Genetics and Genomics

fmo-4 promotes longevity and stress resistance via ER to mitochondria calcium regulation in *C. elegans*

Reviewed Preprint
v1 • August 27, 2024
Not revised

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Abstract

Flavin-containing monooxygenases (FMOs) are a conserved family of xenobiotic enzymes upregulated in multiple longevity interventions, including nematode and mouse models. Previous work supports that *C. elegans fmo-2* promotes longevity, stress resistance, and healthspan by rewiring endogenous metabolism. However, there are five *C. elegans* FMOs and five mammalian FMOs, and it is not known whether promoting longevity and health benefits is a conserved role of this gene family. Here, we report that expression of *C. elegans fmo-4* promotes lifespan extension and paraquat stress resistance downstream of both dietary restriction and inhibition of mTOR. We find that overexpression of *fmo-4* in just the hypodermis is sufficient for these benefits, and that this expression significantly modifies the transcriptome. By analyzing changes in gene expression, we find that genes related to calcium signaling are significantly altered downstream of *fmo-4* expression. Highlighting the importance of calcium homeostasis in this pathway, *fmo-4* overexpressing animals are sensitive to thapsigargin, an ER stressor that inhibits calcium flux from the cytosol to the ER lumen. This calcium/*fmo-4* interaction is solidified by data showing that modulating intracellular calcium with either small molecules or genetics can change expression of *fmo-4* and/or interact with *fmo-4* to affect lifespan and stress resistance. Further analysis supports a pathway where *fmo-4* modulates calcium homeostasis downstream of activating transcription factor-6 (*atf-6*), whose knockdown induces and requires *fmo-4* expression. Together, our data identify *fmo-4* as a longevity- promoting gene whose actions interact with known longevity pathways and calcium homeostasis.

eLife assessment

This **important** study offers **convincing** evidence that fmo-4 plays essential roles in established lifespan interventions and downstream of its paralog fmo-2, a beneficial advancement in our understanding of this enzyme family that underscores their importance in longevity and stress resistance. The study also suggests a connection between fmo-4 and dysregulation of calcium signalling. The authors' conclusions and interpretations were generally based on **solid** genetic methodology and evidence.

https://doi.org/10.7554/eLife.99971.1.sa3

Introduction

Aging is a major risk factor for the onset of cancer, cardiovascular disease, dementia, and many other serious illnesses. Studying the aging process is crucial because a more comprehensive understanding can lead to the development of therapeutics that treat multiple diseases simultaneously. The use of model organisms, from yeast to mammals, has helped define genetic and environmental pathways that influence aging[1]. Many of these pathways, such as dietary restriction (DR), defined as a decrease in nutrient intake without malnutrition, involve active modification of metabolism that robustly and reproducibly improve health and longevity across species[2–4]. Similarly, the response to oxygen deficiency, or hypoxic response, can lead to increased longevity, healthspan, and stress resistance[5]. Interestingly, in *C. elegans*, both DR and the hypoxic response converge upon a single gene, *fmo-2*, that is necessary and sufficient to improve health and longevity[6].

Flavin-containing monooxygenases (FMOs) are a family of enzymes that use oxygen and NADPH to oxygenate nucleophilic substrates. FMOs oxygenate a wide array of xenobiotic substrates and were discovered ~50 years ago for their role in drug metabolism[7,8].

Consequently, a majority of published data on FMOs relate to this role. Recently, studies have begun to focus on the endogenous role(s) of FMOs. Results show that multiple mammalian FMO proteins are involved in systemic metabolism[9,10]. We initially discovered a role for *C. elegans fmo-2* in regulating stress resistance and longevity downstream of DR and hypoxia and have continued to identify role(s) for FMO-2 in *C. elegans* metabolism and longevity[10].

However, the conserved mechanisms for FMO-mediated health benefits are still unclear, as are the roles of individual FMO enzymes. Thus, to best understand how FMOs could be leveraged to improve health, we need to understand the conserved mechanism of these enzymes in longevity and metabolism.

There are five *C. elegans* FMOs, and three of the five share significant structural overlap with mammalian FMOs. This overlap involves a predicted endoplasmic reticulum (ER) localization and a membrane spanning domain, which are each found in *fmo-1, fmo-2*, and *fmo-4.* In comparison to the more well-studied longevity gene *fmo-2*, *fmo-4* in particular shares 88% identity in the catalytic domain amino acids, suggesting that FMO-2 and FMO-4 may bind similar substrates and could plausibly have overlapping endogenous roles. Published data also show that DR induces both *fmo-2* and *fmo-4* gene expression, implying a role for *fmo-4* in longevity regulation[8]. Based on this, we hypothesized that *fmo-4* may play a role in *C. elegans* longevity and that studying this role can help to understand this gene family and its relevance to aging.

FMOs are transmembrane enzymes thought to primarily reside in the ER. While mainly studied for its involvement in protein and lipid synthesis, the ER also plays a key role in metabolism and maintaining homeostasis within the cell. For instance, the ER responds to misfolded proteins by eliciting its unfolded protein response (UPR^{ER}), thereby removing dysfunctional proteins and restoring homeostasis[11]. The ER is also responsible for storing and releasing calcium in a coordinated manner with the cytosol and mitochondria[12]. The ER establishes calcium homeostasis through processes involving 1) sequestering calcium in the lumen with the calreticulin chaperone[12,13], 2) releasing calcium into the cytosol through the inositol triphosphate receptor (IP_3R)[12,14], and 3) bringing calcium into the ER lumen through the sarcoplasmic endoplasmic reticulum ATPase (SERCA) pump[12,15]. Interestingly, the ER's role in regulating metabolic and calcium homeostasis in the cell has recently been linked to longevity in *C. elegans*. Knocking out activating transcription factor (*atf*)-6, one of the three branches of the UPR^{ER}, modulates calcium signaling from the ER to the mitochondria, resulting in increased mitochondrial turnover and lifespan extension in *C. elegans*[16].

Given that 1) *fmo-4* is induced by a longevity-promoting pathway (DR), 2) *fmo-4* is predicted to be an ER transmembrane protein, and 3) the ER's role in cellular homeostasis is linked to aging, we hypothesized that *fmo-4* regulates longevity by modulating ER-related processes. Here, we test this hypothesis by modifying *fmo-4* expression and interrogating interactions between *fmo-4*, stress resistance, longevity, and the ER. Our resulting data show that *fmo-4* promotes longevity and paraquat stress resistance downstream of mTOR and DR through calcium homeostasis. We find that not only is *fmo-4* a regulator of multiple longevity promoting pathways, but it is also sufficient to extend lifespan and confer paraquat stress resistance when overexpressed either ubiquitously or in the hypodermis. Transcriptomics data and stress assays reveal that calcium signaling is altered downstream of *fmo-4* expression, and that *fmo-4* interacts with calcium regulation between the ER and mitochondria to promote longevity and paraquat stress resistance. Together, these results establish *fmo-4* as a longevity promoting gene and provide evidence as to how *fmo-4* extends *C. elegans* lifespan through calcium-mediated ER to mitochondrial processes.

Results

Cefmo-4 is required for DR and mTOR-mediated lifespan extension

C. elegans fmo-4 shares significant similarity to its family member and longevity gene, *fmo-2*, including 88% conservation in catalytic residues (Supplementary Fig 1), a transmembrane domain (**Fig 1A**) and predicted subcellular localization in the ER (Supplementary Fig 2)[17]. Additionally, *fmo-4* is induced by the longevity intervention DR[8]. These structural similarities and DR-mediated induction led us to hypothesize that *fmo-4* may play a role in aging. To test this, we first asked whether *fmo-4* is required for well-studied longevity pathways, including DR. We utilized *fmo-4* (*ok294*) knockout (KO) animals[18] on five conditions reported to extend lifespan in *C. elegans*. Since *fmo-4* is induced by dietary restriction, we started with fed and DR (sDR[19]) conditions. Our results show that loss of *fmo-4* has no significant effect on control fed worms but prevents nearly all the lifespan extension seen under DR (**Fig 1B**). Wild-type (WT) worms on DR experience a ~35% lifespan extension compared to fed WT worms, but when *fmo-4* is knocked out this extension is reduced to ~10% and this interaction is significant by cox regression (p-value < $4.50e^{-6}$). These data support that *fmo-4* is required for the DR longevity pathway (**Fig 1B**). Having established this role, we continued lifespan analyses of *fmo-4* KO worms exposed to RNAi knockdown of the S6-kinase gene *rsks-1* (mTOR signaling)[20], the von hippel lindau gene *vhl-1* (hypoxic signaling)[21], the insulin receptor *daf-2* (insulin-like signaling)[22], and the cytochrome c reductase gene *cyc-1* (mitochondrial electron transport chain, cytochrome c reductase)[23] (**Fig 1C-F**). The resulting data show that *fmo-4* is fully required for *rsks-1*-mediated longevity as marked by a complete abrogation of the *rsks-1* RNAi lifespan extension when *fmo-4* is knocked out (**Fig 1C**), placing *fmo-4* downstream of mTOR. *fmo-4* was not required for the hypoxic response

(**Fig 1D**), insulin- like signaling (**Fig 1E**), or cytochrome c reductase inhibition pathways (**Fig 1F**) to increase lifespan. It is notable that, unlike *fmo-4*, *fmo-2* is required for *vhl-1*-mediated longevity[6]. This result, coupled with *fmo-4* and *fmo-2* each being required for DR, suggests that these two genes in the same family are overlapping but distinct in their requirement. Together, these data suggest that *fmo-4* plays a necessary role in *C. elegans* longevity regulation downstream of at least two pathways: DR and mTOR signaling.

fmo-4 is required for *fmo-2*-mediated longevity, stress resistance, and healthspan

Having established that, like *fmo-2*, *fmo-4* is required for DR-mediated longevity, we next hypothesized that *fmo-2* and *fmo-4* may be acting in the same pathway. To test this, we crossed *fmo-2* overexpressing (OE) worms with *fmo-4* KO worms to create an *fmo-2* OE; *fmo*-4 KO strain (*fmo-2*OE;4KO). We validated this strain via qPCR analysis (Supplementary Table 1).

Upon measuring their lifespan compared to *fmo-2* OE animals, we find that knocking out *fmo-4* completely abrogates the lifespan extension from *fmo-2* OE (**Fig 2A**). This result suggests that *fmo-4* is required for *fmo-2*-mediated longevity and thus may act downstream of *fmo-2* to promote longevity in *C. elegans.* We previously showed that *fmo-2* OE is not just sufficient for lifespan extension but is also sufficient to promote resistance to multiple forms of stress, including oxidative stress (paraquat), heat stress, and ER stress (tunicamycin)[6]. Since *fmo-4* is required for *fmo-2*- mediated longevity, we hypothesized that *fmo-4* would also be required for *fmo-2*-mediated stress resistance. Following exposure to 5mM paraquat, 37°C heat stress, or 5µg/mL tunicamycin, we find that the *fmo-2*OE;4KO worms survive similar to WT worms and are no longer resistant to these stresses (**Fig 2B-D**). Thus, knocking out *fmo-4* blocks *fmo-2*-mediated broad stress resistance.

Healthspan is a crucial component to longevity, and we previously found that *fmo-2* OE is sufficient to promote healthspan benefits with age in *C. elegans*[6]. As *fmo-4* is required for both the lifespan extension and stress resistance observed with *fmo-2* OE, we hypothesized that *fmo-4* would also be required for *fmo-2*-mediated healthspan benefits. To test this, we measured the thrashing rate of *fmo-2* OE;4KO worms at day 2 and day 10 of adulthood. We find that knocking out *fmo-4* abrogates the healthspan benefits of the *fmo-2* OE worms with age (**Fig 2E-F**). Thus, *fmo-4* is required for *fmo-2*-mediated health and resilience benefits, further supporting its acting downstream of *fmo-2* to promote health and longevity.

Overexpression of *fmo-4* promotes longevity, healthspan, and paraquat stress resistance

While being necessary for increased healthspan, lifespan, and stress resistance shows that *fmo-4* is required for these benefits, it does not test whether it can modulate aging directly.

Therefore, to better understand *fmo-4's* role in longevity and health, we next asked whether *fmo-4* is sufficient for lifespan extension, stress resistance, and healthspan benefits. We created a ubiquitous *fmo-4* OE worm strain with *fmo-4* expressed under the *eft-3* promoter via multicopy extrachromosomal array followed by random integration. qPCR analysis shows that *fmo-4* is overexpressed ~150 fold over WT in the ubiquitous *fmo-4* OE worms (Supplementary Table 1), and their development time is slightly (~1.5 hours) but statistically significantly delayed compared to WT (Supplementary Fig 3). Interestingly, we find that ubiquitous *fmo-4* OE is sufficient to reproducibly extends lifespan by ~20% (**Fig 3A**). Epistatic analysis with *fmo-2* shows that *fmo-4* OE lifespan extension is not additive with *fmo-2* OE (Supplementary Fig 4A), as determined by comparing the individual OE strains to the *fmo-2* OE;*fmo*-4 OE (*fmo- 2*OE;4OE) worm strain (Supplementary Table 1). This further supports that these two genes in the same family act in the

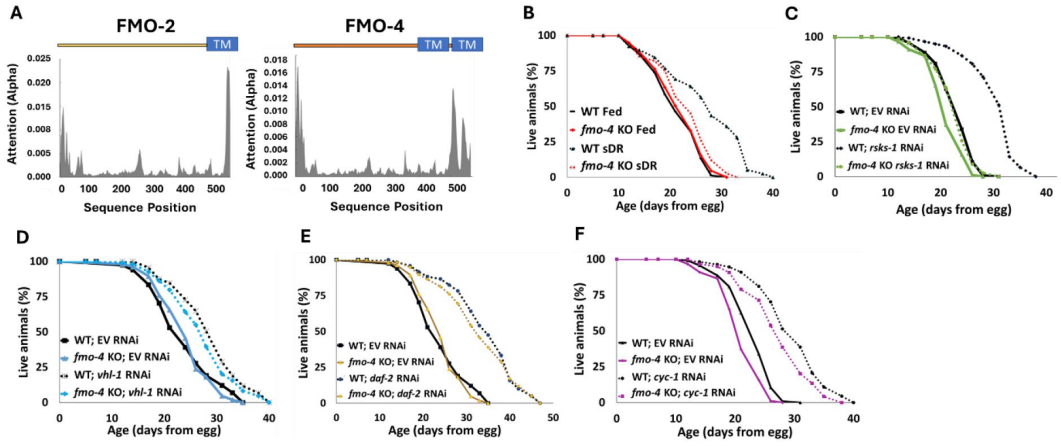

Figure 1.

Cefmo-4 regulates mTOR and dietary restriction mediated longevity.

(A) Hydropathy plots of FMO-2 and FMO-4 predicting endoplasmic reticulum (ER) transmembrane (TM) domains based on protein sequence analysis. Analysis was done using Deeploc-2.0. (B) Lifespan analysis of wild-type (WT) worms and *fmo-4* knockout (*fmo-4* KO) worms on fed and dietary restricted (DR) conditions. (C) Lifespan analysis of WT and *fmo-4* KO worms on empty vector (EV) RNAi and *rsks-1* RNAi. (D) Lifespan analysis of WT and *fmo-4* KO worms on EV and *vhl-1* RNAi. (E) Lifespan analysis of WT and *fmo-4* KO worms on EV and *daf-2* RNAi. (F) Lifespan analysis of WT and *fmo-4* KO worms on EV and *cyc-1* RNAi. For each lifespan, n = ~120 worms per condition per experiment, and three replicate experiments were performed. Significance was determined at $p < 0.05$ using log-rank analysis and significant interactions between the condition of interest and genotype was determined at $p < 0.01$ using Cox regression analysis.

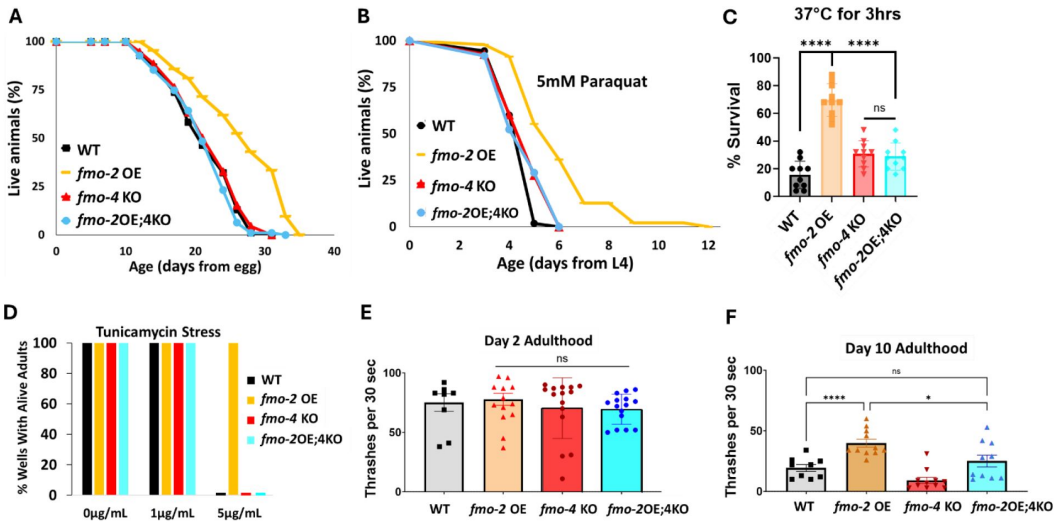

Figure 2.

fmo-4 is required for the health benefits of *fmo*-2 overexpression.

(A) Lifespan analysis of wild-type (WT), *fmo-2* overexpressing (*fmo-2* OE), *fmo*-4 knockout (*fmo-4* KO), and *fmo*-2 OE;*fmo-4* KO (*fmo*-2OE;4KO) worms on *E. coli* OP50 (n = ~120 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log-rank analysis. (B) Survival of WT, *fmo*-2 OE, *fmo*-4 KO, and *fmo*-2OE;4KO worms exposed to 5mM paraquat at L4 stage (n = ~90 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log-rank analysis. (C) Survival of worms exposed to 37°C heat shock for 3 hours (hrs) at L4 stage (n = 100 worms per condition, three replicate experiments). (D) Survival of worms exposed to 0, 1, and 5µg/mL tunicamycin from egg until day 1 of adulthood (n = ~60 eggs per condition, three replicate experiments). (E) Healthspan analysis of worm thrashing in a drop of M9 solution for 30 seconds on day 2 of adulthood (n = 10 worms per condition, three replicate experiments). (F) Healthspan analysis of worm thrashing in a drop of M9 solution for 30 seconds on day 10 of adulthood (n = 10 worms per condition, three replicate experiments). For heat stress, tunicamycin stress, and healthspan assessments, * denotes significant change at $p < 0.05$ using unpaired two-tailed t test or one-way ANOVA. N.S. = not significant.

same pathway. To test whether *fmo-4* requires *fmo-2*, we overexpressed *fmo-4* in the context of *fmo-2* knockout (*fmo-4* OE;2KO) (Supplementary Table 1). The results show that *fmo-4* OE does not require *fmo-2* for its longevity benefits (Supplementary Fig 5A). This further validates that *fmo-4* likely acts downstream of *fmo-2*.

Increased longevity is frequently observed in tandem with increased healthspan and resistance to toxic stress[24]. We assessed the healthspan of the ubiquitous *fmo-4* OE worms at days 2 and 10 of adulthood by measuring thrashing rates (movement). We find that ubiquitous *fmo-4* OE is sufficient for an improvement ($p = 0.011$) in healthspan benefits with age (Fig B-C). We next measured the stress resistance of the ubiquitous *fmo-4* OE worms to 5mM paraquat (oxidative stress), 37°C heat stress, 5µg/mL of tunicamycin (ER glycosylation stress), and 1mg/mL thapsigargin (ER calcium stress). We included two ER stresses because FMO-4 is predicted to be an ER transmembrane protein. We find that ubiquitous *fmo-4* OE is sufficient to convey paraquat stress resistance (**Fig 3D**) but does not offer any protection from heat (**Fig 3E**) or tunicamycin stress (**Fig 3F**). Interestingly, ubiquitous *fmo-4* overexpressing animals are sensitive to thapsigargin, as shown by the worms' inability to develop compared to WT control worms (**Fig 3G-I**). These results suggest a narrow stress resistance and even some sensitivity for *fmo-4* in comparison to other longevity genes. To test whether *fmo-4* OE would interact with *fmo-2* OE, we combined the strains and measured their stress resistance. We find that the *fmo- 2* OE strain is still resistant to paraquat, heat, and tunicamycin stress in the presence of increased *fmo-4*, suggesting that overexpression of *fmo-4* does not affect resistance to these stresses (Supplementary Fig 4B-D). Interestingly, the *fmo-2* OE strain is sensitive to thapsigargin stress in the presence of increased *fmo-4*, suggesting that *fmo-4* likely also acts downstream of *fmo-2* in this stress pathway (Supplementary Fig 4E-I). Overall, these results suggest that *fmo-4* 1) is sufficient to improve healthspan, 2) is both necessary and sufficient to promote paraquat resistance, 3) is necessary but not sufficient to improve heat and tunicamycin resistance, and 4) promotes sensitivity to calcium-mediated ER stress from thapsigargin. As expected under the hypothesis that *fmo-4* acts downstream of *fmo-2*, the *fmo-4*OE;2KO strain phenocopies the *fmo-4* OE strain in all stress assays (Supplementary Fig 5B-I). Together, these data suggest that *fmo-4* OE is sufficient to promote lifespan extension and paraquat stress resistance as well as a healthspan benefit, while exhibiting sensitivity to ER calcium stress.

Hypodermal overexpression of *fmo-4* is sufficient for longevity and paraquat resistance

While using the ubiquitous overexpressing worm strain is helpful to probe into *fmo-4's* involvement in longevity, stress resistance, and healthspan, it is also important to note that *fmo- 4* is normally localized and expressed primarily in the hypodermis (**Fig 4A-C**)[25]. Thus, we asked whether hypodermal-specific *fmo-4* overexpression is sufficient for these health benefits. We created a hypodermal-specific *fmo-4* OE worm strain expressing *fmo-4* under the *dpy-7* promoter via multicopy extrachromosomal arrays followed by random integration. qPCR data show that *fmo-4* is expressed ~45 fold over WT in the hypodermal-specific *fmo-4* OE worms (Supplementary Table 1), and their developmental time does not differ from WT (Supplementary Fig 3). We measured lifespan, resistance to paraquat, heat, tunicamycin and thapsigargin stress, as well as healthspan of the hypodermal-specific *fmo-4* OE worms (*fmo-4* OE^{Hyp}). We find that the *fmo-4* OE^{Hyp} strain exhibits a lifespan extension (**Fig. 4D**), resistance to paraquat (**Fig. 4E**) but not heat or tunicamycin stress (**Fig. 4F-G**), and sensitivity to thapsigargin stress (**Fig. 4H-J**). However, the *fmo-4* OE^{Hyp} strain did not have a statistically significant effect on healthspan unlike the ubiquitous *fmo-4* OE strain (**Fig 4K-L**). Overall, these results agree with the *C. elegans* single cell atlas as well as previously published work[25] showing that *fmo-4* is mostly localized to the hypodermis (**Fig 4A**) and are consistent with the ubiquitous strain primarily benefiting from expressing additional *fmo-4* in the hypodermis. Thus, ubiquitous or hypodermal-specific

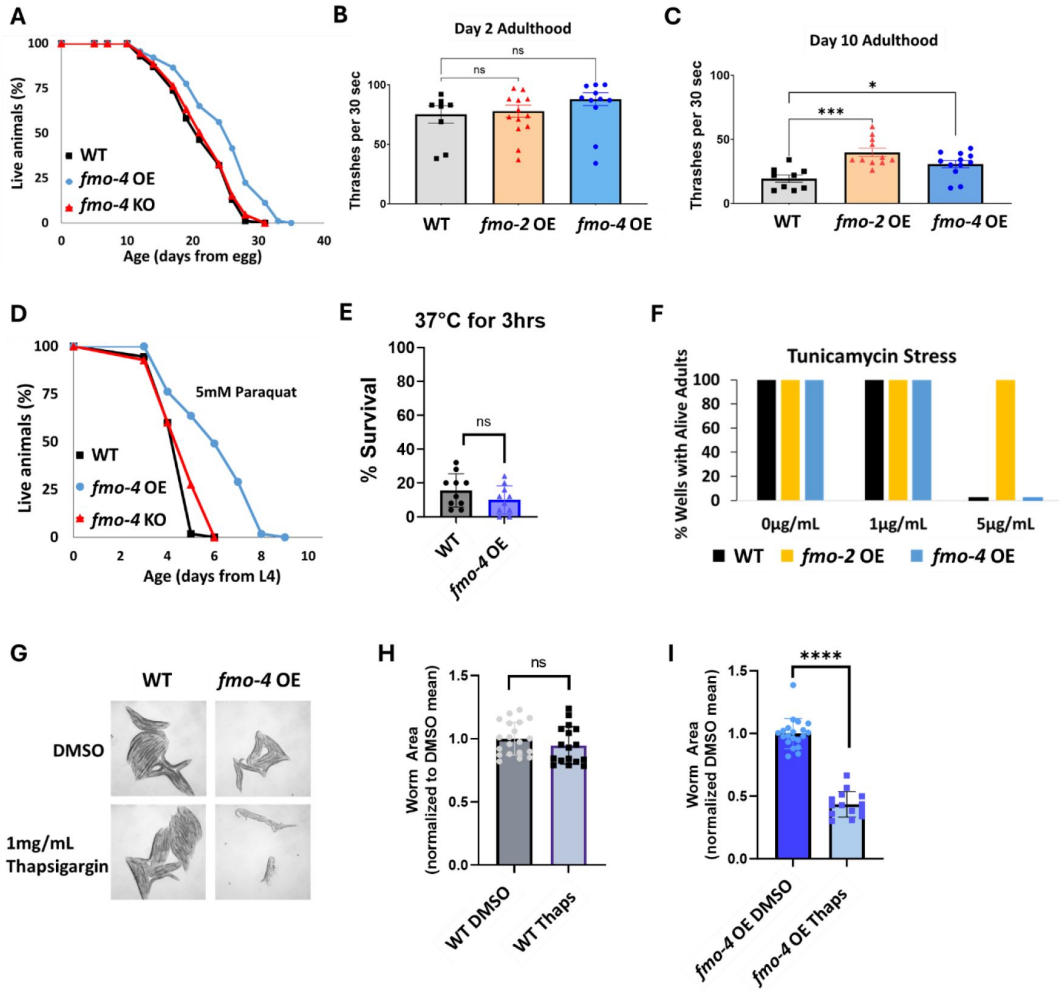

Figure 3.

Overexpressing *fmo*-4 is sufficient for lifespan extension, paraquat stress resistance and improved healthspan.

(A) Lifespan analysis of wild-type (WT), *fmo*-4 overexpressing (*fmo-4* OE), and *fmo*-4 knockout (*fmo*-4 KO) worms on *E. coli* OP50 (n = ~120 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log-rank analysis. (B) Healthspan analysis of worms thrashing in a drop of M9 solution for 30 seconds (sec) on day 2 of adulthood (n = 10 worms per condition, three replicate experiments). (C) Healthspan analysis of worms thrashing in a drop of M9 solution for 30 seconds on day 10 of adulthood (n = 10 worms per condition, three replicate experiments). (D) Survival of worms exposed to 5mM paraquat starting from L4 stage (n = ~90 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log-rank analysis. (E) Survival of worms exposed to 37°C for 3 hours (hrs) at L4 stage (n = 100 worms per condition, three replicate experiments). (F) Survival of worms exposed to 0, 1, and 5µg/mL tunicamycin starting from egg until day 1 of adulthood (n = ~60 eggs per condition, three replicate experiments). (G) Brightfield images of WT and ubiquitous *fmo-4* OE worms exposed to DMSO or 1mg/mL thapsigargin (n = ~20 worms per condition, three replicate experiments). Quantifications of (G) images in (H-I). For heat stress, tunicamycin stress, thapsigargin stress, and healthspan assessments, * denotes significant change at $p < 0.05$ using t test. N.S. = not significant.

overexpression of *fmo-4* is sufficient for longevity and paraquat stress resistance (**Fig 3A,D**, **Fig 4D-E**), does not affect heat or tunicamycin resistance (**Fig 3E-F**, **Fig 4F-G**), and results in thapsigargin sensitivity (**Fig 3G-I**, **Fig 4H-J**).

fmo-4 OE transcriptomics reveal a link to calcium regulation

To identify the downstream effects of *fmo-4* expression, we analyzed the transcriptome of *fmo-4* OE animals and compared them with control animals (**Fig 5A**). Based on a p-value of < 0.05, we find ~800 transcripts are upregulated and ~500 transcripts are downregulated when *fmo-4* is overexpressed (Supplemental Data 1). Of the upregulated and downregulated transcripts unique to the *fmo-4* OE profile, we noticed that some of the significant pathways include transcription, Wnt signaling, TGFβ signaling, protein processing in the ER, and other subsets of signaling, as determined by the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID) Functional Annotation Bioinformatics Microarray Analysis (**Fig 5A**). Based on its known role as a xenobiotic enzyme, we were intrigued to find that *fmo-4* OE is modulating signaling pathways. We were further intrigued to find that one of the signaling pathways affected by *fmo-4* is calcium signaling (Supplementary Table 2). This was interesting considering that *fmo-4* OE worms are sensitive to thapsigargin (**Fig 3G-I**), implicating an interaction between calcium signaling and *fmo-4*. To further verify this possibility, we used another tool called PANTHER Classification System Protein Class, which identified FMO-4 as a putative transmembrane signal receptor (G-protein coupled receptor), ion transporter, and/or calcium-binding protein (Supplemental Data 2). Together, these results support the possibility that FMO-4 is involved in calcium-related processes.

To begin exploring this interaction, we determined how changes in intracellular calcium levels impact *fmo-4* expression and lifespan. We first manipulated calcium levels by supplementing the acetylcholine agonist, carbachol. Carbachol activates acetylcholine receptors, increasing overall intracellular calcium levels[26]. We measured *fmo-4* gene expression upon exposure to carbachol using an *fmo-4p::mCherry* transcriptional reporter strain.

Interestingly, we find that supplementation of 300μM carbachol induces *fmo-4* promoter expression fluorescence nearly 2-fold over a water control (**Fig 5B-C**). This was similar to the level of induction of *fmo-4* in fasted (DR-like) conditions (**Fig 5B-C**). We postulate that *fmo-4* is induced by carbachol because increased intracellular calcium activates *fmo-4* gene expression. Since carbachol supplementation induces *fmo-4*, we hypothesized that carbachol may affect lifespan and would interact with *fmo-4*. We measured lifespan of worms supplemented with 50μM carbachol and find that while WT lifespan is significantly extended by carbachol, this extension is not additive with *fmo-4* OE (**Fig 5D**), and requires *fmo-4*, as the *fmo-4* KO is shorter-lived when exposed to carbachol (**Fig 5E**).

To test whether reducing calcium has an opposing effect to increasing it, we utilized EthyleneDiamineTetraAcetic acid (EDTA) to deplete calcium levels[27]. First, we placed *fmo-4p::mCherry* reporter worms on plates containing 10mM EDTA and measured fluorescence. Surprisingly, we find that EDTA also significantly induces *fmo-4* expression (**Fig 5B-C**). We then assessed the survival of WT, *fmo-4* OE and KO worms on plates supplemented with 50μM EDTA. We find that EDTA significantly extends WT lifespan without further extending *fmo-4* OE lifespan (**Fig 5F**), while shortening the lifespan of *fmo-4* KO animals (**Fig 5G**). Overall, these data suggest that *fmo-4* is highly sensitive to changing calcium levels in either direction, and that both increasing and depleting calcium 1) induces *fmo-4*, 2) extends WT lifespan, 3) is not additive with *fmo-4* OE lifespan, and 4) is deleterious to *fmo-4* KO animals. Thus, these results support an interaction between *fmo-4* and calcium signaling.

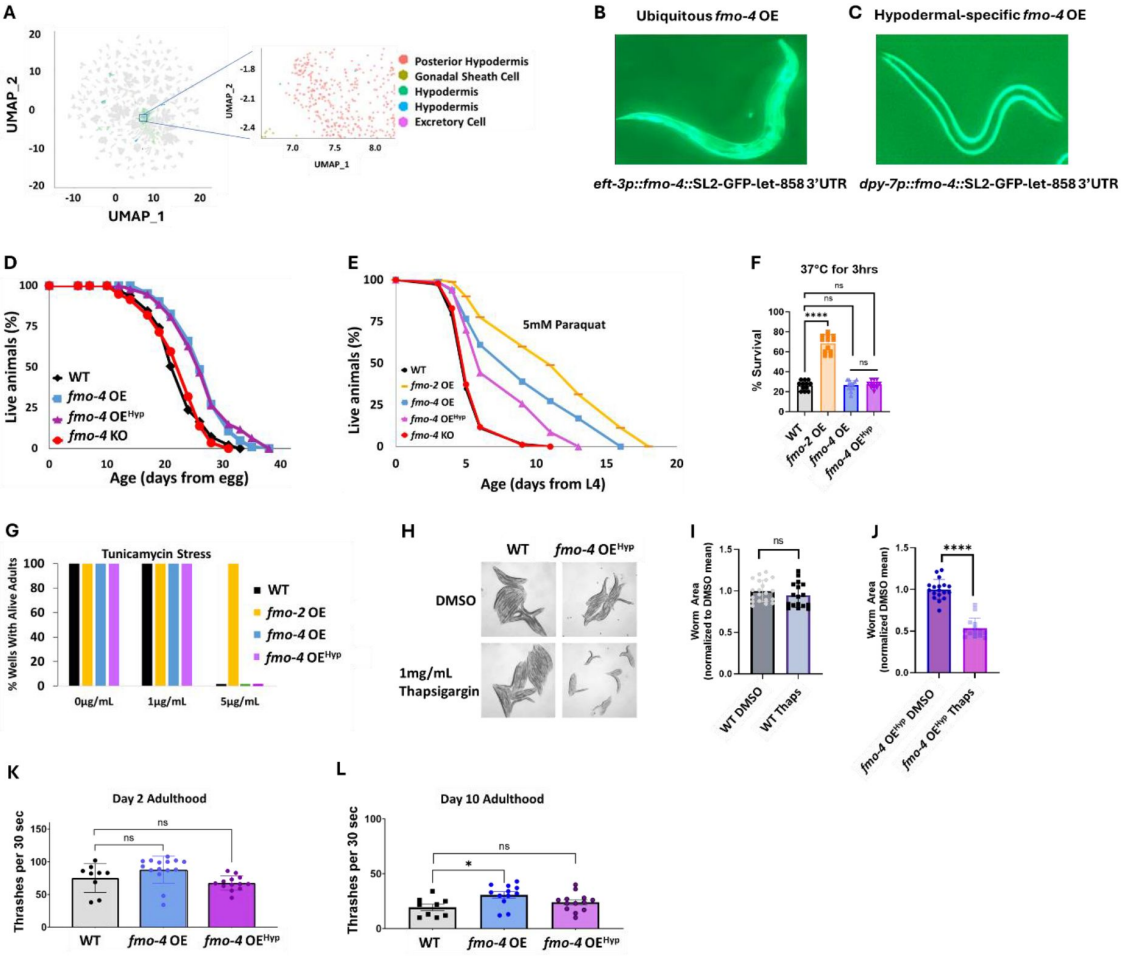

Figure 4.

Overexpressing *fmo*-4 in the hypodermis is sufficient for lifespan extension and paraquat stress resistance.

(A) Complete cell atlas of *C. elegans* aging cluster map highlighting regions of *fmo-4* gene expression. (B) Image of the ubiquitous *fmo*-4 overexpressing (*fmo-4* OE) worm by the *eft-3* promoter showing expression throughout its body. (C) Image of the hypodermal-specific *fmo-4* OE worm by the *dpy-7* promoter showing expression in the hypodermis. (D) Lifespan analysis of wild-type (WT), *fmo-4* OE, hypodermal- specific *fmo-4* OE (*fmo-4* OE^{Hyp}), and *fmo*-4 knockout (*fmo-4* KO) worms (n = ~120 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log-rank analysis. (E) Survival of worms exposed to 5mM paraquat starting at L4 stage (n = ~90 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log- rank analysis. (F) Survival of worms exposed to 37°C heat for 3 hours (hrs) at L4 stage (n = 100 worms per condition, three replicate experiments). (G) Survival of worms exposed to 0, 1, and 5ug/mL tunicamycin starting at egg until day 1 of adulthood (n = ~60 eggs per condition, three replicate experiments). (H) Brightfield images of WT and *fmo*-4 OE^{Hyp} worms exposed to DMSO or 1mg/mL thapsigargin (n = ~20 worms per condition, three replicate experiments). Quantification of (H) in (I-J). (K) Healthspan analysis of worms thrashing in a drop of M9 solution for 30 seconds (sec) on day 2 of adulthood (n = 10 worms per condition, three replicate experiments). (L) Healthspan analysis of worms thrashing in a drop of M9 solution for 30 seconds on day 10 of adulthood (n = 10 worms per condition, three replicate experiments). For heat stress, tunicamycin stress, thapsigargin stress, and healthspan assessments, * denotes significant change at $p < 0.05$ using t test. N.S. = not significant.

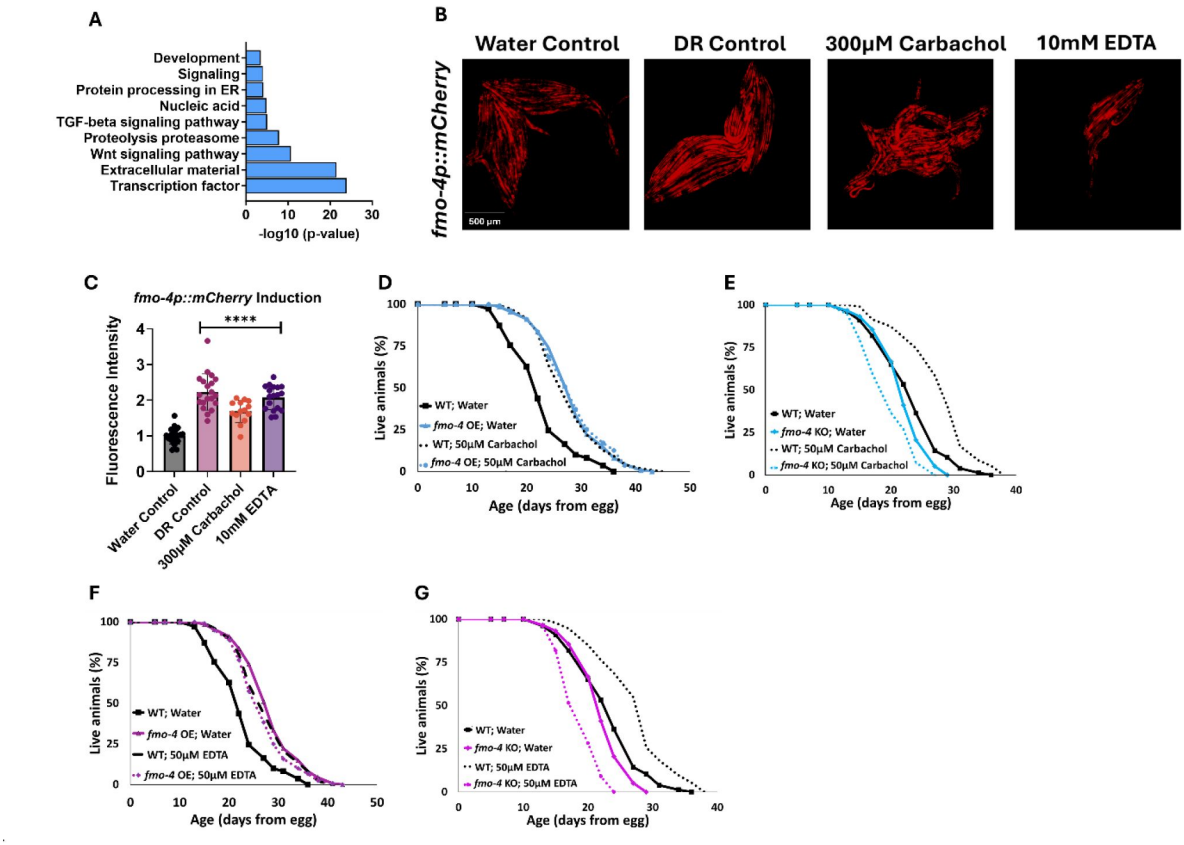

Figure 5.

fmo-4 OE transcriptomics reveals a link to calcium regulation.

(A) Gene ontology (GO) analysis of significantly regulated pathways unique to *fmo*-4 overexpression (*fmo-4* OE). (B) Fluorescence images of *fmo-4p::mCherry* reporter worms exposed to a water control, dietary restriction (DR) control, 300µM carbachol, or 10mM EDTA, which is quantified in (C) (n = ~20 worms per condition, three replicate experiments). (D) Lifespan assessment of wild-type (WT) and *fmo-4* OE worms exposed to a water control or 50µM carbachol. (E) Lifespan assessment of WT and *fmo-4* knockout (*fmo-4* KO) worms exposed to a water control or 50µM carbachol (F) Lifespan assessment of WT and *fmo*-4 OE worms exposed to a water control or 50µM EDTA. (G) Lifespan assessment of WT and *fmo-4* KO worms exposed to a water control or 50µM EDTA. For all lifespan analyses, n = ~120 worms per condition, three replicate experiments performed. Significance was determined at $p < 0.05$ using log-rank analysis and significant interactions between the condition of interest and genotype was determined at $p < 0.01$ using Cox regression analysis. For imaging experiments, * denotes significant change at $p < 0.05$ using unpaired two-tailed t test. N.S. = not significant.

fmo-4 genetically interacts with calcium signaling to promote longevity and paraquat resistance

Having established an interaction between *fmo-4* and calcium perturbations, we next asked if *fmo-4* interacts with genes involved in calcium signaling. These genes include calreticulin (*crt-1*), inositol triphosphate receptor (IP_3R, *itr-1*), and the mitochondrial calcium uniporter (*mcu-1*)[16]. Calreticulin is responsible for binding and sequestering calcium in the ER lumen[12,13]. It was previously shown that FMO protein extracted from rabbit lung can form a complex with calreticulin, suggesting a physical interaction[28]. When testing for genetic interactions between *crt-1* and *fmo-4*, we find that *crt-1* RNAi extends WT lifespan, as previously reported[16], and that this lifespan extension is not additive with *fmo-4* OE (**Fig 6A**). Treating *fmo- 4* KO worms with *crt-1* RNAi can significantly extend lifespan (Supplementary Fig 6A), but this result was inconsistent (Supplemental Data 3). Overall, this suggests that *fmo-4* and *crt-1* are acting in the same genetic pathway. IP_3R, or *itr-1* in *C. elegans*, is located in the ER membrane and is critical for exporting calcium from the ER lumen into the cytosol[12,14]. Previous results suggest that loss of *itr-1* decreases WT lifespan[16], which our results confirm (**Fig 6B**).

Importantly, we also find that *itr-1* RNAi decreases lifespan of *fmo-4* KO worms similarly (Supplementary Fig 6B) and *fmo-4* OE (**Fig 6B**) worms to a greater extent than WT, negating the relative longevity of *fmo-4* OE worms compared to WT. Thus, *itr-1* is required to increase lifespan downstream of *fmo-4*. MCU (*mcu*-1) is located in the inner mitochondrial membrane and allows cytosolic calcium into the inner mitochondrial matrix[12,15]. We also find that *mcu-1* is required downstream of *fmo-4* OE, as *mcu-1* RNAi abrogates the *fmo-4* OE lifespan (**Fig 6C**). The *fmo-4* KO lifespan is slightly decreased on *mcu-1* RNAi (Supplementary Fig 6C). These data support that *fmo-4* extends lifespan through its interactions with the calcium signaling genes, *crt-1*, *itr-1*, and *mcu-1*.

In addition to lifespan assessment, we were curious whether these genes also interact with *fmo-4* expression to modify resistance to paraquat. We exposed WT, *fmo-4* OE, and *fmo-4* KO worms to 5mM paraquat plates with RNAi for *crt-1*, *itr-1* or *mcu-1* and assessed survival over time. Interestingly, we find that *crt-1* RNAi promotes resistance in WT worms and this effect is not additive in *fmo-4* OE worms (**Fig 6D**). Further, *crt-1* RNAi extends the lifespan of *fmo-4* KO worms (Supplementary Fig 6D), suggesting that *crt-1* and *fmo-4* act in the same pathway and that *crt-1* may act downstream of *fmo-4* to promote paraquat stress resistance. Neither *itr-1* RNAi nor *mcu-1* RNAi confers resistance to paraquat, and *fmo-4* OE resistance is lost when exposed to these RNAi (**Fig 6E-F**). The *fmo-4* KO worms show a decrease in paraquat survival when treated with *itr-1* and *mcu-1* RNAi (Supplementary Fig 6E-F). These data support a model where *fmo-4* OE converges onto a shared pathway mediating paraquat stress resistance and longevity regulation. Together, we conclude that *fmo-4* interacts with ER to mitochondrial calcium signaling through *crt-1*, *itr-1*, and *mcu-1*, to promote longevity and paraquat stress resistance.

atf-6 KD regulates *fmo-4*-mediated longevity and paraquat resistance

Our data establish that *fmo-4* interacts with ER and mitochondrial calcium signaling to promote lifespan extension and resistance to paraquat. A previous study linked many of these components to a major regulator of UPR^{ER}, activating transcription factor-6 (*atf-6*)[16]. When *atf-6* is lost, lifespan is extended through changes in calcium signaling between the ER and mitochondria requiring *crt-1*, *itr-1* and *mcu-1*[16]. Based on our transcriptomics data and *fmo-4's* interactions with calcium signaling genes, we hypothesized that *fmo-4* could interact with *atf-6* to promote longevity and paraquat resistance. To test whether *atf-6* is regulating *fmo-4*, we utilized our *fmo-4p::mCherry* transcriptional reporter strain. We measured *fmo-4* gene expression after RNAi knockdown of *atf-6* and find that *fmo-4p::mCherry* worms on *atf-6* RNAi show a consistent ~2-fold increase in fluorescence (**Fig 7A-B**), similar to what we observe from calcium perturbations (**Fig**

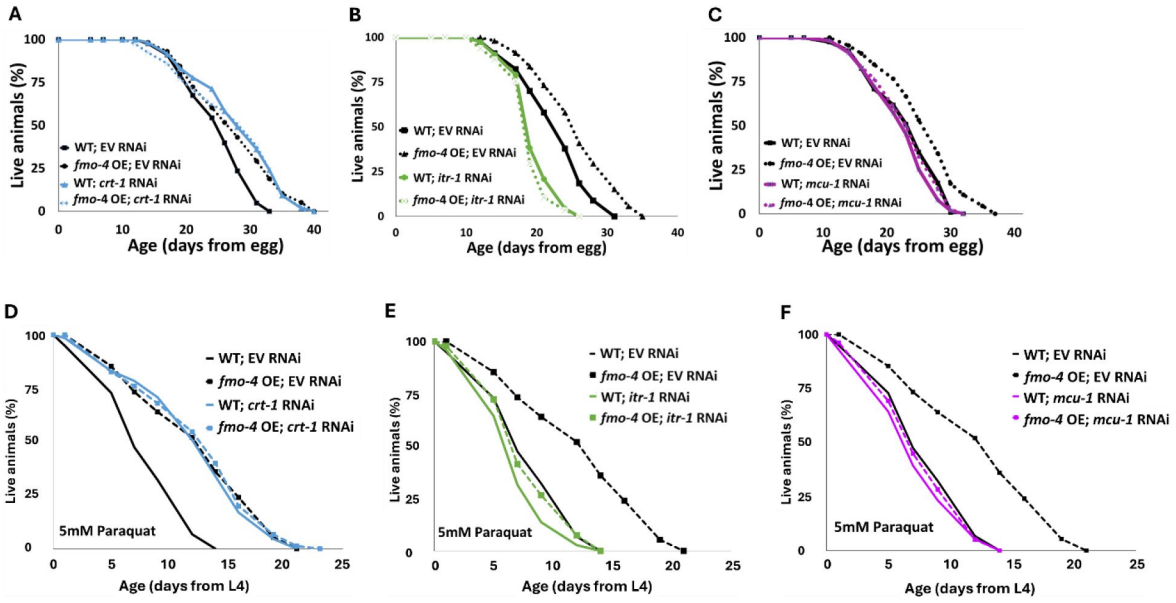

Figure 6.

fmo-4 interacts with calcium signaling genes to promote longevity and paraquat resistance.

(A) Survival analysis of wild-type (WT) and *fmo-4* overexpressing (*fmo-4* OE) worms on empty vector (EV) and *crt-1* RNAi. (B) Survival analysis of worms on EV and *itr-1* RNAi. (C) Survival analysis of worms on EV and *mcu-1* RNAi. For all lifespan analyses, n = ~120 worms per condition, three replicate experiments performed. (D) Survival of WT and *fmo-4* OE worms on EV and *crt-1* RNAi exposed to 5mM paraquat at L4 stage. (E) Survival of worms on EV and *itr-1* RNAi exposed to 5mM paraquat at L4 stage. (F) Survival of worms on EV and *mcu-1* RNAi exposed to 5mM paraquat at L4 stage. For all paraquat survival assays, n = ~90 worms per condition, three replicate experiments performed. Significance was determined at $p < 0.05$ using log-rank analysis and significant interactions between the condition of interest and genotype was determined at $p < 0.01$ using Cox regression analysis.

5B-C). Since *atf-6* is one of three branches of UPR^{ER}, we were curious to see if *fmo-4* interacts specifically with *atf-6* or also with the other two branches, *ire-1*/*xbp-1* and *pek- 1*/*atf-4*[11]. We treated the *fmo-4p::mCherry* reporter worms with *ire-1*, *xbp-1*, *pek-1*, or *atf-4* RNAi and measured fluorescence. We find that knocking down the components of the IRE-1 or PEK-1 branches does not induce *fmo-4* gene expression (Supplementary Fig 7A-B). Thus, only knockdown of the ATF-6 branch of UPR^{ER} induces *fmo-4* expression.

We hypothesized that *atf-6* limits *fmo-4* expression and thus *fmo-4* acts downstream of *atf-6* knockdown to modulate lifespan extension. To test this, we assessed survival and find that while *atf-6* RNAi extends the lifespan of WT worms, as reported[16], this effect is abrogated when *fmo-4* is knocked out (**Fig. 7C**). This result suggests that *fmo-4* is indeed acting downstream of *atf-6* to promote longevity. To determine if *atf-6* is also involved in *fmo-4*-mediated paraquat resistance, we exposed WT and *fmo-4* KO worms to 5mM paraquat plates with *atf-6* RNAi. We find that *atf-6* RNAi promotes stress resistance to paraquat, but this effect is lost when *fmo-4* is knocked out, supporting the hypothesis that *atf-6* is involved in *fmo-4*-mediated paraquat resistance (**Fig 7D**). Together, these data suggest that *fmo-4* modulates lifespan and paraquat stress resistance downstream of the reduction in *atf-6*, ultimately regulating ER to mitochondria calcium signaling (**Fig 7E**).

Discussion

Together, our data present a model where *fmo-4* acts downstream of DR and mTOR to positively affect healthspan and longevity (**Fig 1B-C**). *fmo-4* overexpression is sufficient to provide these benefits (**Fig 3**) and does not require the DR-mediating family member, *fmo-2* (**Fig 2**). Our results also suggest that *fmo-4* gene expression is upregulated upon changes in intracellular calcium, and that *fmo-4* interacts with calcium signaling through key genes like *crt- 1*, *itr-1,* and *mcu-1*, to promote longevity and paraquat stress resistance (**Fig 5**-**6**). The relationship between *fmo-4* and calcium is further illustrated by *fmo-4* OE's susceptibility to thapsigargin, a calcium-mediated ER stress. Furthermore, we find that knocking down the UPR^{ER} transcription factor and calcium regulator, *atf-6,* induces *fmo-4* gene expression and requires *fmo-4* to promote longevity (**Fig 7**). Collectively, our data suggest that *fmo-4* promotes longevity and paraquat stress resistance by regulating calcium homeostasis between the ER and mitochondria (**Fig 7E**).

Previous studies from our lab have elucidated how *C. elegans fmo-2* promotes longevity through endogenous metabolism[10]. Based on the conservation within the *fmo* gene family and that multiple Fmos are induced in long-lived worms and mammals, it was reasonable to hypothesize that Fmos could play overlapping roles in longevity regulation. Our data here support the broader hypothesis that Fmos have a conserved role in aging, but interestingly, that the mechanisms by which they modulate aging are separable. Importantly, we find that *fmo-4* is required for multiple longevity-promoting pathways, including DR and mTOR, but is not required for lifespan extension through the hypoxic response, insulin-like signaling, or cytochrome c reductase pathways. We also find that *fmo-4* does not require *fmo-2*, but that *fmo-2* does require *fmo-4*. This is interesting because not only do these data tell us that *fmo-4* is important in the context of longevity, but also that it acts distinctly from *fmo-2*.

After creating ubiquitous and hypodermal-specific *fmo-4* overexpressing strains, we find that *fmo-4* is sufficient for longevity and paraquat stress resistance but not heat, tunicamycin, or thapsigargin stress. These results further differentiate *fmo-4* from *fmo-2* and also provide insight into *fmo-4's* potential roles in the cell. For instance, *fmo-4* OE worms' resistance to paraquat could suggest a role in responding to or controlling reactive oxygen species (ROS) production in the mitochondria. Additionally, *fmo-4* OE worms' sensitivity to thapsigargin likely points towards an involvement in ER calcium regulation. These are both interesting plausible roles for *fmo-4,* and may go hand in hand, as calcium levels are known to impact ROS levels[29], and changes in ROS

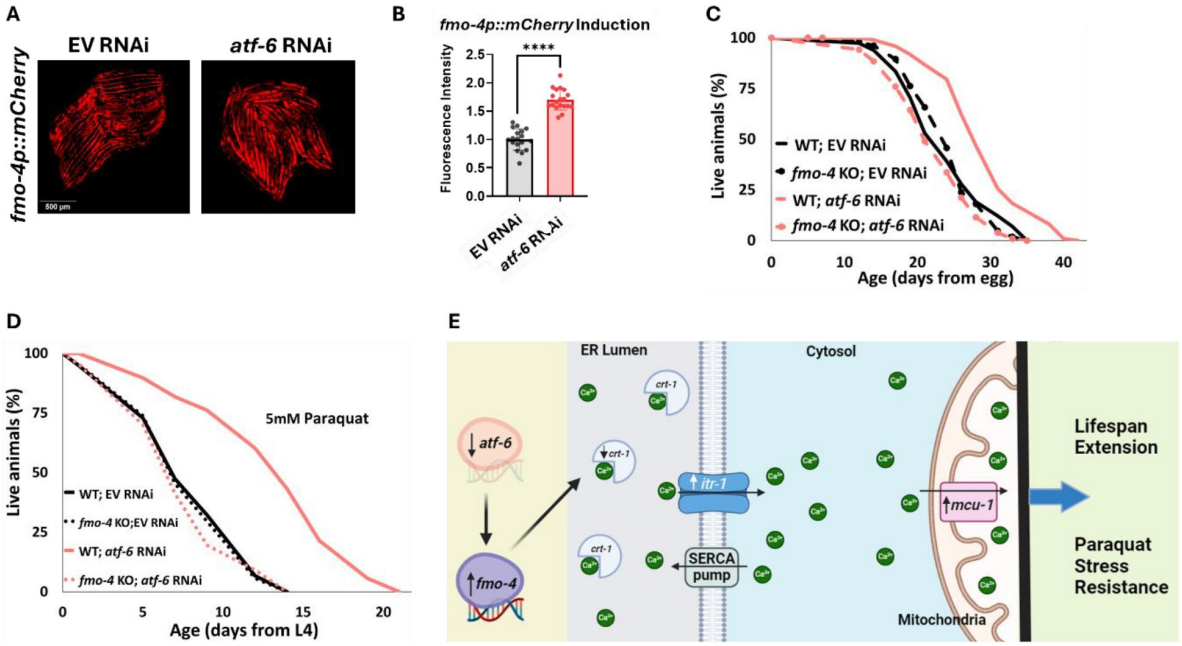

Figure 7.

atf-6 KD regulates *fmo*-4-mediated longevity and paraquat stress resistance

(A) Fluorescence imaging of the *fmo-4p::mCherry* reporter worms on empty vector (EV) and *atf-6* RNAi. (B) Quantification of (A) (n = ~20 worms per condition, three replicate experiments). (C) Lifespan analysis of wild-type (WT) and *fmo*-4 knockout (*fmo-4* KO) worms on EV and *atf-6* RNAi (n = ~120 worms per condition, three replicate experiments). (D) Survival of WT and *fmo*- 4 KO worms on EV and *atf-6* RNAi exposed to 5mM paraquat at L4 stage (n = ~90 worms per condition, three replicate experiments). Significance was determined at $p < 0.05$ using log-rank analysis and significant interactions between the condition of interest and genotype was determined at $p < 0.01$ using Cox regression analysis. (E) Working model showing reduced expression of *atf-6* induces *fmo-4* expression, which regulates calcium regulation from the endoplasmic reticulum (ER) to the mitochondria to promote lifespan extension and paraquat stress resistance. For imaging experiments, * denotes significant change at $p < 0.05$ using unpaired two-tailed t test. N.S. = not significant.

levels are known to impact ER calcium release[29]. Based on this and the data with calcium perturbations, we speculate that FMO-4 acts as a calcium sensor, and when calcium levels are too high or too low, FMO-4 responds by regulating downstream calcium signaling proteins to restore homeostasis in the cell. As FMO-4 is a predicted ER transmembrane protein, we hypothesize that FMO-4 is sensing changes in calcium levels in the ER and/or cytosol.

We note that our data reveal genetic interactions between *fmo-4* and other longevity pathways like DR, mTOR, FMO-2, and ATF-6. However, it is important to also understand on the protein level how FMO-4 is interacting with these pathways and how FMO-4 is regulating downstream calcium signaling components to promote longevity and paraquat stress resistance. For instance, it was previously shown that rabbit FMO protein forms a complex with calreticulin[28]. It is possible that an increase in FMO-4 leads to more binding between FMO-4 and calreticulin, which ultimately prevents calreticulin from binding ER calcium. This would then allow for proper calcium flux from the ER to the mitochondria via the IP_3R and MCU, respectively. Our future work will look into these protein interactions so that we can further tease apart the FMO-4-mediated longevity pathway.

While our transcriptomics analysis and calcium manipulations suggest *fmo-4* involvement in calcium regulation, there are other measures that can better assess an interaction between *fmo-4* and calcium. Carbachol and EDTA supplementation are not perfect assessments of altered calcium levels, as carbachol does not act in every tissue and EDTA may be depleting more than just calcium from the cell. Thus, future studies should measure calcium levels and calcium flux in *fmo-4* OE and KO worms using GCaMP expressing worms to determine the direct link between *fmo-4* expression and calcium in the cell. Additionally, to determine if FMO-4 is acting as a calcium sensor or interacting with one to regulate calcium homeostasis, development of FMO-4 antibodies for immunoprecipitation assays would be useful[32]. Together, it will be important to establish the biochemical parameters of the FMO-4 calcium interaction.

As calcium signaling occurs between the ER and the mitochondria, our results could have interesting implications in ER-mitochondrial metabolism. We would expect that FMO-4 regulates ER-mitochondria metabolism because 1) FMO-4 is a predicted ER transmembrane protein and blocking the expression of another ER transmembrane protein, ATF-6, induces the expression of *fmo-4*, 2) *fmo-4* OE worms are highly sensitive to ER calcium stress but resistant to ROS induced stress, 3) lifespan extension driven by *fmo-4* depends on mitochondrial calcium import (*mcu-1*), and 4) Fmo gene expression is induced by mitochondrial inhibitors[33].

Additionally, changes in mitochondrial calcium import affect various mitochondrial measures like respiration, dynamics (i.e., fission and fusion), membrane potential, and TCA cycle activity[34].

Future studies will delve into how *fmo-4* perturbations impact each of these measures and if they act in the *fmo-4*-mediated longevity pathway. Based on our data, we speculate that *fmo-4* OE may increase mitochondrial calcium by regulating *mcu-1* activity, and that *fmo-4* OE worms may in turn be regulating ROS production in the mitochondria. We hypothesize that changes in *fmo-4* expression will alter mitochondrial respiration, dynamics, membrane potential, and TCA cycle activity. Taken together, future experiments will shed light on the role that FMOs play in calcium regulation and mitochondrial metabolism and will help to further tease apart the FMO- mediated longevity pathway.

Methods and Materials

Strains and Growth Conditions

Standard *C. elegans* cultivation procedures were used as previously described[6]. Briefly, all worm strains were maintained on solid nematode growth media (NGM) using *E. coli* OP50 throughout life except where double stranded (ds) RNAi (*E. coli* HT115) were used. Worms were transferred using a platinum wire. All worm strains were kept at 20°C. RNAi used is listed in Supplementary Table 3. Worm strains are listed in Supplementary Table 4. Genotyping primers are listed in Supplementary Table 5.

Development Assays

Animals were synchronized by placing 10 gravid adult worms on NGM plates seeded with *E. coli* OP50 to lay eggs for 1 hour at 20°C. The gravid adult worms were then removed, and the eggs were allowed to hatch and develop at 20°C until larval stage 2 (L2). At this point the L2 worms were moved to individual 35mm NGM plates seeded with *E. coli* OP50, one worm per plate. This was done for each worm strain tested and ten total 35mm plates per worm strain were prepared. The worms were then followed through development and watched hourly during young adulthood to score the time of the first egg lay, as previously described[6]. Development time was reported in hours since egg lay. Three replicate experiments were performed.

Stress Resistance Assays

Paraquat stress assay

Paraquat (Methyl viologen dichloride hydrate, 856177, Sigma-Aldrich) was used to induce oxidative stress. Worms were synchronized from eggs on either NGM plates seeded with *E. coli* OP50 or RNAi plates seeded with HT115 strain expressing dsRNAi for a particular gene. At L4 stage, 30 worms were transferred to either NGM plates or RNAi-FUdR (40690016, Bioworld) plates containing 5mM paraquat dissolved in water. Three plates per strain per condition were prepared, for a total of 90 worms per condition. As previously described, worms were then scored every other day and considered dead when they did not move in response to prodding under a dissection microscope[6]. Worms that crawled off the plate were not considered, but ruptured worms were noted and considered. Three replicate experiments were performed. Log-rank test was used to derive p-value for paraquat stress resistance survival assays using $p < 0.05$ cut-off threshold compared to EV or wild-type controls. Cox regression was also used to assess interactions between genotype and condition for paraquat stress resistance survival assays using $p < 0.01$ cut-off threshold compared to controls.

Heat stress assay

Worms were synchronized from eggs on NGM plates seeded with *E. coli* OP50. Once the worms reached day 1 of adulthood, 25 worms of each strain were transferred to 35mm NGM plates seeded with *E. coli* OP50[16]. Four plates per strain per condition were prepared, for a total of 100 worms per condition. The plates were placed in a single layer on a tray and incubated at 37°C for 3 hours. After 3 hours, the plates were removed and placed at 20°C for 24 hours to give the worms time to recover. After 24 hours, the number of dead and alive worms was counted. % Alive was calculated as (# alive / # total)×100 and graphed in Graphpad Prism using unpaired two-tailed t tests with Welch's correction as well as one-way ANOVA to determine significance. Three replicate experiments were performed.

Tunicamycin stress assay

E. coli OP50 was cultured at 37°C shaking overnight and then centrifuged and concentrated to 100x. 100µL of bacteria was added to 9.9mL of S-media to create a 1x mixture. 100µL of this bacteria/S-media mixture was added to the wells of a Falcon non tissue culture treated clear flat

bottom 96 well plate (Falcon, 351172). Approximately 20 eggs per worm strain were added to the wells of the 96 well plate as previously described[6]. Three replicates per worm strain were added to the 96-well plate, for a total of 60 eggs per worm strain. Then either 0, 1, 2, or 5µg/mL of tunicamycin (Sigma, T7765) diluted in dimethyl sulfoxide (DMSO) (Sigma, D2650) was added to the wells. Total volume per well was 100µL. The plate was incubated covered at room temperature on an orbital shaker for 72 hours. After 72 hours, each well containing worms was rinsed with 100µL M9 and then added to individual 35mm NGM plates seeded with *E. coli* OP50 to sit covered at room temperature overnight. The following day, plates were assessed for live adults. % Alive Adults was calculated by determining how many of the three technical replicates of a given strain showed at least one live adult and then graphed in Microsoft Excel. Three replicate experiments were performed.

Thapsigargin stress assay

To assess development of worms during chronic ER calcium stress, NGM plates seeded with *E. coli* OP50 were spotted with 25µL 1mg/mL thapsigargin in DMSO (Sigma) or DMSO only directly on to the *E. coli* OP50 lawns and allowed to dry overnight. Then, 30 synchronized L1's per worm strain were added directly to the spotted lawns[16]. Forty-eight hours later, worms were picked off these plates, added to a 2% agarose pad on a glass slide, anesthetized in 0.5M sodium azide (Sigma), and imaged at 6.3x magnification (brightfield) with the LASx software and Leica scope. Three replicates were performed. Each worm was recorded in ImageJ. Data were analyzed in GraphPad Prism using unpaired two-tailed t tests with Welch's correction.

Thrashing Assays

Animals were synchronized by placing 10 gravid adult worms on NGM plates seeded with *E. coli* OP50 and allowing them to lay eggs for 2 hours at 20°C. The gravid adult worms were then removed, and the eggs were allowed to hatch and develop at 20°C until day 1 adulthood.

Worms were placed in a drop of M9 solution, as previously described[6]. The individual body bends were counted at maximum rate for 30 seconds. Thrashing was assayed on day 2 and day 10 of adulthood. The animals that were not used for the day 2 assay were transferred to fresh fed FUdR plates 4 times until they were ready to be assayed. Three replicates were performed. Data were analyzed in GraphPad Prism using unpaired two-tailed t tests with Welch's correction as well as one-way ANOVA.

Lifespan Assays

Gravid adults were placed on NGM plates containing 1mM β-D-isothiogalactopyranoside (IPTG), 25µg/ml carbenicillin, and the corresponding RNA interference (RNAi) clone from the Vidal or Ahringer RNAi library. 200µL of HT115 bacteria expressing double stranded (ds) RNA of either the control empty vector (EV) or the RNAi of interest at optical density (OD) of 3.0 and concentration of 3X was added to each plate. After 3 hours, the adults were removed, and the eggs were allowed to develop at 20°C until they reached late L4/young adult stage. From here, 70 worms were placed on each RNAi plate and transferred to fresh RNAi + FUDR plates on day 1, day 2, day 4, and day 6 of adulthood. A minimum of two plates per strain per condition were used per replicate experiment. Experimental animals were scored every 2-3 days and considered dead when they did not move in response to prodding under a dissection microscope. Worms that crawled off the plate were not considered, but ruptured worms were considered as previously described[6]. A similar method was used for non-RNAi lifespan experiments, except the plates did not contain IPTG and worms were fed *E. coli* OP50. 200µL of *E. coli* OP50 at OD of 3.0 and concentration of 3X was added to each plate. A similar method was also used for carbachol (ThermoFisher, L06674.14) and ethylenediaminetetraacetic acid (EDTA) (ThermoFisher, AM9260G) supplementation lifespan experiments, except 50µM carbachol or 50µM EDTA was added to the NGM plates without IPTG, and worms were fed *E. coli* OP50. 200µL of *E. coli* OP50 at OD of 3.0 and concentration of 3X was

added to each plate. Optimal concentrations of carbachol and EDTA (50μM) were determined by assessing survivability of worms exposed to a range of concentrations. Log-rank test was used to derive p- value for lifespan assays using $p < 0.05$ cut-off threshold compared to EV or wild-type controls. Cox regression was also used to assess interactions between genotype and condition for lifespan assays using $p < 0.01$ cut-off threshold compared to controls.

Dietary Restriction (sDR) Lifespan Assay

Gravid adults were placed on NGM plates seeded with 200μL *E. coli* OP50. After 3 hours, the adults were removed, and the eggs were allowed to develop at 20°C until they reached late L4/young adult stage. From here, 70 worms were transferred to fed FUdR plates seeded with 200μL of *E. coli* OP50 at OD of 3.0 and concentrated 3X on days 1 and 2 of adulthood. On day 3 of adulthood, DR conditions were transferred to plates with 10^9 seeded lawns and transferred every other day four times while the corresponding controls were transferred equally to fed ad- lib plates. This form of DR is termed solid DR (sDR)[19]. 80μL of 10mM palmitic acid (Sigma- Aldrich) dissolved in 100% EtOH was added to the outer rim of the plate to prevent fleeing. A minimum of two plates per strain per condition were used per replicate experiment.

Experimental animals were scored every 2-3 days and considered dead when they did not move in response to prodding under a dissection microscope. Worms that crawled off the plate were not considered, but ruptured worms were considered as previously described[6].

Transcriptomic Analysis

Approximately 600 day 1 adult worms per biological replicate were washed with M9 buffer three times, frozen in liquid nitrogen, and then stored at −80°C. RNA was extracted by adding 500mL of Trizol reagent to the frozen worm pellets, followed by three freeze-thaw cycles with liquid nitrogen and water bath at 42°C. Then, 500μL of ethanol was added to the samples, and RNA was isolated using the Direct-Zol Miniprep Plus Kit (Cat#R2072). Purified RNA was sent to Novogene (Novogene Corporation Inc) for sequencing on the Illumina HWI-ST1276 instrument. Messenger RNA was purified from total RNA by using poly-T oligo-attached magnetic beads.

After fragmentation, first strand cDNA was synthesized using random hexamer primers, followed by second strand cDNA. The library was constructed, which entailed end repair, A- tailing, adapter ligation, size selection, amplification, and purification, and then was sequenced on an Illumina device using paired-end sequencing. Gene ontology analysis was done using the National Institutes of Health DAVID Bioinformatics tool.

Gene Expression Assays

RNA was isolated from day 1 adult worms (approximately 600 worms per strain) following three rounds of freeze-thaw in liquid nitrogen using Invitrogen's Trizol extraction method, similar to the method described above. 1μg of isolated and purified RNA was reverse transcribed to cDNA using SuperScript™ II Reverse Transcriptase (18064071, Invitrogen,). Gene expression levels were measured using 600ng of cDNA and SYBR™ Green PCR Mastermix (A25742, Applied Biosystems) with primers at 10μM concentration. mRNA levels were normalized using Y45F10D.4 as a reference gene[35]. List of qPCR primers used are in Supplementary Table 6.

fmo-4 induction on RNAi

Gravid *fmo-4p::mCherry* transcriptional reporter adult animals were placed on NGM plates containing 1mM β-D-isothiogalactopyranoside (IPTG), 25 μg/ml carbenicillin, and the corresponding RNAi clone from the Vidal or Ahringer RNAi library. After 3 hours, the adults were removed, and the eggs were allowed to develop at 20°C until they reached late L4/young adult stage. Then 40-50 worms per plate were transferred to similar plates that contain FUdR for overnight. The following day, ~20 worms per condition were picked off these plates, added to a 2%

agarose pad on a glass slide, anesthetized in 0.5M sodium azide (Sigma), and imaged at 6.3x magnification with the LASx software and Leica scope using the mCherry fluorescence channel[36]. Three replicates were performed. Fluorescent intensity in the mCherry channel was measured in ImageJ. Brightness of images within a dataset was increased to the same level. Data were analyzed in GraphPad Prism using unpaired two-tailed t tests with Welch's correction.

fmo-4 induction on Carbachol

Gravid *fmo-4p::mCherry* transcriptional reporter adult animals were placed on NGM plates seeded with *E. coli* OP50. After 3 hours, the adults were removed, and the eggs were allowed to develop at 20°C until they reached late L4/young adult stage. Then 30 worms were transferred to NGM plates seeded with *E. coli* OP50 also containing either 300µM carbachol (ThermoFisher, L06674.14) or water. An optimal concentration of carbachol was determined by first assessing survivability of worms exposed to a range of concentrations and then by assessing fluorescence of the *fmo-4p::mCherry* reporter worms exposed to a range of concentrations. The worms were incubated for 24 hours at 20°C. After 24 hours, ~20 worms per condition were then picked off these plates and added to unseeded NGM plates, anesthetized in 0.5M sodium azide (Sigma), and imaged at 6.3x magnification with the LASx software and Leica scope using the mCherry fluorescence channel[36]. Three replicates were performed. Each worm was measured for fluorescence in ImageJ. Data were analyzed in GraphPad Prism using t tests.

fmo-4 Induction Assay on EDTA

Gravid *fmo-4p::mCherry* transcriptional reporter adult animals were placed on NGM plates seeded with *E. coli* OP50. After 3 hours, the adults were removed, and the eggs were allowed to develop at 20°C until they reached late L4/young adult stage. Then 30 worms were transferred to NGM plates seeded with *E. coli* OP50 topically spotted with 150µL of 0.5M ethylenediaminetetraacetic acid (EDTA) (ThermoFisher, AM9260G) or 150µL of water. An optimal concentration of EDTA was determined by first assessing survivability of worms exposed to a range of concentrations and then by assessing the fluorescence of the *fmo- 4p::mCherry* reporter worms exposed to a range of concentrations. The worms were incubated for 24 hours at 20°C. After 24 hours, ~20 worms per condition were then picked off these plates and added to unseeded NGM plates, anesthetized in 0.5M sodium azide (Sigma), and imaged at 6.3x magnification with the LASx software and Leica scope using the mCherry fluorescence channel[36]. Three replicates were performed. Each worm was recorded in ImageJ. Data were analyzed in GraphPad Prism using t tests.

Statistical Analyses

Log-rank test was used to derive p-value for lifespan and stress resistance survival assays using $p < 0.05$ cut-off threshold compared to EV or wild-type controls. Cox regression was also used to assess interactions between genotype and condition for lifespans and stress resistance survival assays using $p < 0.01$ cut-off threshold compared to controls. Supplemental Data 3 provide the results of the Log-rank test and Cox regression analysis, which were run in Rstudio.

Acknowledgements

This work was supported by grants from NIH. AMT was supported by NIH T32AG000114 and the University of Michigan Rackham Research Grant. SFL was supported by R01AG075061 and the Glenn Foundation for Medical Research.

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Reviewer #1 (Public Review):
Summary:
This interesting and well written article by Tuckowski et al. summarizes work connecting the flavin-containing monooxygenase FMO-4 with increased lifespan through a mechanism involving calcium signaling in the nematode Caenorhabditis elegans.

The authors have previously studied another fmo in worms, FMO-2, prompting them to look at additional members of this family of proteins. They show that fmo-4 is up in dietary restricted worms and necessary for the increased lifespan of these animals as well as of rsks-1 (s6 kinase) knockdown animals. They then show that overexpression of fmo-4 is sufficient

to significantly increase lifespan, as well as healthspan and paraquat resistance. Further, they demonstrate that overexpression of fmo-4 solely in the hypodermis of the animal recapitulates the entire effect of fmo-4 OE.

In terms of interactions between fmo-2 and fmo-4 they show that fmo-4 is necessary for the previously reported effects of fmo-2 on lifespan, while the effects of fmo-4 do not depend on fmo-2.

Next the authors use RNASeq to compare fmo-4 OE animals to wild type. Their analyses suggested the possibility that FMO-4 was modulating calcium signaling, and through additional experiments specifically identified the calcium signaling genes crt-1, itr-1, and mcu-1 as important fmo-4 interactors
in this context. As previously published work has shown that loss of the worm transcription factor atf-6 can extend lifespan through crt-1, itr-1 and mcu-1, the authors asked about interactions between fmo-4 and atf-6. They showed that fmo-4 is necessary for both lifespan extension and increased paraquat resistance upon RNAi knockdown of atf-6.

Overall this clearly written manuscript summarizes interesting and novel findings of great interest in the biology of aging and suggests promising avenues for future work in this area.

Strengths:
This paper contains a large number of careful, well executed and analysed experiments in support of its existing conclusions, and which also point toward significant future directions for this work. In addition it is clear and very well written.

Weaknesses:
Within the scope of the current work there are no major weaknesses. That said, the authors themselves note pressing questions beyond the scope of this study that remain unanswered. For instance, the mechanistic nature of the interactions between FMO-4 and the other players in this story, for example in terms of direct protein-protein interactions, is not at all understood yet. Further, powerful tools such as GCaMP expressing animals will enable a much more detailed understanding of what exactly is happening to calcium levels, and where and when it is happening, in these animals.

https://doi.org/10.7554/eLife.99971.1.sa2

Reviewer #2 (Public Review):

Summary:
Members of a conserved family of flavin-containing monooxygenases (FMOs) are necessary and at least partly sufficient for lifespan extension induced by diet restriction and hypoxia. Of 5 FMOs in C. elegans, fmo-2 has received the majority of attention, but this study identifies that fmo-4 is also an important, positive modulator of lifespan. Based on differential requirements of fmo-2 and fmo-4 in stress resistance and lifespan extension paradigms, the authors conclude that fmo-4 acts through mechanisms that are overlapping, but distinct from fmo-2. Ultimately, the authors place fmo-2 genetically within a pathway involving atf-6, calreticulin, the IP3 receptor, and mitochondrial calcium uniporter, which was previously shown to link ER calcium homeostasis to mitochondrial homeostasis and longevity. Because the known enzymatic activity of FMOs involves oxygenating xenobiotic and endogenous metabolites, these findings highlight a potential new link between redox/metabolic homeostasis and ER-mitochondrial calcium signaling, while revealing that different FMO family members regulate stress resistance and lifespan through distinct mechanisms.

Strengths:
The authors have used genetics to discover an interesting and unanticipated new link

between conserved FMOs and ER calcium pathways known to regulate lifespan.

The genetic epistasis patterns for lifespan and stress resistance phenotypes are generally clean and compelling.

Weaknesses:
The effects of carbachol and EDTA on intracellular calcium levels are inferred, especially in the tissues where fmo-4 is acting. Validating that these agents and fmo-4 itself have an impact on calcium in relevant subcellular compartments is important to support conclusions on how fmo-4 regulates and responds to calcium.

Experiments are generally reliant on RNAi. While in most cases experiments reveal positive results, indicating RNAi efficacy, key conclusions could be strengthened with the incorporation of mutants.

While FMO-4 is clearly placed in the ER calcium pathway genetically, a putative molecular mechanism by which FMO-4 would alter ER calcium remains unclear. Notably, Tuckowski et al. highlight this gap in the discussion as well.

https://doi.org/10.7554/eLife.99971.1.sa1

Reviewer #3 (Public Review):

Summary:
The authors assessed the potential involvement of fmo-4 in a diverse set of longevity interventions, showing that this gene is required for DR and S6 kinase knockdown related lifespan extension. Using comprehensive epistasis experiments they find this gene to be a required downstream player in the longevity and stress resistance provided by fmo-2 overexpression. They further showed that fmo-4 ubiquitous overexpression is sufficient to provide longevity and paraquat (mitochondrial) stress resistance, and that overexpression specifically in the hypodermis is sufficient to recapitulate most of these effects.

Interestingly, they find that fmo-4 overexpression sensitizes worms to thapsigargin during development, an effect that they link with a potential dysregulation in calcium signalling. They go on to show that fmo-4 expression is sensitive to drugs that both increase or decrease calcium levels, and these drugs differentially affect lifespan of fmo-4 mutants compared to wild-type worms. Similarly, knockdown of genes involved in calcium binding and signalling also differentially affect lifespan and paraquat resistance of fmo-4 mutants.

Finally, they suggest that atf-6 limits the expression of fmo-4, and that fmo-4 is also acting downstream of benefits produced by atf-6 knockdown.

Strengths:
• comprehensive lifespans experiments: clear placement of fmo-4 within established longevity interventions.
• clear distinction in functions and epistatic interactions between fmo-2 and fmo-4 which lays a strong foundation for a longevity pathway regulated by this enzyme family.

Weaknesses:
• no obvious transcriptomic evidence supporting a link between fmo-4 and calcium signalling: either for knockout worms or fmo-4 overexpressing strains.
• no direct measures of alterations in calcium flux, signalling or binding that strongly support a connection with fmo-4.
• no measures of mitochondrial morphology or activity that strongly support a connection with fmo-4.
• lack of a complete model that places fmo-4 function downstream of DR and mTOR signalling

(first Results section), fmo-2 (second Results section) and at the same time explains connection with calcium signalling.

https://doi.org/10.7554/eLife.99971.1.sa0