HAAO-1 INHIBITION ENHANCES C. ELEGANS OXIDATIVE STRESS RESPONSE

By

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Abstract

The kynurenine pathway is the sole *de novo* biosynthetic pathway that produces nicotinamide adenine dinucleotide (NAD^+) from available tryptophan. Previous work in our lab has shown that elevating endogenous levels of the kynurenine pathway metabolite 3-hydroxyanthranilic acid (3HAA) through either inhibition of 3HAA dioxygenase (HAAO) or 3HAA supplementation increases the lifespan of the nematode *Caenorhabditis elegans*. HAAO metabolizes 3HAA into 2-amino-3-carboxymuconate-6-semialdehyde (ACMSA), a precursor of NAD^+. However, the mechanism of lifespan extension through this pathway is still unknown. Here, we investigated the relationship between *haao-1* and oxidative stress in *C. elegans.* Animals with reduced HAAO expression are resistant to multiple forms of oxidative stress. We found that *haao-1(tm4627)* animals (HAAO KO) have elevated endogenous reactive oxygen species (ROS) and activation of the NRF2/SKN-1 oxidative stress response pathway. Treating *haao-1(tm4627)* animals with the glutathione precursor N-Acetyl Cysteine (NAC) rescues the increase in ROS, but only partially rescues activation of NRF2/SKN-1 in animals with mutant *haao-1* or *haao-1* RNAi. This demonstrates that activation of SKN-1 in the *haao-1(tm4627)* mutant background is partially dependent on ROS, but also there are also likely non-ROS-dependent mechanisms.

Introduction

Oxidative stress is a normal cellular process that occurs in the cell. Cells respond to elevated ROS by activating the oxidative stress response pathway. Increase in oxidative stress plays an important role in the development of several age-related diseases and is associated with aging. Oxidative stress occurs due to the imbalance between an accumulation of reactive oxygen species (ROS) and how well the system is able to remove these reactive products. Some ROS are superoxide radicals ($O_2•^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH•^-$), singlet oxygen (O_2), lipid peroxides, among others. One major source of ROS is the mitochondria where they are produced as byproducts of normal oxygen metabolism in the electron transport chain (ETC) and other biological systems including innate immune responses (Pizzino, et al, 2017; Murphy, 2009). However, the dichotomic role of ROS in the cell as damaging or signaling molecules, demonstrate that the duality in molecular behavior between these downstream effects is based on a fine tuning of the spatial and temporal localization, therefore generating particular effects base on specific thresholds (Ludovico and Burhans, 2014).

The kynurenine pathway converts ingested tryptophan to NAD^+, which is a cofactor involved in several enzymatic redox reactions and mitochondrial energy production. Decreases in NAD^+ levels have been associated in many age-associated diseases (Covarrubias, et al., 2020). In this pathway, the enzyme 3-hydroxyanthranilate dioxygenase (HAAO) metabolizes 3-hydroxyanthranilic acid (3HAA), which is a precursor of NAD^+ (Castro-Portuguez and Sutphin, 2020). Previous work in our lab has shown that inhibition of the *haao-1* enzyme or supplementation with 3HAA leads to a lifespan extension in the nematode, *Caenorhabditis elegans* (Dang, et al., 2021). However, the mechanism by which this lifespan extension occurs in *C. elegans*, remains unknown.

Due to preliminary data pointing at the antioxidant effects of 3HAA from our lab and others, we investigated the relationship between *haao-1* and oxidative stress in *C. elegans*. We have found that inhibition of *haao-1* leads to elevated endogenous ROS and in turn, the activation of the NRF2/SKN-1 oxidative stress response pathway. This pathway is known to play a role in promoting longevity in *C. elegans*, by reducing the harmful ROS species in the nematodes and increasing resistance to other secondary stressors (Blackwell et al., 2015; Staab et al., 2013). We suggest that elevated ROS activates specific transcriptional activity that mediates the NRF2/SKN-1 pathway and leads to an enhanced oxidative stress response.

Materials and Methods

Strains

The following strains were obtained from the Caenorhabditis Genetic Center (CGC) at the College of Biological Sciences at the University of Minnesota:
ldIs7 [skn-1b/c::GFP + rol-6(su1006)] (LD1); ldIs3 [gcs-1p::GFP + rol-6(su1006)] (LD1171); wgIs341 [skn-1::TY1::EGFP::3xFLAG + unc-119(+)] (OP341); N2; jrIs1 [rpl-17p::HyPer + unc-119(+)] (JV1); jrIs2 [rpl-17p::Grx1-roGFP2 + unc-119(+)] (JV2); skn-1(zj15) IV (QV225); hif-1(ia4) V (ZG31); hsf-1(sy441) I (PS3551); daf-16(mu86) I (CF1038); skn-1(zu135) IV/nT1 [unc-?(n754) let-?] (IV;V) (EU31); skn-1(zu67) IV/nT1 [unc-?(n754) let-?] (IV;V) (EU1); skn-1(mg570) IV (GR2245). Strain *haao-1(tm4627)* V (FX04627; backcrossed 6x to N2 to create strain GLS130) were obtained from the C. elegans National Bioresource Project (NBRP) at the School of Medicine at the Tokyo Women's Medical University. Wild-type (N2) worms were originally obtained from Dr. Matt Kaeberlein (University of Washington, Seattle, WA, USA). Strain *haao-1(tm4627)* V; *ldIs3 [gcs-1p::GFP + rol-6(su1006)]* (GLS333) was generated by crossing GLS130 to LD1171. Strain *haao-1(tm4627)* V; *ldIs7[skn-1b/c::GFP* + *rol-6(su1006)]* (GLS334) was generated by crossing GLS130 to LD1.

C. elegans culture and maintenance

We kept the worms on 60 mm plates of Nematode Growth Media (NGM) spotted with OP50 *E. coli* (Sutphin and Kaeberlein, 2009) unless described differently. The worms were maintained at 20°C and transferred to new plates at least twice per week to prevent starvation. For RNAi experiments, OP50 was replaced with different RNAi containing plasmids in the HT115 *E. coli* strain. Finally, 50 µL of carbenicillin and 100 µL of IPTG per 100 mL of NGM were added to RNAi plates (Sutphin and Kaeberlein, 2009).

Age synchronization

The bleaching technique was used for age synchronization of *C. elegans*. To do this, we washed the desired plate with sterile H_2O to loosen the eggs and worms that were stuck to the plate. Then, we transferred this liquid into a sterile centrifuge tube. We prepared a mixture of 10M NaOH, d_2H_2O, and bleach. Next, we placed the tube in a centrifuge for 60 seconds at 1700 RPM. We added 5 mL of the bleach solution and placed it on a nutator for no more than 3 minutes. We aspirate the supernatant and repeat the

previous step one more time. Then we added 10 mL of sterile d_2H_2O and placed it in the centrifuge for 60 seconds at 1700 RPM. We removed the excess liquid and placed the desired number of eggs on the selected plate (Porta-de-la-Riva, et. al., 2012).

Acute juglone toxicity assay

Juglone is an ROS-generating compound that induces oxidative stress commonly used to assess the sensitivity to oxidative stress in *C. elegans* (Senchuk, et al., 2017).
We use juglone in NGM plates at a concentration of 200 μM. These plates were poured on the same day as the experiment and were left to dry for approximately 20 minutes. They were then spotted with empty vector (EV, control RNAi) or *haao-1* RNAi in the HT115. These were again left to dry for approximately one hour. Finally, 40-60 worms at the L4 stage were placed on the plate and left for 16 hours. At the end of the 16-hour incubation, the worms were prodded to assess for survival. We also used juglone in NGM plates at a concentration of 250 μM. They were poured and set up in the same way and spotted with OP50 unless noted differently. They were then prodded every hour for 10 hours to assess for survival.

ROS measurements

2',7'-dichlorofluorescein diacetate (H_2DCFDA) is used to detect the presence of ROS in *C. elegans*. It penetrates the cellular membrane and then is deacetylated by esterases which turns it into H_2DCF, a non-fluorescent compound. This compound is rapidly oxidized to DCF, which is highly fluorescent in the presence of ROS (Yoon, et. al., 2018). After we treated our worms (approximately 50), we washed the worms from the agar plate with M9 buffer. This was transferred to a centrifuge tube and then washed three times with M9 buffer. These worms were transferred to an OP50 agar plate for 5-10 minutes, so they were able to crawl from the liquid. Approximately 15 animals were transferred onto an agarose pad with a drop of levamisole. Pictures were taken with a Leica microscope to measure the fluorescence signal.

Results

Animals with reduced *haao-1* are resistant to oxidative stress

We first sought to determine the effects of *haao-1* knockdown in response to oxidative stress inducers and the potential mechanism mediating this particular

response. Previous experiments in our lab showed that *haao-1(tm4627)* mutants (Figure 1A) and *haao-1(RNAi)* treated worms (Figure 1B) that were exposed to 250 µM juglone showed an increase in survival compared to wildtype worms or *EV(RNAi)* treated worms, demonstrating that worms without active *haao-1* are resistant to oxidative stress. To assess potential downstream effectors of this response, we selected 4 transcription factors that mediate stress response as potential mediators of oxidative stress resistance in *haao-1* deficient animals. In *C. elegans*, SKN-1 is the transcription factor that is orthologous to NRF2 in humans (Edwal et al, 2017) and DAF-16 is the ortholog of FOXO in mammals (Senchuck, et al, 2018), and they are used for oxidative stress response. HSF-1 is the ortholog to HSF1 in mammals (Brunquell et al, 2016) and is used in heat shock response, and finally HIF-1 is the ortholog to HIF1A in mammals (Hwang et al, 2014) which is used in hypoxic response.

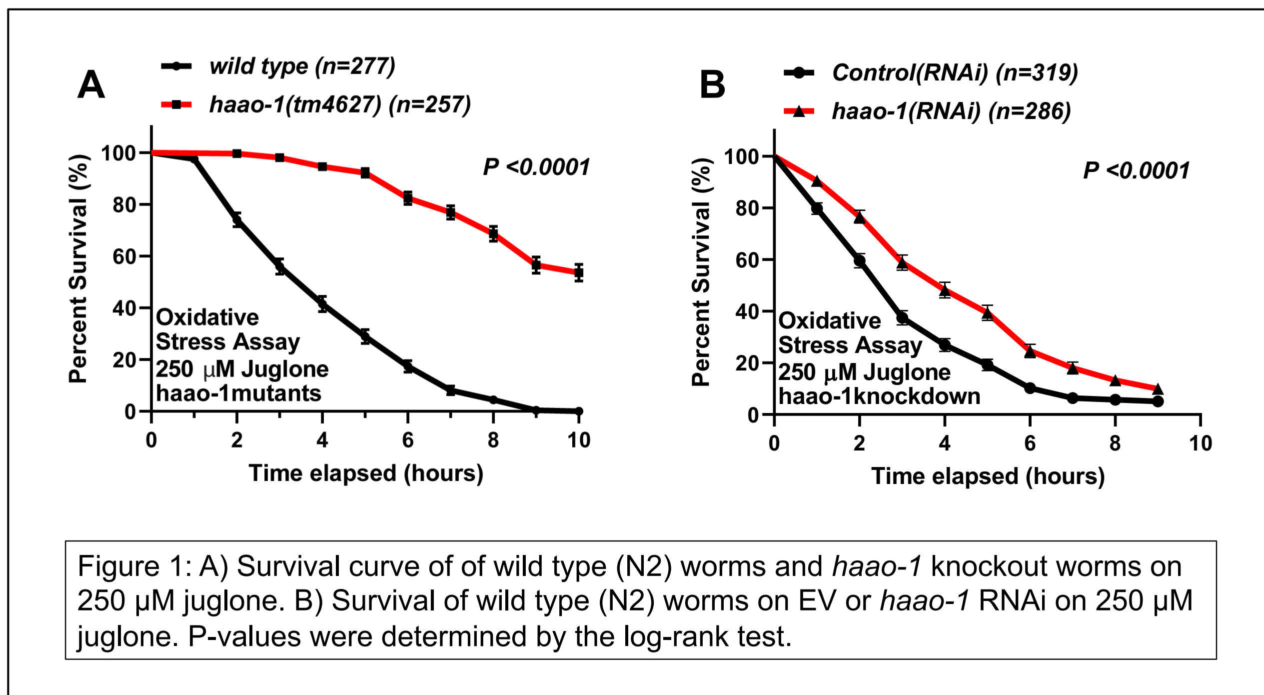

Figure 1: A) Survival curve of of wild type (N2) worms and *haao-1* knockout worms on 250 µM juglone. B) Survival of wild type (N2) worms on EV or *haao-1* RNAi on 250 µM juglone. P-values were determined by the log-rank test.

To see which transcription factors were required for an enhanced stress response upon *haao-1* inhibition, we compared wild type worms to strains with loss-of-function mutations in each transcription factor under *EV(RNAi)* or *haao-1(RNAi)*. We place each strain under acute juglone stress plates for approximately 16 hours. We observed that all the *haao-1(RNAi)* treated worms had an increased survival compared to the *EV(RNAi)* (Figure 2), except for the hsf-1(sy441) mutants. However, we also saw that the *hsf-1 EV(RNAi)* treated worms were still alive after the 16 hours, meaning that the worms were already resistant to juglone and unable to determine if the increase in resistance was due to the *haao-1(RNAi)* treatment. The involvement of HSF-1 in HAAO-1-mediated oxidative stress response could therefore not be assessed. This shows that

SKN-1, DAF-16, and HIF-1 are not required for oxidative stress resistance conferred by *haao-1* inhibition.

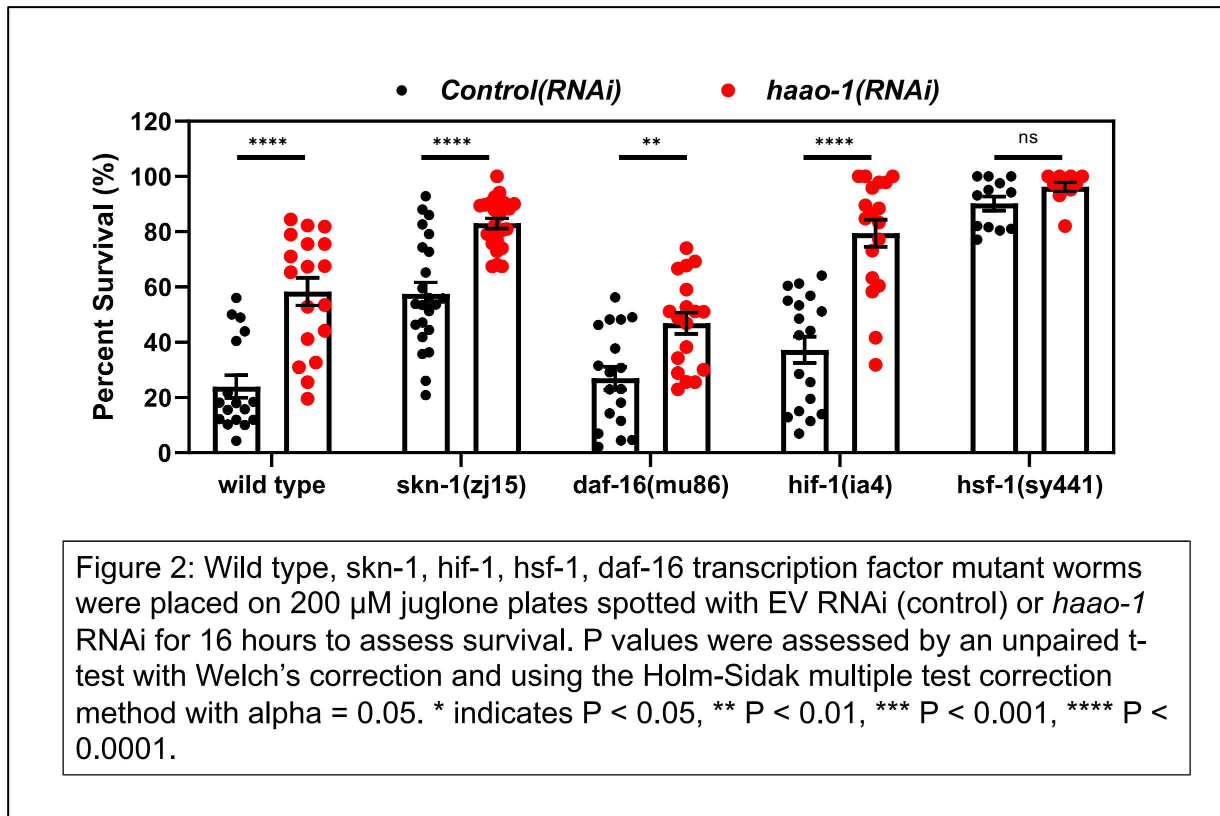

Figure 2: Wild type, skn-1, hif-1, hsf-1, daf-16 transcription factor mutant worms were placed on 200 μM juglone plates spotted with EV RNAi (control) or *haao-1* RNAi for 16 hours to assess survival. P values were assessed by an unpaired t-test with Welch's correction and using the Holm-Sidak multiple test correction method with alpha = 0.05. * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

haao-1 inhibition induces the activation of the transcription factor skn-1

We next investigated the idea that *haao-1* inhibition may rely on ROS to induce the overall stress response pathway and protect against oxidative stress. First, in order to see if ROS levels were increased upon *haao-1* inhibition, we used H_2DCFDA to measure the amount of ROS in *C. elegans* at day 7 from egg (Figure 3). We observed that *haao-1* RNAi increases ROS by day 7 and we wanted to further investigate this effect. To do this we used N-acetylcysteine (NAC), a precursor of glutathione (GSH) which reduces endogenous ROS (Halasi et al, 2013), with 3HAA, *haao-1* RNAi, a *haao-1* knockout, and wildtype. We found that the H_2DCFDA first increased the ROS levels in the 3HAA treated worms as well as the *haao-1(RNAi)* worms and *haao-1* inhibited worms in comparison to the wildtype. However, we also found that the worms treated with NAC, with the exception of 3HAA, had lower ROS levels (Figure 4). We believe that the 3HAA may have interfered with the NAC or vice versa by direct interaction into the agar plates, and therefore causing higher variability in the observed results.

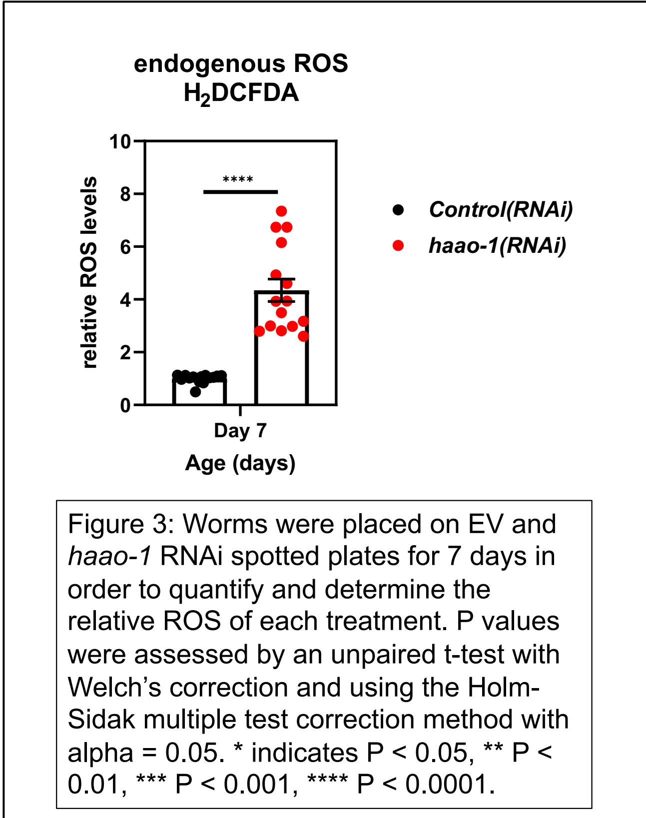

Figure 3: Worms were placed on EV and *haao-1* RNAi spotted plates for 7 days in order to quantify and determine the relative ROS of each treatment. P values were assessed by an unpaired t-test with Welch's correction and using the Holm-Sidak multiple test correction method with alpha = 0.05. * indicates P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

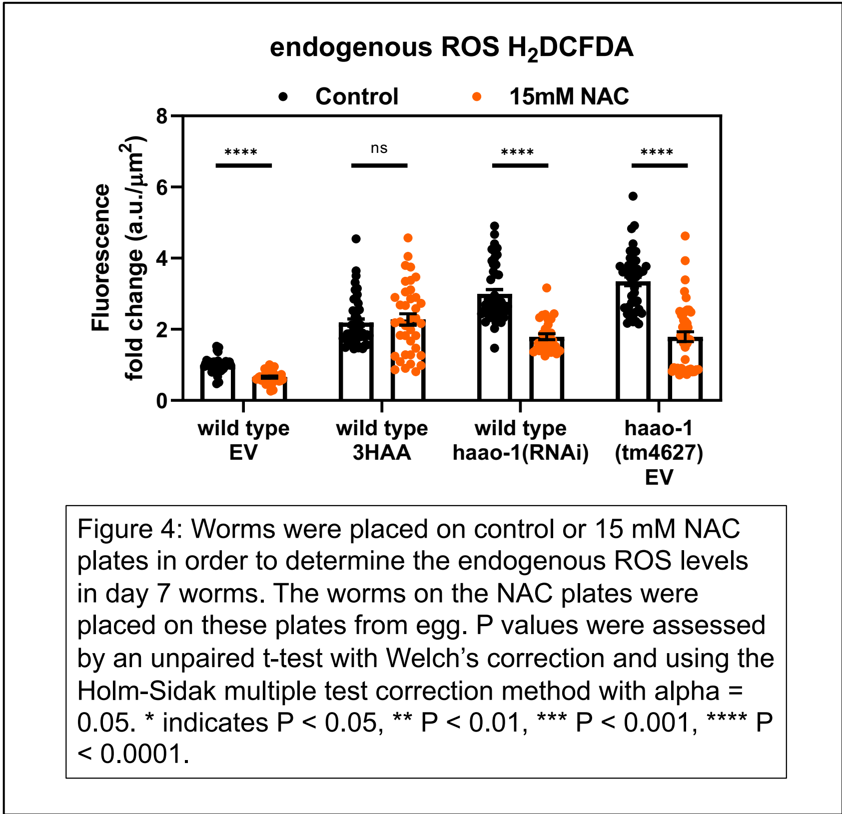

Figure 4: Worms were placed on control or 15 mM NAC plates in order to determine the endogenous ROS levels in day 7 worms. The worms on the NAC plates were placed on these plates from egg. P values were assessed by an unpaired t-test with Welch's correction and using the Holm-Sidak multiple test correction method with alpha = 0.05. * indicates P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

One of the pathways known to be activated by haao-1 inhibition is the NRF2/SKN-1 oxidative stress response pathway. To determine the mechanism behind this activation, we first used a HyPer oxidative stress reporter strain, which expresses a fluorophore with an altered excitation wavelength in the presence of hydrogen peroxide, with 15 mM NAC. We saw that with this strain, the NAC was able to bring the ROS down to basal levels that were similar to the control (Figure 5). From there we used a SKN-1::GFP fluorescent fusion reporter and a *gsc-1p::GFP* transcriptional reporter. SKN-1::GFP directly reports SKN-1 levels and *gcs-1p::GFP* is transcriptionally upregulated by active SKN-1, so both allowed us to observe SKN-1 activity. With both reporters, the ROS levels decreased, but the SKN-1 transcriptional activity did not return to basal levels (Figure 6). Therefore, we conclude that the activation of SKN-1 is partially, but not, entirely driven by increased ROS when *haao-1* is inactive.

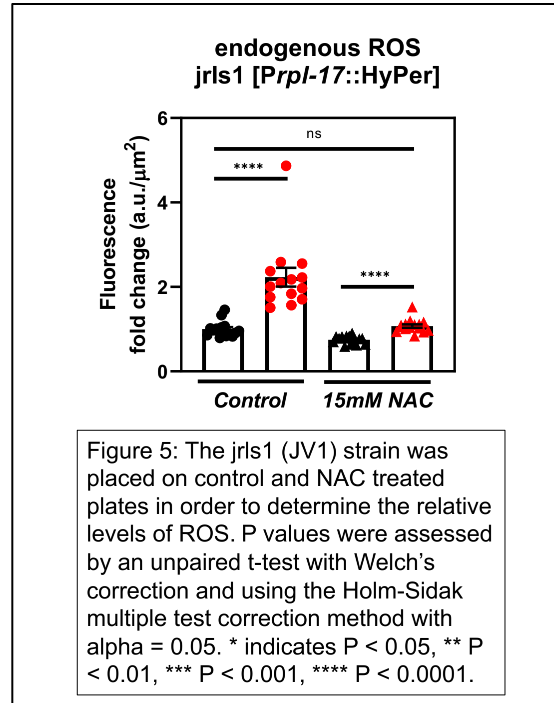

Figure 5: The jrls1 (JV1) strain was placed on control and NAC treated plates in order to determine the relative levels of ROS. P values were assessed by an unpaired t-test with Welch's correction and using the Holm-Sidak multiple test correction method with alpha = 0.05. * indicates P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

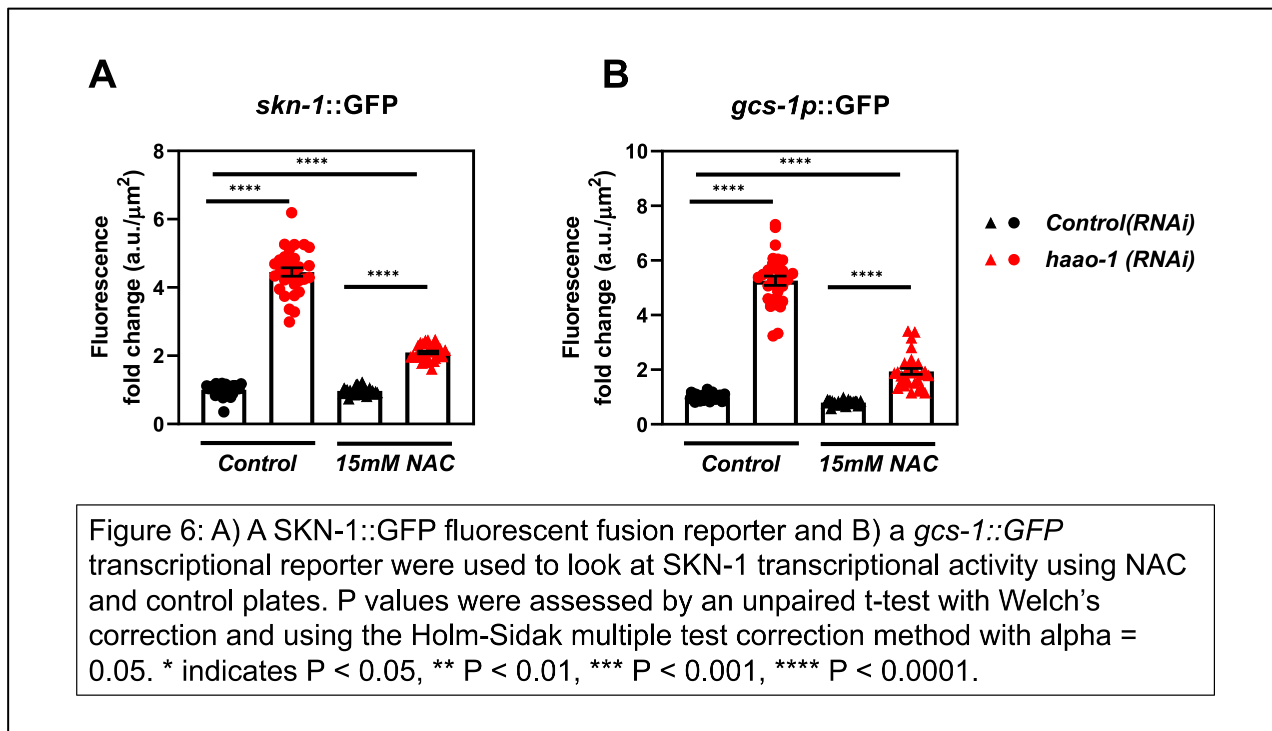

Figure 6: A) A SKN-1::GFP fluorescent fusion reporter and B) a *gcs-1::GFP* transcriptional reporter were used to look at SKN-1 transcriptional activity using NAC and control plates. P values were assessed by an unpaired t-test with Welch's correction and using the Holm-Sidak multiple test correction method with alpha = 0.05. * indicates P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

To figure out the other means of activation of the SKN-1 pathway, previous work in the lab used the *haao-1* knockdown worms crossed with the *skn-1::GFP* and placed them on plates with different RNAi of genes that had the ability to activate the pathway. This led to 11 genes being selected that were seen to have the potential to reduce the ROS activity back to basal levels in the worms (Figure 7). From there, we did another screening with those specific 11 gene RNAis but this time we used the *haao-1* knockout crossed with the *skn-1::GFP* (Figure 8) and also *haao-1* knockout crossed with *gcs-1p:GFP* promoter-driven GFP skn-1 reporter (Figure 9). We again used 15mM NAC to treat the worms and found that two genes, *aco-2* and *rho-1*, were able to bring the ROS levels back down to basal level, suggesting that these genes may mediate the non-ROS activation of SKN-1 in the absence of active *haao-1*.

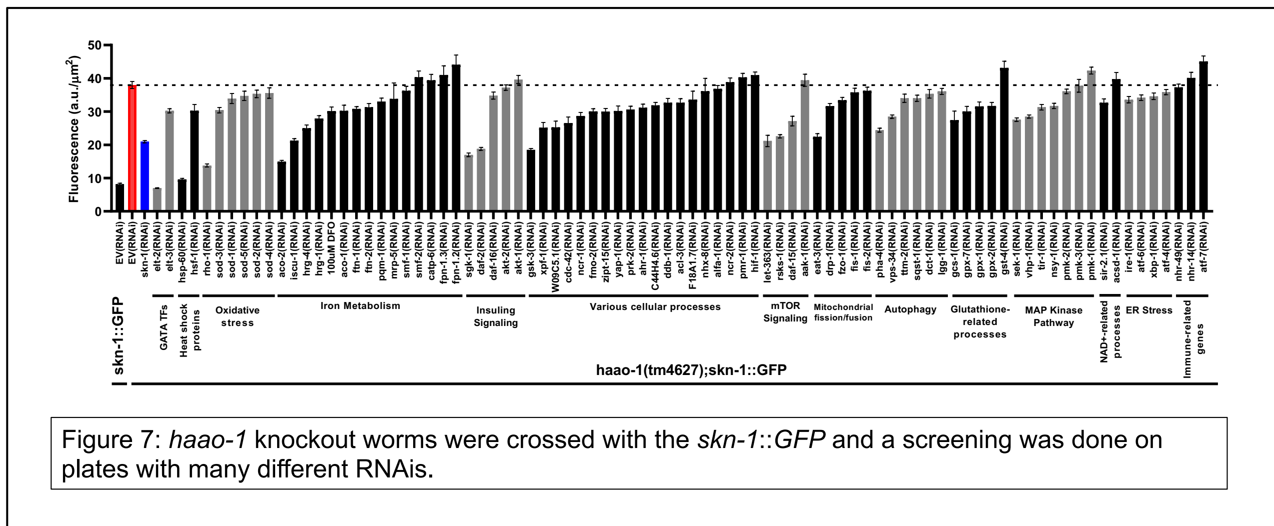

Figure 7: *haao-1* knockout worms were crossed with the *skn-1*::*GFP* and a screening was done on plates with many different RNAis.

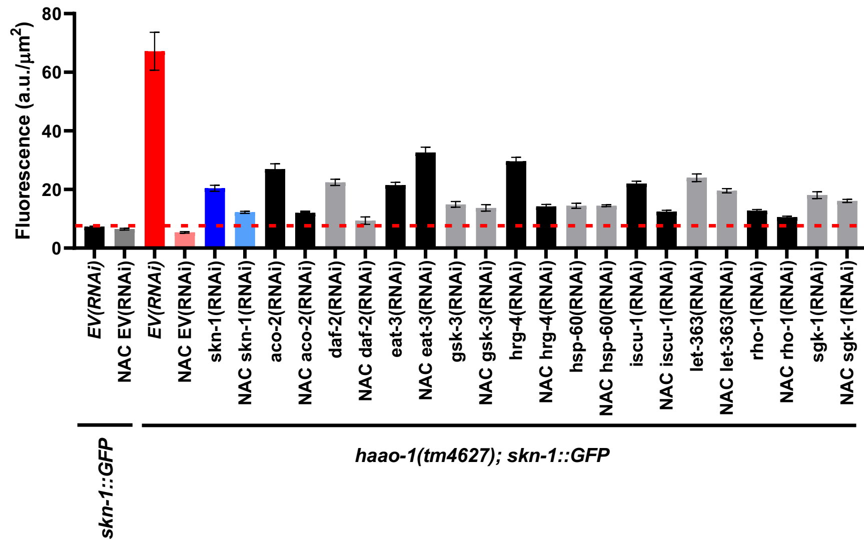

Figure 8: The secondary screening was performed with a cross of *haao-1* knockout and *skn-1*::*GFP* on the 11 selected RNAis.

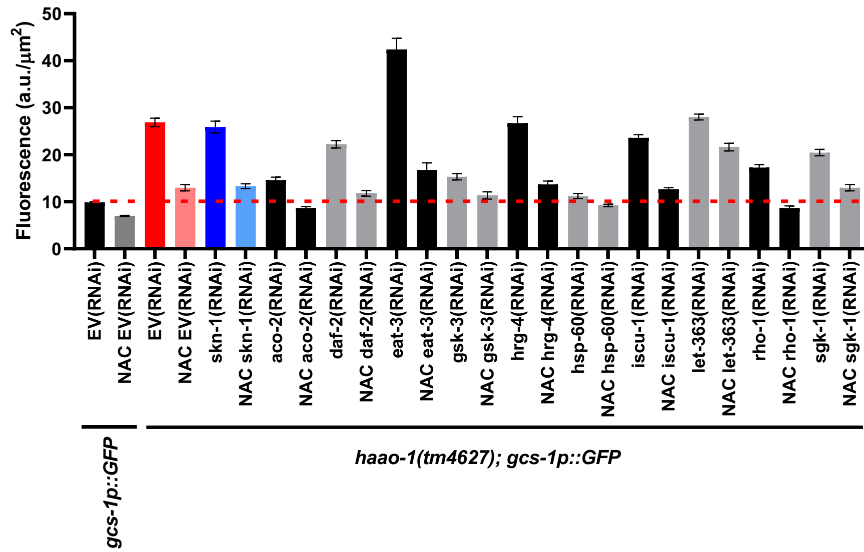

Figure 9: The secondary screening was performed with a cross of *haao-1* knockout and *gcs-1p*::*GFP* on the 11 selected RNAis.

Discussion

In this study we explore how the inhibition of certain parts of the kynurenine pathway leads to enhanced oxidative stress response in *C. elegans*. When *haao-1* is knocked down, the worms are able to better respond to ROS. We found that *haao-1* knockdown lead to an increased lifespan as well as the activation of certain transcription factors. These transcription factors, SKN-1, HSF-1, HIF-1, and DAF-16, play an important role in the oxidative stress response. Specifically, the SKN-1 pathway may be required for an enhanced stress response under *haao-1* inhibition. However, we also found that this pathway is likely not fully activated by an increase in ROS due to the *haao-1* inhibition.

To fully activate SKN-1, we found that two genes *aco-2* and *rho-1* are required. *aco-2* is located in the mitochondrial matrix which is significant as the mitochondria is implicated in aging with declining function. This is due to the accumulation in ROS which leads to oxidative damage and mutations (Chistiakov et al. , 2014). *rho-1* is a part of the Rho family which are small GTPases (McMullan and Nurrish, 2011). It is associated with oxidase bil-3 which promotes ROS production which is why inhibition improves oxidative stress (Ewald et al., 2017). Overall, both of these genes show that there are non-ROS dependent mechanisms of activation in the SKN-1/NRF2 pathway in the *haao-1(tm4627)* mutant background.

These two genes are necessary for the full activation of SKN-1, however, we still do not know yet whether they are sufficient or the mechanism of action of how this occurs. In the future, we need to examine the mechanism linking *haao-1* to non-ROS mediated SKN-1 activation. In addition, it may be significant to figure out whether these mechanisms are also present in mammalian cells.

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