Inhibition of *acn-1*, an ACE ortholog, reduces intestinal GST-4 expression in *Caenorhabditis elegans*

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Abstract

We investigated the role of <u>acn-1</u>, the <u>C. elegans</u> ortholog of human ACE and ACE2, on the expression of <u>GST-4</u>, an antioxidant molecule produced in response to oxidative stress. <u>acn-1</u> RNAi exposure significantly reduced <u>GST-4</u> reporter expression after 24 and 48 hours. The difference in <u>gst-4</u> expression appears most prevalent in the intestine. Our findings suggest that <u>acn-1</u> function positively influences <u>gst-4</u> expression in <u>C. elegans</u>.
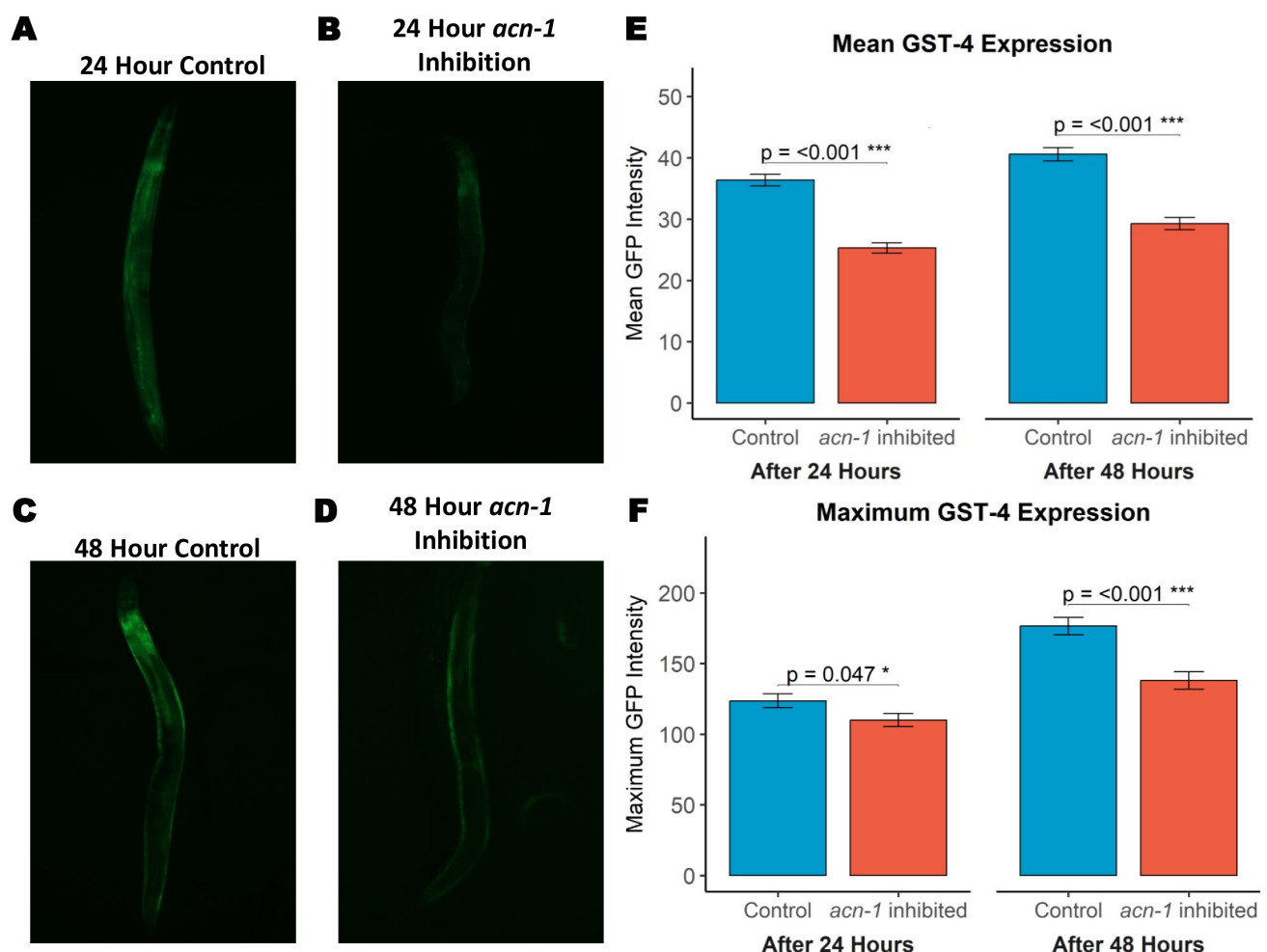
Figure 1. Basal GST-4 expression diminishes when *acn-1* is inhibited:

(A) Representative images showing GFP expression in worms exposed to *E. coli* containing an empty vector for 24 hours. Worms in this group appear to express high intestinal GFP. (B) Representative images showing GFP expression in worms exposed to <u>acn-1</u> RNAi for 24 hours. (C) Representative images showing GFP expression in worms exposed to *E. coli* containing an empty vector for 48 hours. Worms in this group appear to express high intestinal GFP. (D) Representative images showing GFP expression in worms exposed to <u>acn-1</u> RNAi for 48 hours. Worms in this group often lacked detectable GFP expression in the intestine region. (E) Mean GFP intensity throughout the whole body after 24 hours ($p < 1e^{-14}$; N=80 control, N=82 <u>acn-1</u> RNAi, ± SEM across 3 experiments) and 48 hours (Mean $p < 1e^{-11}$; N=76 control, N=72 <u>acn-1</u> RNAi, ± SEM across 2 experiments). F. Maximum GFP intensity throughout the whole body after 24 hours ($p < 4.68e^{-02}$; N=80 control, N=82 <u>acn-1</u> RNAi, ± SEM across 3 experiments) and 48 hours ($p < 6.10e^{-06}$; N=76 control, N=72 <u>acn-1</u> RNAi, ± SEM across 2 experiments).

Description

While previous studies have linked _acn-1_ to life extension and stress resistance in _C. elegans_, the mechanism by which this is done remains unclear. Inhibition of _acn-1_ is reported to increase lifespan and oxidative stress resistance. There is a significant gap in scientific literature in regard to interactions between _acn-1_ and regulatory reporters, like GST-4. GST-4 is an antioxidant that is released in response to oxidative stress (Pohl et al., 2019).

Our quantitative image analysis revealed a drastic decrease in GFP mean fluorescence after 24 hours ($p < 1e^{-14}$; N=80 control, N=82 _acn-1_ RNAi, 3 experiments) and an even larger difference after 48 hours ($p < 1e^{-11}$; N=76 control, N=72 _acn-1_ RNAi, 2 experiments) (Figure 1). To determine whether the signal reduction was more localized or uniform, we also analyzed the maximum intensities of fluorescence. A similar reduction was observed after both 24 hours ($p < 4.68e^{-02}$) and 48 hours ($p < 6.10e^{-06}$), with the brightest signals frequently being located in the caudal intestine region.

Our experimental results reveal that when _acn-1_ is inhibited, _gst-4_ is expressed less. This indicates that _acn-1_ regulates _gst-4_ expression, but the pathways and interactions through which it does so are still not clear. Despite being frequently used as a marker of oxidative stress, _gst-4_ can be expressed through multiple pathways that are not all exclusively induced by oxidative challenge (Detienne et al., 2016). While the mechanism by which _acn-1_ regulates _gst-4_ is unclear, literature indicates life extension mediated by _acn-1_ requires the transcription factor _daf-16_. _gst-4_ is known as a target gene of _daf-16_. However, based on current evidence, _acn-1_ does not appear to impact the nuclear localization of DAF-16 (Kumar et al., 2016). This suggests that _acn-1_ either affects specific target genes of _daf-16_ or works in parallel with _daf-16_. Because of the compensatory nature of detoxification molecules (Back et al., 2010), we suspect that the decreased _gst-4_ expression during _acn-1_ inhibition reflects a redistribution of stress response gene expression where _gst-4_ is reduced and offset by the increase of other _daf-16_ target genes like _gst-10_, which increases lifespan when overexpressed (Shields et al., 2021).

In summary, inhibition of _acn-1_ decreases _gst-4_ expression, and this suggests a positive regulatory role. This reduction in _gst-4_ may reflect a compensatory shift in gene expression where other _daf-16_ regulated genes associated with life extension are upregulated (Back et al., 2010; Park et al., 2009). _acn-1_ shows some homology with human ACE, and ACE inhibitors affect aging in various organisms (Brar et al., 2018; Egan et al., 2022). These findings may point to a conserved mechanism worth further investigation. Despite being homologous to human ACE at the sequence levelc, however, ACN-1 lacks several histidine residues that are necessary for the peptide-cleaving functions of ACE. ACN-1 plays an essential yet unclear role in nematode larval molting, a functionality that is unrelated to the role of human ACE in the renin-angiotensin system (Egan et al., 2022). Due to the functional differences, these results should not be interpreted as directly applicable to human ACE functions and human ACE inhibitors. Further investigation in mammalian models is required to establish any potential parallels.

Methods

RNAi inhibition of _acn-1_

A CL2166 strain of _C. elegans_ with _gst-4p_::GFP reporter was ordered from the University of Minnesota's Caenorhabditis Genetics Center (_Genotype: dvls19 III. 2024_). Samples of _E. coli_ containing _acn-1_ RNAi clones from the Ahringer RNAi library were received from the Kornfeld Lab (Egan et al., 2024). Gravid _C. elegans_ populations were synchronized using a bleaching protocol (Stiernagle, 2006). The resulting eggs were allowed to hatch on NGM plates with no food for 30 hours. Simultaneously, _E. coli_ was induced for 30 hours with 1 mM IPTG. The _E. coli_ containing _acn-1_ was grown alongside _E. coli_ containing pPD129.36, an empty vector used for control. After 30 hours, the hatched L1s were washed onto NGM plates containing _E. coli_ (OP50-1). After 25 hours, the now synchronized L3-L4s were washed to the IPTG NGM plates containing RNAi. Half of the worms were transferred to plates of _E. coli_ containing _acn-1_ RNAi, and the other half were transferred to plates of _E. coli_ containing pPD129.36.

Fluorescent assessment of GST-4 expression

gst-4 expression was assessed under the Leica M205 FCA fluorescence stereo microscope. _C. elegans_ were imaged under NaN₃ (20 mM) on NGM plates. A set of worms was analyzed after 24 hours of _acn-1_ RNAi exposure (N=80 control, N=82 _acn-1_ inhibited), and another set was analyzed after 48 hours of RNAi exposure (N=76 control and N=72 _acn-1_ inhibited). 24-hour procedures were replicated on 3 different days, and 48-hour procedures were replicated on 2 different days. ImageJ was used to analyze all the images by first splitting the images into separate color channels and selecting out only green fluorescence. The entirety of each worm body was selected using the polygon tool. Then the fluorescent intensity was assessed on a normalized scale of 0-255. Finally, the mean and maximum fluorescent intensity were measured and analyzed statistically using Pearson's t-test.

Reagents

Strain	Genotype	Available From

CL2166	*dvIs19 [gst-4p::GFP::NLS]* III	CGC (Caenorhabditis Genetics Center)
Bacteria/RNAi constructs	*Description*	**Available From**
OP50-1	Wild type used as a food source.	CGC
E. coli HT115(DE3) + *acn-1* RNAi	*E. coli that contains dsRNA plasmid targeting acn-1*	Kornfeld Lab, Washington University in St. Louis
E. coli HT115(DE3) + pPD129.36	*Empty vector used as RNAi control*	Addgene or CGC RNAi library
Plasmid	*Genotype*	**Description**
pPD129.36	*Empty vector*	Standard L4440-based RNAi control plasmid used for feeding RNAi.
Tool	*Purpose*	**Source**
ImageJ	*Fluorescence intensity analysis*	NIH
UCSC Genome Browser	*RNA-seq visualization*	UCSC Genomics Institute
NCBI BioProject	*RNA-seq data acquisition*	NCBI

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Author Contributions: Josiah King: conceptualization, formal analysis, investigation, methodology, writing - original draft, validation, visualization, writing - review editing. Kristopher Schmidt: data curation, formal analysis, funding acquisition, methodology, investigation, resources, validation, supervision, writing - review editing, project administration.

Reviewed By: Anonymous

Nomenclature Validated By: Anonymous

WormBase Paper ID: WBPaper00068561

Citation: King J, Schmidt K. 2025. Inhibition of *acn-1*, an ACE ortholog, reduces intestinal GST-4 expression in *Caenorhabditis elegans*. microPublication Biology. 10.17912/micropub.biology.001682