysates from wild-type, *brap-2(ok1492)*, *pmk-1(km25)*, and wild-type worms treated with arsenite probed with an α -phospho-p38 antibody. (Right) Relative intensity of bands corresponding to phospho-PMK levels were compared to α -tubulin levels using ImageJ and normalized to wild type. The experiment was carried out three times and the relative intensity of the phospho-PMK bands were pooled and graphed. (B) As a measure of PMK-1 activity, transcript levels of two *pmk-1* targets (left, *F08G5.6*; and right, *F35E12.5*) in *brap-2*, *pmk-1*, and *brap-2;pmk-1* mutants were measured using qRT-PCR (log₁₀ scale). *brap-2(ok1492)* animals showed higher levels of transcripts that were dependent on functional *pmk-1*. (C) PMK-1 is required to promote phase II detoxification gene expression in *brap-2(ok1492)* worms. The mRNA levels of (top left) *gst-4*, (top right) *gst-10*, (bottom left) *gsto-2*, and (bottom right) *sdz-8* were quantified using qRT-PCR. The *brap-2;pmk-1* double mutant showed a reduction of mRNA expression for each gene tested. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

complexity of *SKN-1*'s role in life-span extension. Since the transcription factor *ELT-3* is involved in the regulation of aging and is needed for phase II detoxification gene expression, we sought to determine whether *ELT-3* is required to coactivate the *SKN-1* regulated pathway for life-span extension. To test this, we used the moderately expressing *SKN-1* transgenic strain (LD1250; Tullet *et al.* 2008), knocked down

elt-3 by RNAi, and performed a life-span assay. Overexpressing *SKN-1* significantly increased life span compared to wild type, while RNAi-mediated knockdown of *elt-3* in this strain prevented this increase (Figure 7 and Table S6). This may indicate that the enhanced life span observed in transgenic animals overexpressing *SKN-1* is dependent on functional *ELT-3*, although it is possible that the decrease in life span

Figure 5 Loss of *elt-3* reduced *gst-4* expression in *brap-2(ok1492)* mutants. (A) Synchronized *gst-4p::gfp* and *brap-2(ok1492); gst-4p::gfp* worms were grown on control (L4440) or *elt-3* RNAi plates and GFP expression was examined at L4 stage of development. *brap-2(ok1492)* worms show a reduced *gst-4p::gfp* expression when *elt-3* is knocked down. (B) Synchronized worms were grown on NGM plates followed by RNA extraction and qRT-PCR. The double mutant *brap-2(ok1492); elt-3(vp1)* shows a 70% reduction in *gst-4* mRNA expression in comparison to *brap-2(ok1492)*. *** $P < 0.001$, ** $P < 0.01$.

could be due to the *elt-3* mutation making the animals less healthy in general. Since *brap-2(ok1492)* activates *SKN-1*, we also asked if the *brap-2(ok1492)* animals had an increased life span. However, *brap-2(ok1492)* worms demonstrated a shorter life span compared to wild type (Figure S5 in File S1), possibly due to either an insufficient amount of activated *SKN-1*, inactivation of other detoxification enzymes (such as phase I detoxification enzymes), and/or modification of some as-yet-unidentified pathway or physiological process in the *brap-2* mutant that prevents life span extension.

Discussion

The work presented here identifies new roles for **BRAP-2** and **ELT-3** in oxidative stress response through the transcription factor *SKN-1*. Using *C. elegans* as a model organism, we have shown that the expression of the phase II detoxification genes is increased in the *brap-2(ok1492)* deletion mutant. This strain was used to perform a transcription factor-specific RNAi screen which identified the GATA transcription factor **ELT-3** as a regulator of *gst-4* expression. We also demonstrated

Figure 6 ELT-3 is required for *gst-4* expression during oxidative stress and synergistically activates *gst-4* promoter expression through interaction with SKN-1. (A) Synchronized worms were collected and treated with (left) 5 mM sodium arsenite (As) or (right) 100 mM paraquat (PQ) for 2 hr followed by RNA isolation. qRT-PCR was performed to measure the *gst-4* mRNA transcript levels. (B) Survival assays of N2 and *elt-3(vp1)* strains were determined in (left) arsenite and (right) paraquat. Results indicated ELT-3 is essential for *gst-4* expression during oxidative stress and that *elt-3* mutant worms are more susceptible to arsenite exposure compared to wild type. (C) Luciferase assay was performed and the transcriptional activity was determined by the activation of the *gst-4* promoter/luciferase reporter vector. Maximum activation occurs upon coexpression of both ELT-3 and SKN-1 constructs. (D) A pull down of GST-ELT-3 fusion protein using glutathione sepharose and Western blot using anti-GFP antibody to detect GFP-SKN-1 fusion protein. This assay reveals that EGFP::SKN-1 and GST::ELT-3 physically interact *in vitro*. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

that BRAP-2 and the PMK-1 pathway are involved in regulating the SKN-1/ELT-3 response to oxidative stress. Recently, the related GATA transcription factor ELT-2 was shown to control *C. elegans* immune responses in a p38-dependent manner (Block *et al.* 2015), indicating that the p38 MAPK pathway may play a general role in regulating GATA transcription factor function in response to different types of stress.

Previously, we demonstrated that the *brap-2(ok1492)* mutant worms are sensitive to oxidative stress, as they undergo L1 larval arrest when exposed to paraquat (Koon and Kubiseski 2010). Paradoxically, we find and report here that these animals

have higher mRNA expression of SKN-1-dependent phase II detoxification enzymes, which would presumably lead to an enhancement of the cellular mechanisms to deal with ROS and a reduction in oxidative stress. One possibility to explain this paradox is that SKN-1-dependent phase II detoxification enzymes by themselves are insufficient in completely removing oxidative stress, and alternate signaling pathways or mechanisms are also required. These alternate pathways may be compromised in *brap-2(ok1492)* mutants, preventing them from fully detoxifying ROS compared to wild-type animals, and enhancing their sensitivity to ROS-producing compounds such as paraquat.

Figure 7 Extended life span in overexpressing SKN-1 requires ELT-3. Survival curves of N2 and worms containing the SKN-1 transgene [strain LD1250 (*ldEx7*) expressing *skn-1b/c::gfp*] fed on control bacteria (L4440) or bacteria expressing *elt-3* RNAi from L1 worms at 20°. While *skn-1b/c::gfp* has a significantly longer life span than N2 (mean life spans are 16.7 ± 0.79 days and 13.6 ± 0.62 days, respectively; $n = 40$), less extension of life span was observed in overexpressing SKN-1B/C in *elt-3* RNAi (*skn-1b/c::gfp* mean = 11.1 ± 0.45 days compared to N2 mean = 11.1 ± 0.38 days; $n = 40$). Two independent experiments were performed, data represents one trial.

The *SKN-1* ortholog Nrf2 is a regulator of the major pathways that control detoxification and oxidative stress responses in humans. Defective Nrf2 regulation is commonly found in human diseases, including cancer and Parkinson's disease, and Nrf2 is a candidate target in designing therapeutic agents for attenuation of oxidative stress. Although we do not know if mammalian Brp2 has a similar function in the regulation of Nrf2, our findings in *C. elegans* identify candidate regulatory mechanisms that may also play a role in the oxidative stress response in mammals.

The increased expression of *gst-4* in *brap-2(ok1492)* mutants provided an opportunity to identify novel transcription factors required for phase II detoxification response in *C. elegans* using a genetic suppression screen. We identified *elt-3* as a regulator of *gst-4*. In the worm, GATA transcription factors have well-established roles in directing tissue-specific developmental processes (Block and Shapira 2015). *elt-3* is a member of the GATA family of transcription factors that is expressed in the epidermis during development (Gilleard *et al.* 1999; Gilleard and McGhee 2001; McGhee 2013b). SKN-1A/C::GFP transgenes readily show expression in intestinal cells; however, SKN-1-induced detoxification gene expression can be detected in the hypodermis, suggesting that SKN-1 is active in some tissues when present at low levels (Paek *et al.* 2012). We show that SKN-1 and ELT-3 interact *in vitro* and together enhance transcriptional activity of phase II target genes, indicating that ELT-3 is likely involved in the regulation of this signaling pathway in hypodermal cells. It has been reported that *elt-3* is vital to the regulation of aging, where it is responsible for regulating the expression of specific genes, including *sod-3*; that it is involved in somatic aging; and that *elt-3* is regulated by the DAF-2 insulin-like signaling pathway, where *elt-3* loss of function reverses the life-span extension observed in *daf-2* mutants (Budovskaya *et al.* 2008), although this result is controversial (Kim *et al.*

2012; Tonsaker *et al.* 2012; Kim *et al.* 2013; McGhee 2013a). ELT-2, a GATA transcription factor expressed in the intestine, has also been shown to be deactivated during aging and overexpression of ELT-2 extends life span (Mann *et al.* 2016), so it is possible that ELT-2 and SKN-1 may heterodimerize in the intestine to activate expression of phase II detoxification enzymes as a response to oxidative stress.

Overall, these studies indicate that GATA transcription factors play a role in promoting longevity in *C. elegans*. ELT-3 shows the highest homology to GATA3, a protein that is highly expressed in human epidermal tissues and lymphocytes (Molkentin 2000; Kaufman *et al.* 2003). We would predict that, if this process were conserved between *C. elegans* and humans, Nrf2 and GATA3 (or a closely related GATA transcription factor) would function together to regulate the phase II detoxification response in mammalian cells. Although, to our knowledge, a direct role of GATA3 in regulating response to oxidative stress has not yet been characterized, Nrf2 and GATA3 have been shown to activate cytokine production in response to tert-butylhydroquinone (a synthetic antioxidant) in CD4⁺ T cells (Rockwell *et al.* 2012).

Like mammals, nematodes have well-developed lines of defense for protection against toxic compounds. The *C. elegans* genome encodes a wide range of stress response genes, providing systematic stress response and detoxification strategies. The transcription factor SKN-1 is regarded as a master regulator for the expression of phase II detoxification genes. Our data indicates that BRAP-2 regulates the oxidative stress response by negatively regulating both *skn-1* transcript levels and subcellular localization of SKN-1. While most studies have focused on the post-translational modification of SKN-1 [such as phosphorylation and ubiquitination (An *et al.* 2005; Inoue *et al.* 2005; Tullet *et al.* 2008; Choe *et al.* 2009; Okuyama *et al.* 2010)] as the main mechanism of regulation, our work suggests that *skn-1* transcription is induced upon oxidative stress, as determined by qRT-PCR, and that this induction requires ELT-3.

Oxidative stress is believed to play a critical role in the progression of many diseases including cancer and neurodegenerative diseases. The work presented here provides insight into BRAP2's and SKN-1's roles in the oxidative stress response. We believe that using *C. elegans* to study the coordination of p38 MAPK signaling through proteins such as BRAP-2 enable us to gain a better understanding of diseases caused by stress, as well as identify new targets for future therapies. *C. elegans* mutants lacking BRAP-2 are highly sensitive to oxidative stress, providing us with a valuable genetic tool to reveal a possible new role for BRAP-2 in the regulation of the SKN-1/ELT-3 complex in response to oxidative stress through the p38 MAPK pathway.

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