**New Genes that Influence Longevity and ER Unfolded Protein Response in *C. elegans***

by

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# Dedication

To my family and friends who always give me the unconditional support and freedom to keep doing the stuff I like.

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# Abstract

Insulin-like signaling pathway regulates growth, reproduction, metabolism, and aging. In *C. elegans*, DAF-2/IGFR mutation reduces insulin-like signaling pathway activity and activates DAF-16/FoxO transcription factor to promote life span extension. We and others have shown the DAF-16A and DAF-16F isoforms are the key isoforms that control life span. To elucidate the mechanistic underpinnings of DAF-16/FoxO-dependent life span extension, we performed whole transcriptome profiling and identified isoform-specific DAF-16/FoxO target genes. *acs-3*, which encodes an acyl-CoA synthetase homologous to human ACSL5, was identified as a redundant target gene regulated by both DAF-16A and F. An *acs-3* nonsense mutation significantly suppresses *daf-2* life span extension but does not affect life span in wild-type background. To study the expression pattern of ACS-3, an ACS-3::GFP translational reporter was made and GFP expression was observed in glial, hypodermal, and vulva without obvious expression in the intestine. This ACS-3::GFP expression pattern is biologically relevant because the ACS-3::GFP transgene is able to rescue the shortened life span of *acs-3;daf-2* double mutant to a comparable life span of a *daf-2* single mutant. Tissue-specific experiments reconstituting ACS-3 in the tissues identified by the ACS-3::GFP translational reporter will determine the tissue requirement for rescuing the shortened life span of *daf-2;acs-3* double mutant and provide insight to elucidate the molecular mechanisms of life span regulation mediated by DAF-16/FoxO.

TRAP-1 (translocon-associated protein-1) is a novel regulator of insulin biogenesis in mammalian cells and specifically modulates FoxO activity through the insulin-like signaling

pathway in *C. elegans*. Our studies found TRAP-1 is localized to the ER and loss of *trap-1* activity can increase the expression level of *hsp-4::GFP* reporter which is a proxy for ER unfolded protein response (UPR). A genetic screen was performed to identify mutations that can *m*odify *hsp-4::GFP* expression caused by *trap-1* mutation (*mtro* screen). A *col-75* missense mutant was identified as a *mtro* gene candidate that can increase ER UPR caused by *trap-1* and *col-75* single mutant is sufficient to induce *hsp-4::GFP* expression compared to wild-type. To validate *col-75* as a *bono fide mtro* gene, one independent *col-75* nonsense allele and two independent *col-75* missense alleles were tested to determine if they are able to phenocopy our *col-75* missense mutant emerged from the genetic screen. We found the nonsense allele does not induce *hsp-4::GFP* expression while two independent missense alleles can induce *hsp-4::GFP* expression stronger than wild-type, although to a lesser degree than our mutagenized allele. These results suggest our mutagenized *col-75* mutant is a neomorphic allele and the increased *hsp-4::GFP* phenotype is not due to loss-of-function of COL-75. A *col-75* transcriptional dsRed reporter strain was generated to visualize the expression pattern of COL-75 and we found *col-75p::dsRed* expression in the amphid socket cell, excretory cell, vulva, and phasmid sheath cell. Expression of *col-75p::dsRed* transgene in *col-75;hsp-4::GFP* background does not reveal colocalization of the two transgenes and no *col-75p::dsRed* signal is observed in the intestine where ER UPR is most strongly induced. These suggest *col-75* can induce ER UPR cell non-autonomously. Future experiments expressing mutant collagen cDNA in the tissues identified by the *col-75p::dsRed* can test the ability of missense collagen variants to induce *hsp-4::GFP* expression cell non-autonomously and will shed light on conserved mechanisms through which collagen missense variants may influence organismal health.

# Chapter 1 Introduction

Life is complex and the ultimate goal for the study of life sciences tries to understand complicated questions about living organisms at a molecular level. Fortunately, different organisms, including humans, share similar principles of physiological mechanisms that are evolutionarily conserved to cope with environmental challenges. *Caenorhabditis elegans* is a tiny (larvae are 0.25 millimeter long and adults are 1 millimeter long) non-parasitic nematode that can be isolated from rotten vegetable samples around the world (Frézal and Félix 2015). *C. elegans* has been established as a model organism over 60 years ago thanks to its ease of genetic manipulation. The nematode is a hermaphrodite and under minimal maintenance condition can self-fertilize to produce about 300 progenies, which is particularly suitable for genetic analysis and epistasis studies. In fact, both forward and reverse genetic screens have uncovered fruitful results that deepen our understandings about homologous genes that can cause human diseases as well as genes that are involved in fundamental biological processes. The key discovery of insulin/insulin-like growth factor-1 (IGF-1) signaling (IIS) pathway in *C. elegans* and the demonstration of a single DAF-2/IGFR mutation can extend life span by over two-fold compare to wild-type nematodes have laid the groundwork for studying the biology of aging (Friedman and Johnson 1988a, Kenyon et al. 1993, Kimura et al. 1997, Collins et al. 2007). In addition, forward genetic screen approach identified the alternative splicing of *xbp-1* mRNA, which leads to the activation of ER unfolded protein response (UPR) when ER homeostasis is perturbed, underscores the usefulness of *C. elegans* as a genetic model to study the cellular and molecular processes (Shen et al. 2001, Calfon et al. 2002). This chapter will overview the background

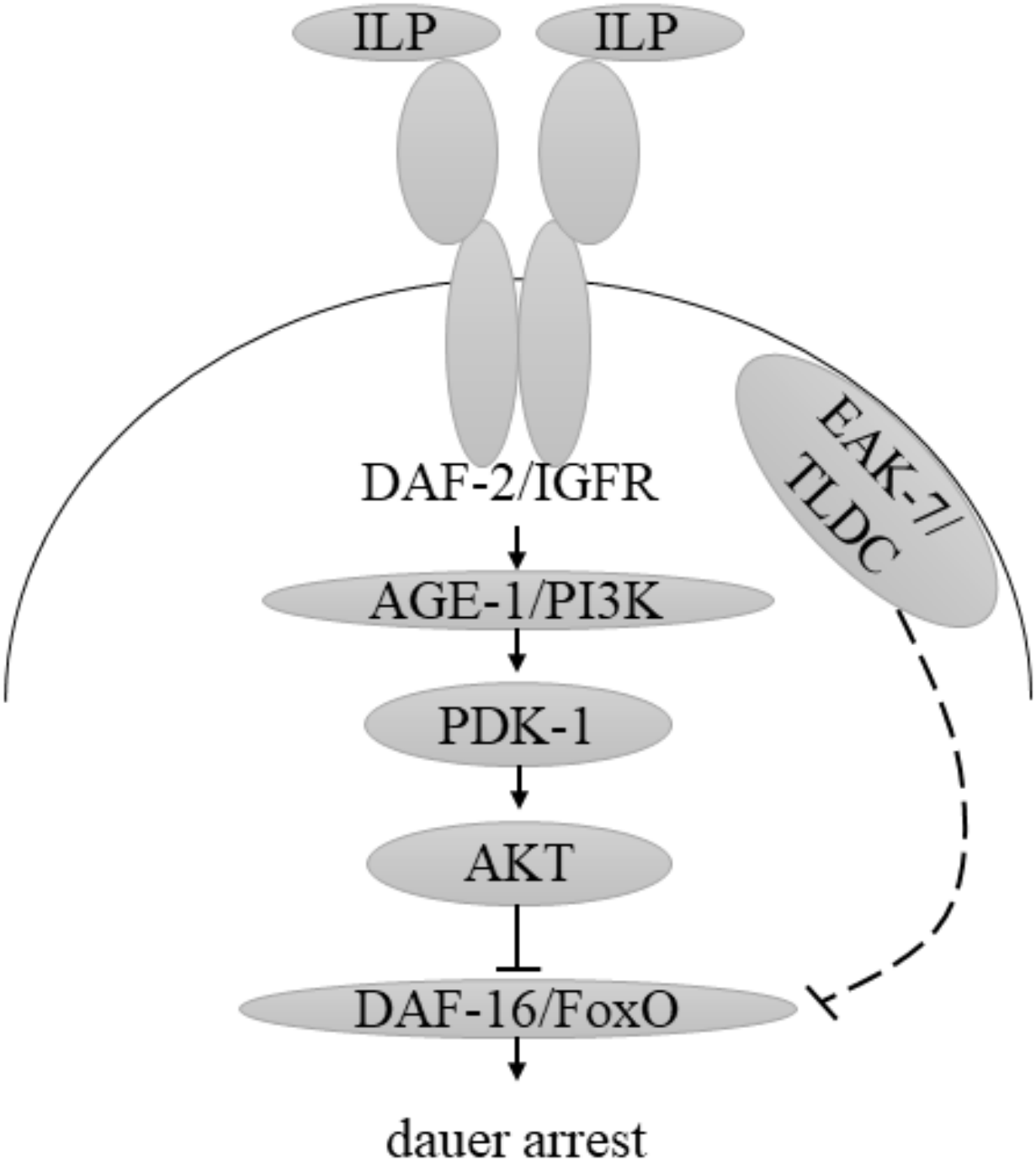
knowledge which enabled our work to identify a novel regulator that promotes longevity in chapter 2 and a previously unknown collagen mutant variant that influences ER UPR in chapter 3.

## Insulin/IGF-1 signaling (IIS) pathway is conserved in *C. elegans* and mammals

Insulin/IGF-1 signaling (IIS) pathway is a central signaling transduction pathway that mediates information about nutrient levels to regulate multiple aspects of a living organism including metabolism, growth, development, longevity and behavior. Previous work from different groups indicated that the major component of the IIS pathway is widely conserved in metazoans (González et al. 2009) and the entire IIS pathway is conserved in *C. elegans* (Engelman et al. 2006). Insulin-like peptides bind to DAF-2/IGFR receptor tyrosine kinase which leads to the autophosphorylation of its intracellular kinase domain. Activation of the DAF-2/IGFR then recruits the insulin receptor substrate and facilitate the activation of AGE-1/PI3Kinase, which catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)=triphosphate (PIP3) (Vanhaesebroeck and Alessi 2000). Activated AGE-1/PI3K then facilitates the phosphorylation and activation of AKT/PKB serine threonine kinase via phosphoinositide-dependent kinase PDK-1 (Paradis et al. 1999). Subsequently, AKT phosphorylates DAF-16/FoxO transcription factors at multiple conserved consensus sites. When FoxO is phosphorylated, 14-3-3 protein then binds to FoxO and promotes the sequestration of FoxO in the cytoplasm which in turn inhibits the downstream gene expression controlled by DAF-16/FoxO transcription factor (Brunet et al. 1999, Cahill et al.

2001, Brunet et al. 2002, Obsilova et al. 2005). Thus, under normal circumstances, IIS suppresses the activity of DAF-16/FoxO transcription factor (Figure 1.1).

**Figure 1.1 Schematic of IIS pathway.**
Adopted from (Baugh and Hu 2020).



## DAF-16/FoxO transcription factor promotes longevity

IIS pathway is the first signal transduction pathway shown to regulate life span. Single genetic mutation in AGE-1/PI3K was originally identified from a forward genetic screen looking for extended adult life span in mutant *C. elegans* strains (Klass 1983, Friedman and Johnson 1988a, Friedman and Johnson 1988b, Johnson 1990, Morris et al. 1996). Further studies determined loss-of-function mutation in DAF-2/IGFR can extend life span and such extension requires the IIS downstream gene *daf-16* (Kenyon et al. 1993, Dorman et al. 1995, Kimura et al. 1997). In addition, loss-of-function mutations in other IIS components *pdk-1*, *akt-1*, and *akt-2* are found to increase life span in a DAF-16/FoxO-dependent manner (Paradis and Ruvkun 1998, Paradis et al. 1999, Alam et al. 2010).

FoxO transcription factors are members of the Forkhead superfamily with conserved winged-helix DNA-binding domain (Kaestner et al. 2000). Mammals have four FoxO proteins: FoxO1, FoxO3, FoxO4, and FoxO6 (Kaestner et al. 2000, Hannenhalli and Kaestner 2009) whereas invertebrate model organisms *Drosophila melanogaster* and *C. elegans* express FoxO orthologs from a single genomic locus. In *C. elegans*, three groups of *daf-16* transcripts (a, b, and d/f/h) are transcribed from distinct promoters (Lin et al. 1997, Ogg et al. 1997, Kwon et al. 2010) and the encoded proteins contain phosphorylation sites conserved in human (Lin et al. 1997, Ogg et al. 1997). The same promoter region modulates the transcription of *daf-16d*, *f*, and *h* but they have distinct 5' regions and translational start sites (Kwon et al. 2010). For ease of reading, the group of d/f/h transcripts are henceforth referred to as *daf-16f* collectively. DAF-16A and DAF-16F have been experimentally verified as the major isoform variants necessary for the life span extension effect caused by reduction in IIS (Kwon et al. 2010, Chen et al. 2015). In long-lived *daf-2/IGFR* mutant background, mutations that reduce *daf-16a* and *f* but not *daf-16b* activity

shortens life span to the same extent as *daf-16* null mutations (Lee et al. 2001), indicating *daf-16b* activity is not sufficient to substitute the role of *daf-16a* and *f* in regulating longevity. Furthermore, overexpression of DAF-16B under its endogenous promoter fails to rescue the shortened life span phenotype of *daf-16(null);daf-2* double mutant (Kwon et al. 2010). Together these data implicate the importance of DAF-16A and F isoforms as downstream effectors to regulate longevity while DAF-16B isoform does not play a significant role in life span control.

In addition to IIS, ablation of germline stem cells (Hsin and Kenyon 1999) and dietary restriction (Greer et al. 2007) also extend life span in a DAF-16/FoxO-dependent manner, suggesting conserved mechanisms that influence aging converge on FoxO transcription factors. The underlying mechanism of life span extension caused by reduced IIS and increased DAF-16/FoxO activity is conserved across multiple species. Mutation in the *Drosophila* insulin receptor substrate *chico* extends life span and this extension depends on the sole *Drosophila* FoxO family member *dFoxO* (Clancy et al. 2001, Yamamoto and Tatar 2011); overexpression of *dFoxO* in the *Drosophila* fat body is sufficient to promote longevity in fruit flies (Hwangbo et al. 2004). IIS can also regulate life span in mammals where adipose tissue-specific knockout of the insulin receptor in mice increases life span and improves resistance to obesity (Bluher et al. 2003) and heterozygous IGF-1 receptor mutant mice live longer than wild-type controls (Holzenberger et al. 2003). Mammalian FoxO transcription factors are associated with many age-related diseases including insulin resistance, osteoporosis, and cancer (Greer and Brunet 2005, Paik et al. 2007, Hesp et al. 2015). Gene association studies of human centenarian also implicate the critical role of FoxO in regulating longevity. Single nucleotide variants in the human IGF-1 receptor and certain FoxO1A and FoxO3A polymorphisms are enriched in aged population with improved health assessment and delayed morbidity, further strengthening the key function of IIS

and FoxO activity in human longevity (Suh et al. 2008, Willcox et al. 2008, Flachsbart et al. 2009, Li et al. 2009, Joshi et al. 2017).

## Dauer arrest as a readout for DAF-16/FoxO activity

IIS has a major function of nutrient sensing and acts as a central signaling pathway to mediate environmental cues to regulate development as well as metabolism. Under optimal conditions at 15-20°C with ample food supply and low population density, *C. elegans* develops from embryos and reaches adulthood in two to three days though four distinct larval stages (L1-L4). Under adverse environmental conditions, such as high ambient temperature, lack of food, and high population density, *C. elegans* can enter an alternative developmental L2 larval phase known as L2d and arrest in a developmental diapause called dauer if the adverse environmental conditions do not improve (Cassada and Russell 1975, Golden and Riddle 1984, Hu 2007). Dauer larvae are morphologically distinct with constricted body shape compared to animals undergoing reproductive development, which can be easily identified under a dissecting microscope. In addition, they have thickened cuticle to survive harsh environments (Blaxter 1993) and their oral orifices are closed by an internal plug (Riddle et al. 1981), and their pharynxes are constricted (Vowels and Thomas 1992) and do not pump (Cassada and Russell 1975). Dauer larvae do not undergo metabolic shift from using lipid in the glyoxylate cycle to aerobic respiration that occur in L2 stage (Wadsworth and Riddle 1989) and dauers are resistant to endogenous oxidative stress (Honda et al. 2008). Dauer diapause can persist for months, which is much longer than typical adult life span (Klass and Hirsh 1976). However, once the environmental condition becomes replete again, dauer larvae can resume reproductive development at L4 and have a normal adult life span.

Dauer arrest phenotype can be used as a proxy for DAF-16/FoxO activity in the control of IIS. Mutations in IIS promotes dauer arrest in a temperature-dependent manner. Strong mutant alleles of *daf-2/IGFR* arrest as dauers at 25°C where wild-type animals can readily undergo reproductive development (Riddle et al. 1981, Thomas et al. 1993, Gottlieb and Ruvkun 1994). Null alleles of *akt-1*, a conserved component of IIS, develop normally at 25°C, but arrest as dauers at 27°C (Ailion and Thomas 2000). Epistasis analysis revealed that loss of *daf-2/IGFR* promotes dauer arrest in a DAF-16/FoxO-dependent manner since *daf-16(null);daf-2* double mutants do not arrest as dauers at 25°C (Riddle et al. 1981, Thomas et al. 1993, Gottlieb and Ruvkun 1994) . Similarly, *daf-16/FoxO* null mutation fully suppress the life span extension phenotype of *daf-2* and *akt-1* mutants (Kenyon et al. 1993, Paradis and Ruvkun 1998, Kwon et al. 2010). Although DAF-16/FoxO likely regulates dauer arrest and life span via distinct mechanisms and dauer arrest is not solely regulated by IIS, dauer arrest phenotype can still provide a conveniently observable readout for assessing DAF-16/FoxO activity. Furthermore, genetic manipulations enhancing or suppressing dauer phenotype can be useful tools to identify novel conserved signaling pathways that mediates DAF-16/FoxO activity to regulate metabolism and longevity.

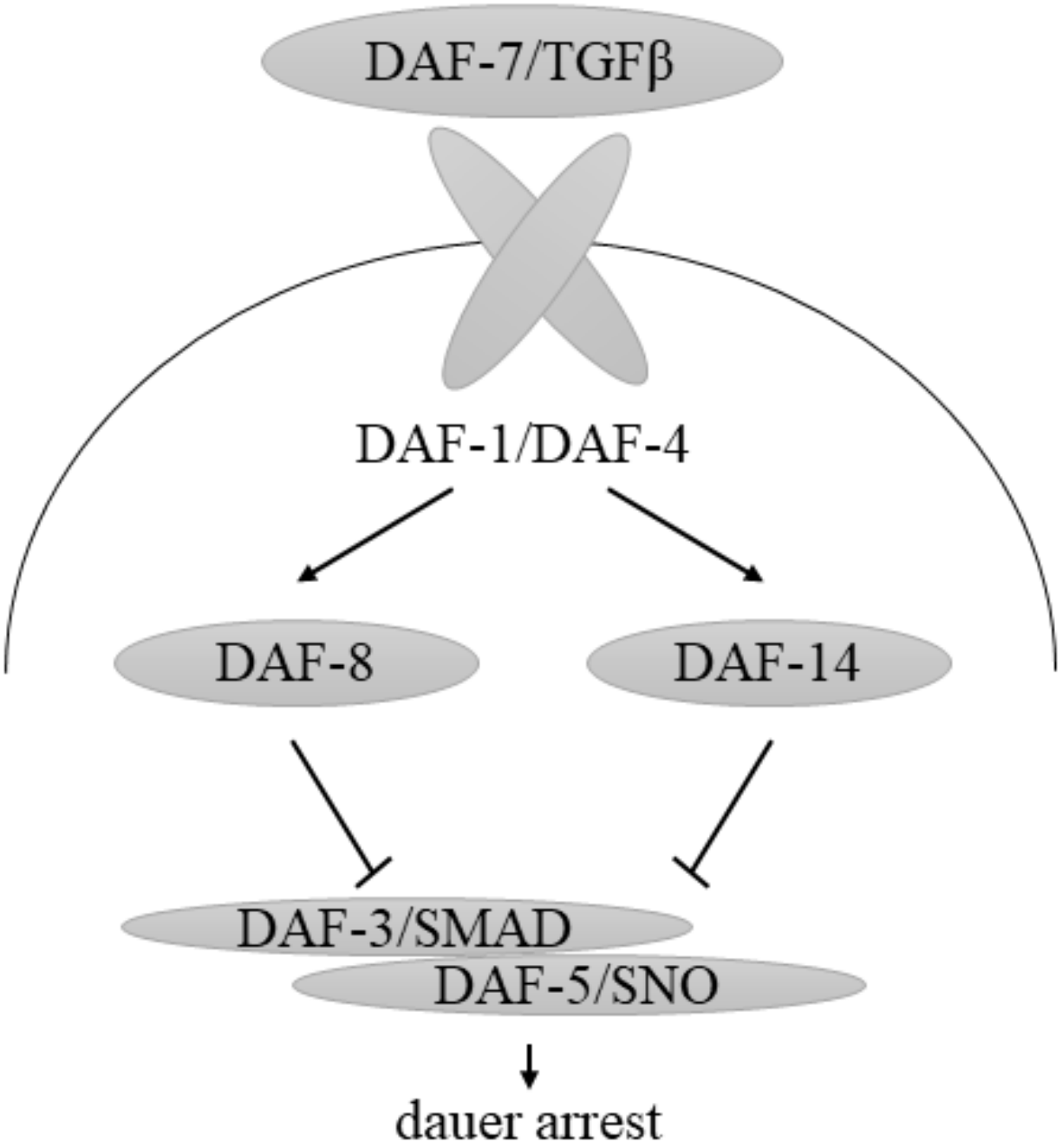
## Pathways that regulate dauer in addition to IIS

Forward genetic screens have mapped out mutations in multiple neuroendocrine signaling pathways that mediate environmental cues to influence dauer formation (Riddle et al. 1981, Vowels and Thomas 1992, Thomas et al. 1993, Gottlieb and Ruvkun 1994, Gerisch et al. 2001, Jia et al. 2002). Two major classes of abnormal dauer formation mutants (daf) have been identified and characterized: dauer-constitutive mutants, or daf-c has high penetrance of dauer formation under favorable conditions, whereas dauer-defective mutants, or daf-d can bypass

dauer arrest when animals would typically arrest under dauer-inducing conditions. The evolutionarily conserved TGFβ-like pathway and steroid hormone pathway also influence dauer formation in addition to IIS.

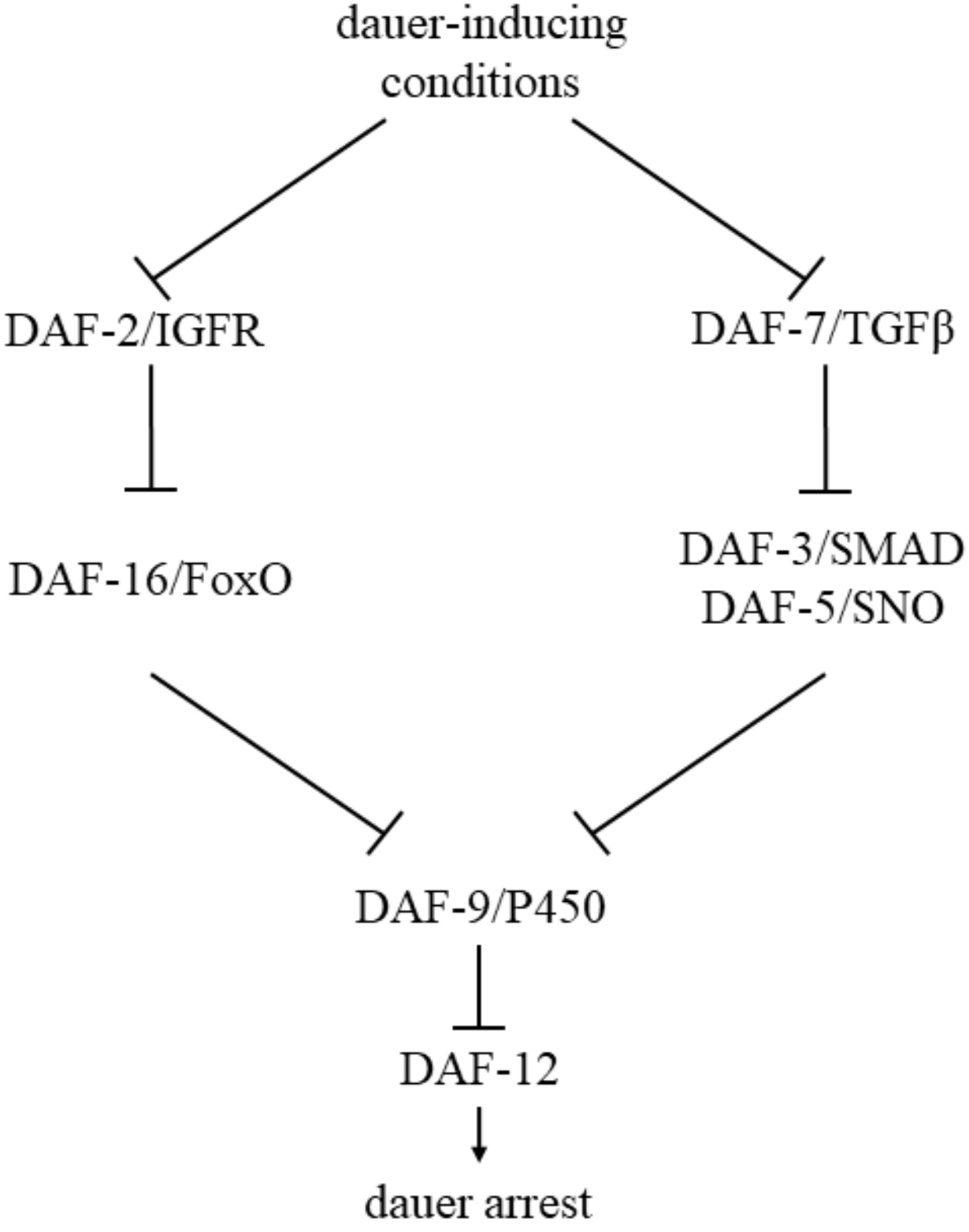
The TGFβ-like signaling pathway is defined by the daf-c genes *daf-1*, *4*, *7*, *8*, and *14* and the daf-d genes *daf-3* and *daf-5* (Patterson and Padgett 2000). DAF-7/TGFβ ligand binds to DAF-1 and DAF-4 heteromeric cell surface TGFβ receptors with serine/threonine kinase activity (Georgi et al. 1990, Estevez et al. 1993, Ren et al. 1996). The TGFβ receptors DAF-1 and DAF-4 then phosphorylate and activate SMAD transcription factors DAF-8 and DAF-14 but inhibit the SMAD/SNO-SKI complex of DAF-3 and DAF-5 (da Graca et al. 2004, Tewari et al. 2004, Park et al. 2010). The daf-c phenotypes of *daf-8* and *daf-14* implicate their necessity for TGFβ pathway activation by DAF-7 to inhibit dauer arrest (Inoue and Thomas 2000), which is consistent with the role of mammalian SMAD transcription factors as positive effectors of TGFβ-like signaling (Shi and Massagué 2003). In contrast, *daf-3* mutation is dauer defective and suppresses daf-c phenotypes of *daf-1*, *4*, *7*, *8*, and *14*, suggesting activation of TGFβ-like signaling pathway inhibits DAF-3 to promote reproductive development (Patterson et al. 1997) (Figure 1.2).

**Figure 1.2 Schematic of TGFβ pathway.**
Adopted from (Baugh and Hu 2020).



The conserved IIS and TGFβ-like signaling pathway converge on the steroid hormone biosynthesis pathway defined by the daf-c gene *daf-9* and daf-d gene *daf-12* (Figure 1.3) (Riddle et al. 1981, Albert and Riddle 1988, Thomas et al. 1993, Antebi et al. 1998, Antebi et al. 2000, Gerisch et al. 2001, Jia et al. 2002). DAF-12, encodes a nuclear hormone receptor homologous to vertebrate vitamin D and thyroid hormone receptors, acts as the most downstream regulator of dauer arrest (Antebi et al. 2000). DAF-9 encodes a cytochrome P450 steroid monooxygenase which participates in the bile-acid like hormone biosynthesis of DAF-12 ligands $\Delta^4$-dafachronic acid and $\Delta^7$-dafachronic acid (Gerisch et al. 2001, Jia et al. 2002, Gerisch and Antebi 2004, Mak and Ruvkun 2004, Motola et al. 2006). While null mutation of *daf-12* inhibits dauer arrest, *daf-12* ligand binding domain mutants have daf-c phenotype, suggesting that unliganded DAF-12 can promote dauer arrest (Antebi et al. 2000). *daf-9* null animals arrest as dauer constitutively and their dauer phenotype can be suppressed by *daf-12* mutation or by supplementation of exogenous dafachronic acids, suggesting that DAF-9 inhibits the dauer promoting activity of DAF-12 by facilitating the biosynthesis of dafachronic acids (Gerisch et al. 2001, Motola et al. 2006, Sharma et al. 2009, Mahanti et al. 2014).

**Figure 1.3 Multiple genetic pathways regulate dauer arrest.**
Conserved signal transduction pathways acting in parallel converge on steroid hormone biosynthesis pathway to promote dauer arrest



## EAK-7 acts parallel to AKT-1 to enhance dauer

The complex regulation of dauer formation by insulin/IGF-1 signaling has been studied extensively, however, multiple lines of evidence suggest additional regulatory inputs may act in parallel to DAF-2/IGFR to influence DAF-16/FoxO activity. AKT phosphorylates FoxO transcription factors on conserved motifs and leads to cytoplasm sequestration of FoxO. Mutation of all four canonical AKT phosphorylation sites causes constitutive DAF-16/FoxO nuclear localization; however, nuclear DAF-16/FoxO is not sufficient to induce dauer arrest nor life span extension (Lin et al. 2001). Furthermore, knockdown of the 14-3-3 protein promotes nuclear localization of DAF-16/FoxO, but does not promote dauer arrest (Li et al. 2007).

To identify the novel regulators of nuclear DAF-16/FoxO that act in parallel to IIS, we performed a forward genetic screen for enhancers of *akt-1* dauer arrest (*eak* screen) (Hu et al. 2006). *akt-1* mutants arrest as dauers at 27°C but undergo reproductive development at 25°C. *eak* screen mutagenized *akt-1* animals and looked for progenies that acquired additional mutations to enhance spontaneous dauer formation in the *akt-1* null background at 25°C. *eak* mutants in wild-type background can develop reproductively at 25°C but exhibit daf-c phenotypes at 27°C, and no combination of *eak* double mutants increases the penetrance of dauer formation of single *eak* mutants, suggesting the *eak* genes act in a single genetic pathway that is independent of IIS (Hu et al. 2006, Zhang et al. 2008, Alam et al. 2010, Dumas et al. 2010). The dauer formation caused by *eak* mutants requires DAF-16/FoxO since *daf-16* null mutation can suppress the daf-c phenotypes of all *eak* mutations and *eak* mutants upregulate the expression of canonical *daf-16* target genes, suggesting EAK pathway normally inhibits DAF-16/FoxO activity (Hu et al. 2006, Zhang et al. 2008, Alam et al. 2010, Dumas et al. 2010).

Six (*eak-2* through *7*) of the seven *eak* mutants have been characterized and all of them are expressed in the endocrine XXX cells, where DAF-16/FoxO is not expressed (Hu et al. 2006, Patel et al. 2008, Zhang et al. 2008, Alam et al. 2010, Dumas et al. 2010, Williams et al. 2010). *eak-2* encodes a hydroxysteroid dehydrogenase known as HSD-1 (Patel et al. 2008, Dumas et al. 2010). *hsd-1* does not affect DAF-16/FoxO localization but regulates DAF-16 target gene expression in a nuclear hormone receptor DAF-12-dependent manner (Dumas et al. 2010). Taken together, these data indicate that *eak* pathway may act cell-non-autonomously to inhibit DAF-16/FoxO via endocrine signaling.

EAK-7 encodes a conserved protein that contains a N-terminal myristoylation domain which promotes binding to the cytoplasm membrane and a C-terminal TBC- and LysM-domain-containing (TLDc) domain with unclear functions but is present in all eukaryotes. EAK-7 is expressed in neurons and intestine in addition to XXX cells (Alam et al. 2010). While DAF-16 is not expressed in the XXX cells, EAK-7 is expressed in multiple tissues that co-express DAF-16/FoxO, suggesting EAK-7 may be the most downstream component of the EAK pathway (Alam et al. 2010). Overexpression of EAK-7::GFP translational fusion protein demonstrated that EAK-7 localizes to the plasma membrane (Alam et al. 2010), suggesting EAK-7 may indirectly regulate nuclear DAF-16/FoxO activity via unknown downstream intermediates (Figure 1.1).

## SEAK screen identified novel regulators of dauer

To further understand the possible links between the EAK-7 bound to the cytoplasm membrane and the DAF-16 localized in the nucleus, we conducted a forward genetic screen for mutations that suppress the daf-c phenotype of *eak-7;akt-1* double mutant at 25°C (*seak* screen) (Dumas et al. 2013). We expected to identify *seak* genes that may act downstream of the *eak*

pathway to control nuclear DAF-16/FoxO activity, in the canonical IIS pathway to modulate DAF-16/FoxO subcellular localization, or downstream of or in parallel to DAF-16. The *seak* screen has identified dosage compensation pathway members *dpy-21* and *set-4* whose functions have not been studied in dauer arrest regulation (Yonker and Meyer 2003, Wells et al. 2012). Two X-linked IIS pathway components *akt-2* and *ins-9* have been shown to be required for the suppression of dauer arrest caused by mutations in *dpy-21* and *set-4*, respectively (Dumas et al. 2013, Delaney et al. 2017). Two other *seak* strains have been identified with causal mutations of known dauer arrest regulators, *akt-2* and *daf-12*, which are both on the X chromosomes. A complex genomic rearrangement that increases the gene dosage of *akt-2* and a *daf-12* loss-of-function mutant with aberrant splicing have been shown to suppress the dauer arrest phenotype of *eak-7;akt-1* (Itani et al. 2016a, Itani et al. 2016b). More recently, we have identified *trap-1* (translocon-associated protein-1) as a *seak* gene which likely acts upstream of IIS pathway and mediates the biosynthesis of insulin-like peptide ligands for DAF-2/IGFR to regulate dauer formation (Li et al. 2019).

## Conclusions and future directions

The study of dauer formation in *C. elegans* was originally set to study the developmental plasticity in response to environmental stimuli. Genetic analyses have unveiled the cellular and molecular underpinnings that regulate dauer arrest and significant progress have been made in defining many conserved components of the intricate signaling transduction pathways governing dauer decision. However, the molecular identity and perception of the environmental signals such as changes in ambient temperature and scarcity in food supply remain obscure. Multiple signaling cascades work together to regulate dauer arrest, and epistasis analysis has revealed that IIS and TGFβ-like pathways converge on the nuclear hormone receptor DAF-12 to control dauer

arrest. Dauer biology offers a relatively easy proxy to study the integrations of these signaling pathways on an organismal level which is not found in other model organisms. Further understanding of the interactions amongst the IIS, TGFβ-like pathway, and the steroid hormone pathway has the potential to improve our knowledge about the interactions of similar signaling pathways in the pathogenesis of human diseases such as cancer and diabetes.

FoxO transcription factor has an unequivocal role in regulating longevity and many aging-related diseases. Substantial work has established IIS as a major regulatory input for controlling DAF-16/FoxO subcellular localization. Dauer phenotype has been a useful genetic tool facilitating the identification of the novel EAK pathway that regulates nuclear DAF-16/FoxO activity including. Continued investigation of the SEAK screen promises to reveal new insights into the intermediate mechanism that transduces signals from cytoplasm membrane to the nucleus. Understanding how EAK-7 inhibits DAF-16/FoxO could lead to a new avenue for therapeutics development that promotes healthy aging and decrease chronic illness in the population.

In chapter 2, we conducted a transcriptome profiling analysis of DAF-16/FoxO downstream targets to identify genes that are critical for life span regulation and genetically characterized one of the DAF-16/FoxO targets. To further study the role of SEAK mutant *trap-1*, we performed a forward genetic screen and uncovered novel collagen variants that modulate ER unfolded response in chapter 3.

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# Chapter 2 ACS-3 Promotes Longevity in *C. elegans*

## Abstract

FoxO transcription factors promote longevity in multiple model organisms. Polymorphisms in human *FoxO* genes are associated with centenarians and FoxO transcription factors may modulate several aging-related diseases. How does FoxO transcription factor regulate its downstream transcriptional programs to influence life span remains poorly understood. In *C. elegans*, A- and F-isoforms of DAF-16/FoxO transcription factor extend life span in the context of reduced insulin-like signaling. To further investigate the mechanisms underlying DAF-16/FoxO-dependent longevity, we used whole transcriptome profiling to define the downstream target genes of isoform-specific *daf-16/FoxO* mutants. We identified *acs-3* as an induced target gene regulated redundantly by both DAF-16A and F isoforms in *daf-2/IGFR* mutant background. *acs-3* is necessary but not sufficient for full life span extension in the context of reduced insulin-like signaling. *acs-3* encodes a conserved acyl-coA synthase but the *in vivo* function has yet to be determined. A functional ACS-3::GFP fusion protein is expressed specifically in glial cells, seam cells, and vulva. Lipid metabolism has been shown to play an important role in aging and fatty acid profile with high mono-unsaturated fatty acid (MUFA) composition is associated with long life. ACS-3 may act on certain MUFA species to influence life span. We propose using metabolomic approach for future studies to identify the fatty acid substrates of ACS-3, which may provide mechanistic insights for the pro-longevity function of

ACS-3 in *C. elegans* and offer new avenues for therapeutic development to treat common diseases in elder population.

# Introduction

DAF-16/FoxO transcription factor is the major regulator for life span extension caused by reduced insulin/IGF-1 signaling (Kenyon et al. 1993, Paradis and Ruvkun 1998). FoxO transcription factors have a conserved role in regulating life span across taxa and influence the pathogenesis of many aging-associated diseases in human (Lin et al. 1997, Clancy et al. 2001, Bluher et al. 2003, Bartke 2011, Yamamoto and Tatar 2011, Shimokawa et al. 2015, Joshi et al. 2017). Elucidating the mechanism how DAF-16/FoxO promotes life span extension can provide insights in to the aging process and offer new approaches to treat and prevent diseases that have high frequencies of affecting the elder population.

Given the general function of DAF-16/FoxO as a transcription factor, many research groups have invested substantial effort and resources to investigate the expression profile of DAF-16/FoxO and collectively identified thousands of DAF-16/FoxO-regulated downstream target genes (McElwee et al. 2003, Murphy et al. 2003, McElwee et al. 2004, Murphy 2006, Chen et al. 2015). Murphy and colleagues (2003) assayed about 60 DAF-16/FoxO target genes for their functions in DAF-16/FoxO-dependent life span extension using RNAi-based knockdown method. Their results suggest that most single gene RNAi knockdowns have relatively small effects on longevity, leading to the conclusion that DAF-16/FoxO promotes longevity through the cumulative regulation of multiple effector genes. However, the vast number of DAF-16/FoxO targets poses a challenge for detailed functional analysis of each individual target in a systematic manner. Several groups have developed various filtering approaches to prioritize a more tractable subset of DAF-16/FoxO target genes that would allow

more careful interrogation of specific genes and study their roles in extending life span but the mechanistic basis for longevity regulation by DAF-16/FoxO remains poorly understood (Lee et al. 2003, Oh et al. 2006, Tepper et al. 2013, Tullet et al. 2014, Chen et al. 2015, Kaletsky et al. 2016).

The *daf-16*/FoxO locus encodes multiple transcripts that are transcribed from distinct promoters in *C. elegans* (Lin et al. 1997, Ogg et al. 1997, Kwon et al. 2010). Two transcripts encode polypeptides predicted to contain only half of the Forkhead DNA binding domain and they probably do not bind DNA to regulate gene expression. One transcript contains a unique N-terminal fragment that includes partial Forkhead DNA binding domain and does not play a major role in life span control (Ogg et al. 1997, Kwon et al. 2010). Two major DAF-16 isoforms, DAF-16A and DAF-16F, extend life span in animals with reduced DAF-2/IGFR signaling (Kwon et al. 2010, Chen et al. 2015). Our previous study has generated loss-of-function DAF-16 isoform-specific mutant alleles to evaluate the effect of individual DAF-16 isoforms on life span extension caused by reduction in IIS (Chen et al. 2015). We found that when both DAF-16A and F isoforms are mutated, *daf-16a/f;daf-2* double mutant has similar longevity phenotype as *daf-16(null);daf-2* mutant in suppressing life span extension caused by *daf-2*/*IGFR* mutant (Chen et al. 2015). This suggests both DAF-16A and DAF-16F are required for the longevity of IIS mutant. In contrast, *daf-16f;daf-2* double mutant has similar life span as a *daf-2* single mutant, indicating that DAF-16A isoform plays a more important role than DAF-16F isoform in life span control (Chen et al. 2015). When we examined the life span shortening effect of *daf-16a* isoform-specific mutant in *daf-2* mutant background, loss of *daf-16a* activity partially reduces the long-lived *daf-2* life span but to a much lesser degree than the reduction observed in *daf-*

*16a/f;daf-2* and *daf-16(null);daf-2* double mutants (Chen et al. 2015). This suggests DAF-16A isoform by itself is not sufficient to fully control life span.

Inactivation of either *daf-16a* or *daf-16f* alone by RNAi modestly shortens the life span in *daf-2/IGFR* mutant background compared to pan-*daf-16*-RNAi that targets all *daf-16/FoxO* isoforms (Kwon et al. 2010). However, when we treated RNAi targeting DAF-16F isoform on *daf-16a;daf-2* double mutant to knockdown DAF-16F activity, we saw significant shortening of life span to a similar extent as treating *daf-16a;daf-2* double mutant with pan-daf-16 RNAi (Chen et al. 2015). Taken together, we think DAF-16/FoxO target genes that are primarily regulated by DAF-16A isoform as well as target genes redundantly regulated by both DAF-16A and DAF-16F isoforms would exert the most influence on life span regulation.

Based on our observations for the differential life span effects caused by DAF-16A and DAF-16F isoforms, we performed whole transcriptome profiling to identify target genes that are regulated by DAF-16A and DAF-16F isoforms (Chen et al. 2015). We first compared the transcriptome of long-lived *daf-2* mutant to wild-type and identify genes that are differentially regulated (Chen et al. 2015). Then we took those genes and ask which ones are regulated in the opposite direction in *daf-16(null);daf-2* and *daf-16a/f;daf-2* double mutants (Chen et al. 2015). This approach defined a set of 399 DAF-16A/F targets, genes that could be regulated by DAF-16A or DAF-16F or both isoforms (Chen et al. 2015). We wanted to determine the relative regulation received from DAF-16A and DAF-16F isoforms for each target genes; therefore, we calculated and empirically assigned a value to reflect the expression level mediated by *daf-16a* mutation and *daf-16f* mutation for each of the 399 target genes (Chen et al. 2015). This filtering strategy delineated amenable subsets of 57 DAF-16A isoform-specific target genes, 8 DAF-16F isoform-specific target genes, and 35 target genes that must be redundantly regulated by DAF-

16A and DAF-16F isoforms (Chen et al. 2015). The remainder 299 shared target genes of DAF-16A and DAF-16F receive varying regulation from both isoforms (Chen et al. 2015). DAF-16A-specific genes likely contribute to the life span difference between *daf-16a;daf-2* and *daf-2* mutants, whereas redundantly regulated genes might be expected to account for the difference in the life spans of *daf-16a/f;daf-2* and *daf-16a;daf-2* double mutants. We identified *acs-3* as one of the redundant target gene that is upregulated by both DAF-16A and DAF-16F isoforms.

# Results

## Acyl-CoA synthase ACS-3 is required for DAF-2 life span extension

Reduction in IIS activates DAF-16/FoxO and extends longevity (Kenyon 2010). We anticipated a target gene upregulated by both DAF-16A and DAF-16F isoforms may contribute significantly to the life span extension in a *daf-2* mutant background. Since *acs-3* expression is increased by both major DAF-16/FoxO isoforms involved in regulating longevity, we hypothesized that loss-of-function mutation in *acs-3* will shorten the extended life span of *daf-2/IGFR* mutant if *acs-3* is necessary for DAF-16/FoxO-dependent life span control as suggested by our filtering strategy. To test this hypothesis, we obtained a publicly available *C. elegans* strain harboring a nonsense *acs-3(gk826522)* mutation in the predicted AMP-binding domain, which causes a premature stop codon and leads to a truncated protein product (Figure 2.1). *daf-2(e1370); acs-3(gk826522)* significantly shortens life span when compared to *daf-2(e1370)* single mutant but *acs-3(gk826522)* does not affect life span in wild-type background (Figure 2.2). This suggests *acs-3(gk826522)* mutation does not shorten life span due to general sickness. The median life span observed in *daf-2(e1370); acs-3(gk826522)* was 25 days compared to *daf-2(e1370)* mutant animals at 37 days.

**Figure 2.1 Schematic of *C. elegans acs-3*.**
(A) *acs-3* genomic locus. *acs-3(gk826522)* is a nonsense allele with a premature stop codon in the AMP-binding domain (B) Domain structure of ACS-3 protein. Domain is based on a sequence search of the Pfam database.

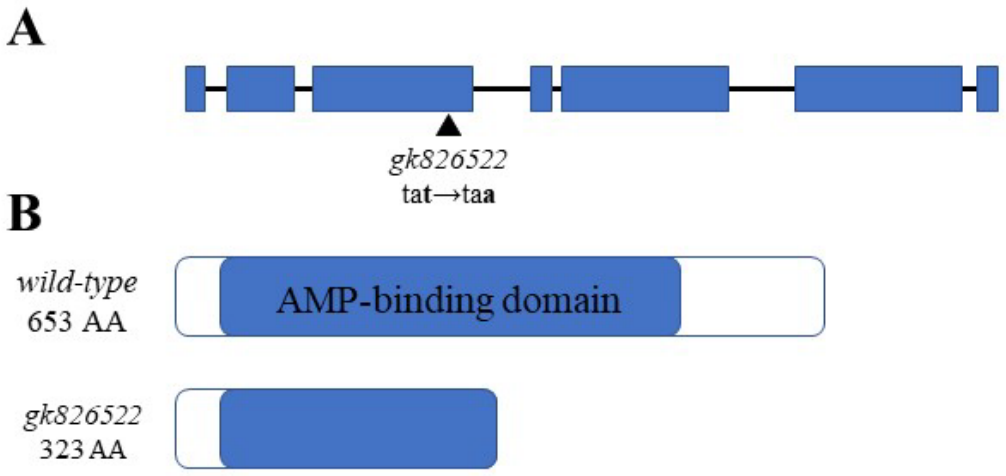
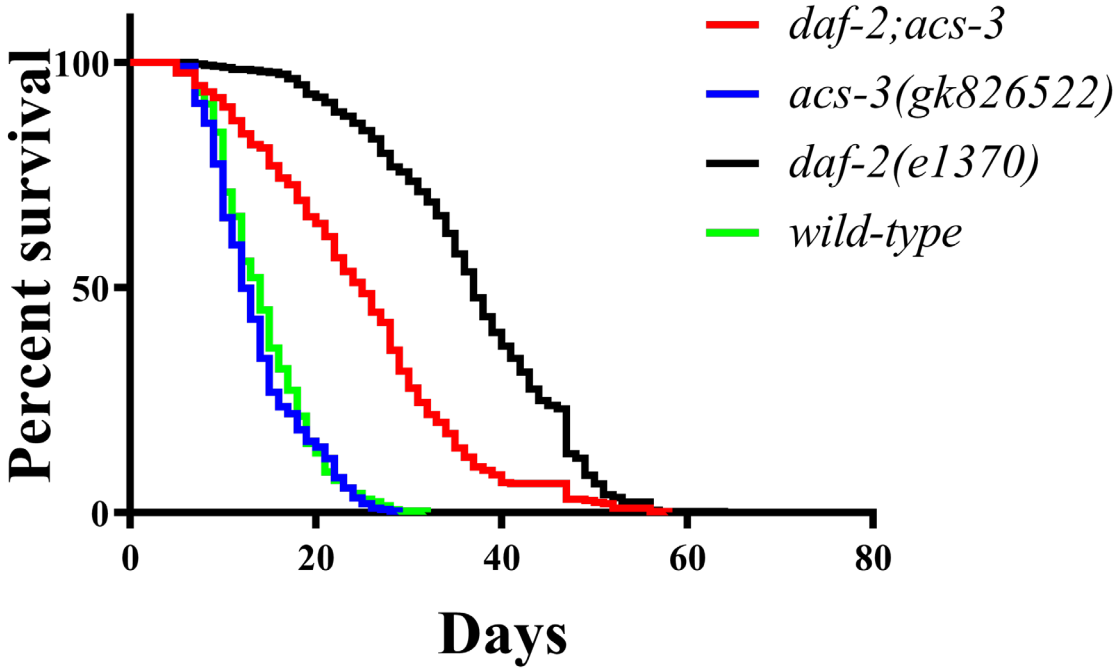



**Figure 2.2 *acs-3* activity is required for life span extension in *daf-2/IGFR* mutant.**
*acs-3(gk826522)* suppresses life span extension phenotype of *daf-2(e1370)*. Aggregated data from three biological replicates. Curves of *daf-2(e1370);acs-3(gk826522)* and *daf-2(e1370)* are significantly different as calculated by the log-rank test; $P < 0.0001$.



## ACS-3::GFP is expressed in glial cells, seam cells, and vulva

To determine the spatiotemporal expression pattern of ACS-3, we generated transgenic animals expressing an ACS-3::GFP fusion protein under the control of the endogenous *acs-3* promoter and *unc-54* 3'UTR. We observed ACS-3::GFP expression in vulva (Figure 2.3A), seam cells (Figure 2.3A and B), and in the nervous system (Figure 2.3C). Coexpression of ACS-3::GFP with the amphid sheath red fluorescence protein (RFP) revealed that ACS-3 is expressed in the amphid sheath glial cells (Figure 2.3D). ACS-3 protein is not predicted to have any transmembrane domain based on its amino acid sequence; therefore, it is likely localized to the inner leaflet of the cell membrane of seam cells (Figure 2.3B) and amphid socket cells (Figure 2.3D).

## ACS-3 is necessary for *daf-2* life span extension but not sufficient to extend life span

Overexpression of the integrated *dpIS29[ACS-3::GFP]* transgene rescued life span extension in *daf-2(e1370);acs-3(gk826522)* double mutant, suggesting that ACS-3::GFP recapitulates endogenous ACS-3 expression and functions in the tissues where it is required for life span extension. However, overexpression of *dpIS29* had no effect on wild-type life span, indicating that ACS-3 is necessary but not sufficient to extend longevity (Figure 2.4)

**Figure 2.3 Tissue-specific expression pattern of a functional ACS-3::GFP transgene.**
(A) Confocal images showing ACS-3::GFP expression in vulva pointed by an arrowhead and seam cells pointed by arrows. (B) Zoom-in view of the dashed rectangle in (A) has characteristic oval shape and one longitudinal syncytium of seam cells. (C) ACS-3::GFP expression is present in the head region where asterisk denotes glial cells and arrowheads point to neurons. (D) ACS-3::GFP colocalizes with the amphid sheath reporter F16F6.9::dsRed.

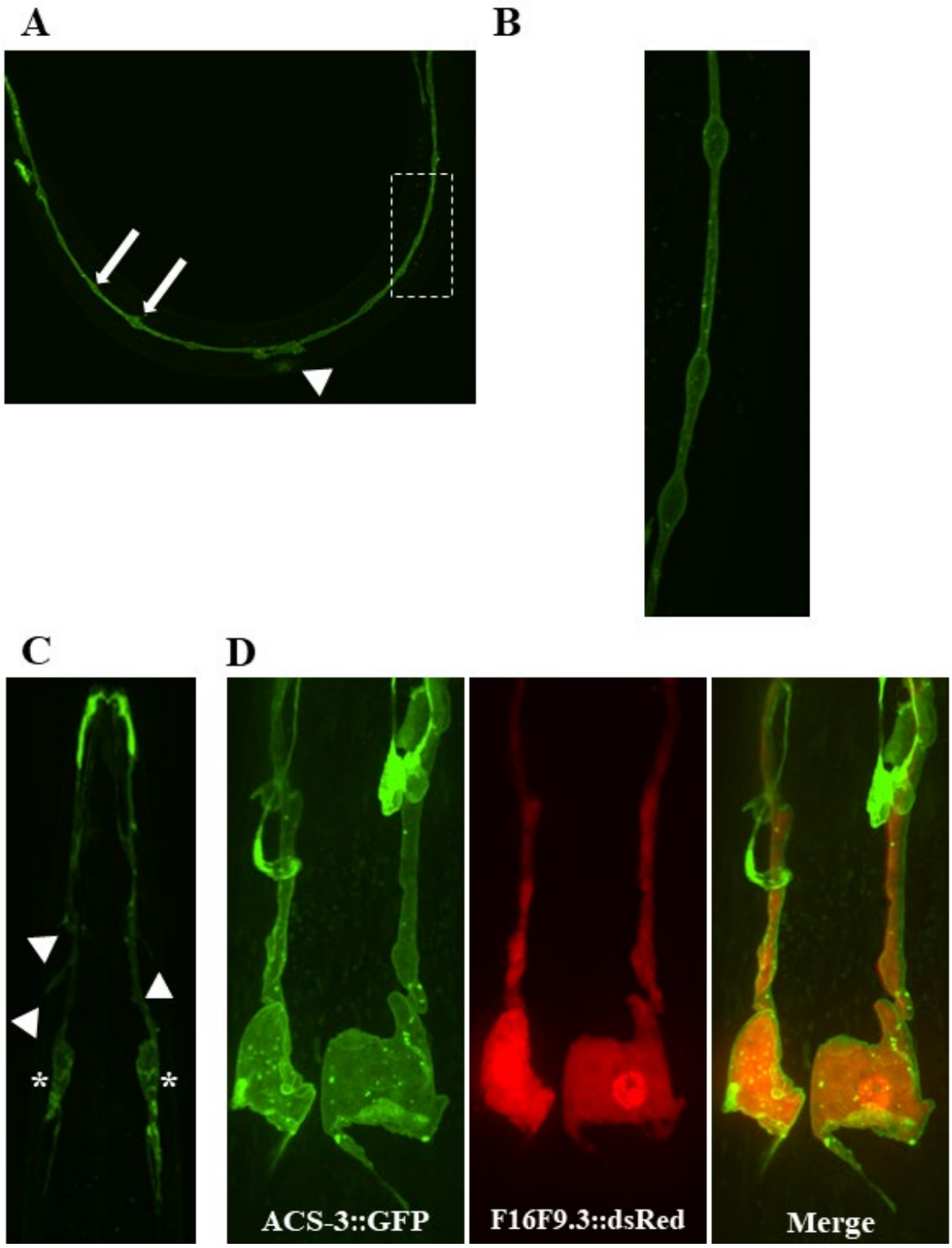
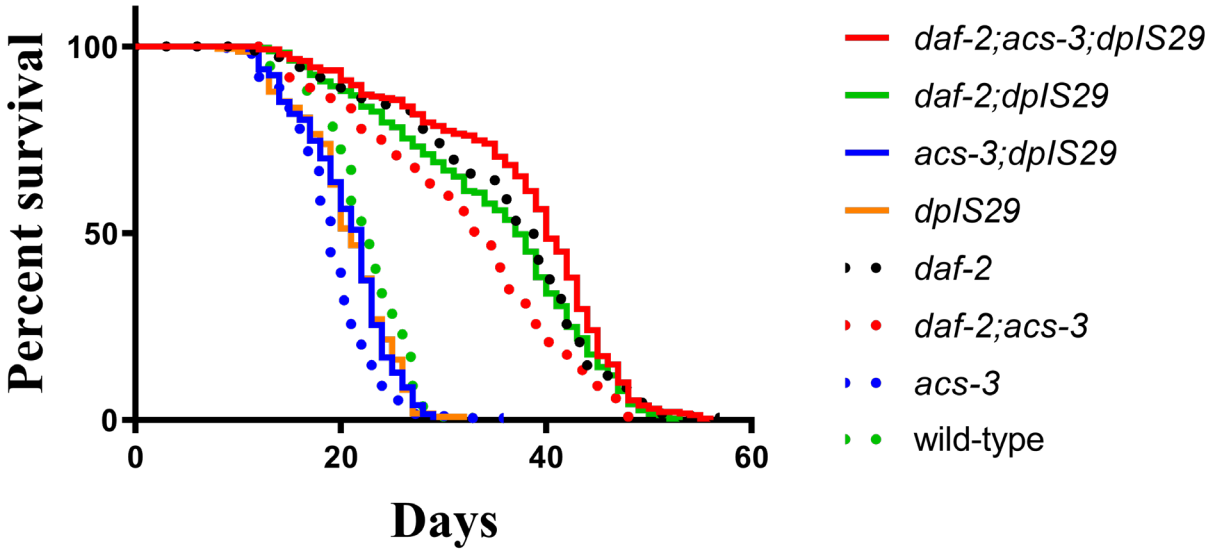


**Figure 2.4 ACS-3::GFP rescues the life span phenotype of *daf-2;acs-3* double mutants.** Overexpression of the integrated *dpIS29[ACS-3::GFP]* transgene in *daf-2(e1370);acs-3(gk826522)* double mutant background rescues life span phenotype but does not influence life span in *daf-2(e1370)* mutant or wild-type background. Results are representative of two experiments. The median life span of *daf-2;acs-3;dpIS29* (red curve) was 40 days and the median life span of *daf-2;acs-3* (red dotted curve) was 33 days; two curves are significantly different as calculated by the log-rank test. $P < 0.0001$.



# Discussion

Aging has been considered as a stochastic process where accumulation of damaged DNA and proteins causes defects in cellular functions and in turn leads to the increased risk of pathologies such as tumor, insulin resistance, atherosclerosis, and neurodegenerative diseases. Isolation of the first long-lived *age-1* mutant in *C. elegans* (Klass 1983) and the discovery of a single *daf-2/IGFR* mutant extends life span in a DAF-16/FoxO-dependent manner (Kenyon 2010) indicate aging is indeed a genetically programed biological process. Interventions such as caloric restriction (Kaeberlein et al. 2004, Greer et al. 2007, Colman et al. 2009, Mattison et al. 2012) and rapamycin treatment (Powers et al. 2006, Harrison et al. 2009, Bjedov et al. 2010, Miller et al. 2011, Robida-Stubbs et al. 2012) have been shown to effectively extend life span in both invertebrates and mammals, suggesting future development of therapies targeting the highly conserved longevity-governing mechanisms has the potential to relieve age-associated morbidities and delay mortality in human.

Multiple physiological regulatory networks including dietary restriction, oxidative stress, nutrient sensing, and germline ablation converge on the DAF-16/FoxO transcription factor to influence life span (Kenyon 2010).We have established a framework for identifying and characterizing genes that regulate life span downstream of DAF-16/FoxO activation. Efforts from multiple groups have tried to identify a subset of DAF-16/FoxO target genes that are critical for controlling life span. However, due to the sheer number of transcriptional changes mediated by DAF-16/FoxO in various biological processes, downstream target genes that are necessary and sufficient to delay aging remain poorly understood. Our strategy utilized the underpinning mechanism of differential life span regulation exerted from DAF-16A and DAF-16F isoforms to facilitate the analysis of the whole transcriptome profiling data and define a

subset of candidate target genes that are responsible for the DAF-16/FoxO-dependent life span effect (Chen et al. 2015). This approach enabled us to identify a previously unknown DAF-16 target gene, ACS-3, as a novel longevity regulator, and lead to a genetic characterization of *acs-3*.

ACS-3 encodes an evolutionarily conserved acyl-coA synthase and it was first identified from a genetic screen looking for altered fat storage phenotypes. Previous work found loss-of-function *acs-3(ft5)* mutant increases lipid uptake, *de novo* fat synthesis, and lipid droplet accumulation in the intestine (Mullaney et al. 2010). However, ACS-3 has not been studied in the context of life span regulation, highlighting our filtering strategy can successfully identify novel regulators that are critical for longevity. Several but not all long-lived *daf-2* mutants exhibit DAF-16/FoxO-dependent elevation of fat storage and synthesis phenotypes (Perez and Van Gilst 2008). Emerging evidence suggests the possibility that IIS may reconfigure fat metabolism to promote longevity where the transcript levels of fat elongase *elo-1*, *elo-2*, and *elo-5* as well as the fatty acid desaturase *fat*-4 are decreased in long-lived *age-1* mutant (Reis et al. 2011). Furthermore, *daf-2* mutant has been found to increase the activity of lysosomal lipase *lipl-4* and RNAi targeting *lipl-4* can reduce the extended life span of *daf-2/IGFR* mutant (Wang et al. 2008).

Prior study has generated a transcriptional *acs-3* reporter and found that *acs-3* transcripts are present in seam cells, vulva, excretory cell, and unidentified cells in the head as well as the tail regions (Mullaney et al. 2010). This data closely corresponds to the expression pattern observed from our translational ACS-3::GFP transgene reporter strain. Subsequent rescuing experiment demonstrated that expressing *acs-3* cDNA in the seam cell of *acs-3(ft5)* mutant animals is sufficient to reverse the increased fat storage phenotype back to wild-type level

(Mullaney et al. 2010). This unexpected result is rather interesting because seam cell has not been implicated in fat storage or regulation. Moreover, the absence of *acs-3* transcript and ACS-3 protein in the intestine, which is the largest adipose tissue as well as a major organ responsible for fat metabolism in *C. elegans*, suggests ACS-3 may act cell non-autonomously to influence lipid storage in the intestine. Therefore, determining the tissue requirement for ACS-3 activity is likely to elucidate the role of fat metabolism in modulating longevity. Future experiments reconstituting functional ACS-3 construct driven by individual tissue-specific promoters in amphid sheath cell, seam cell, vulva, and sensory neurons to test their ability to rescue the shortened life span of *daf-2(e1370); acs-3(gk826522)* double mutant can provide mechanistic insights for life span regulation mediated by DAF-16/FoxO.

The recombinant *C. elegans* ACS-3 protein has been shown to have similar *in vitro* activity as commercially available *Pseudomonas* acyl-coA synthetase (Mullaney et al. 2010). However, the *in vivo* function of ACS-3 remains to be determined. Fatty acid profiling of long-lived mutants revealed that mono-unsaturated fatty acid (MUFA) accumulation is positively correlated with *daf-2* mutant (Reis et al. 2011). In addition, dietary supplementation of oleic acid and palmitoleic acid, both of which are species of MUFAs, is sufficient to extend life span in wild-type animals (Han et al. 2017). ACSL5, the mammalian homolog of ACS-3, was reported to possess higher enzymatic activity metabolizing palmitoleic acid and oleic acid as its preferred substrates in comparison to poly-unsaturated fatty acids (Klett et al. 2017). Collectively, this evidence suggests a plausible hypothesis where *C. elegans* ACS-3 may play an important role in catalyzing MUFAs to influence fat metabolism and regulate longevity. To test this hypothesis, a straightforward experiment is to examine whether the life span extension caused by the supplementation of oleic acid or palmitoleic acid depends on functional ACS-3. If ACS-3

activity is required, we expect supplementing *acs-3(gk826522)* mutant with oleic acid or palmitoleic acid will not increase longevity in contrary to the life span promoting effect observed in wild-type animals.

Fatty acids are building blocks of lipids that constitute biological membranes to protect living organisms from harsh environmental challenges. Lipids are also energy sources and have important functions as signaling molecules mediating communication across different tissues to coordinate fat synthesis, storage, or metabolism that can influence organismal physiology in response to nutrient availability and energy demand (Liu et al. 2014, Sunshine and Iruela-Arispe 2017). Acyl-coA synthase is a critical enzyme that activates fatty acid for entry into pathways of degradation, complex lipid formation, esterification to proteins, and eicosanoids synthesis (Grevengoed et al. 2014). It remains unclear how ACS-3 activity promotes longevity. While ACS-3::GFP is only expressed in certain tissues, it may play a role in channeling or partitioning specific fatty acid substrates into signaling transduction pathways to regulate life span. Future studies utilizing unbiased metabolomic approach (Edison and Schroeder 2010) to identify ACS-3 substrates by comparing the lipid profiles differentially regulated in *daf-2*, *daf-2;acs-3*, and *daf-2;acs-3;dpIS29* may generate hypotheses about pro-longevity functions of ACS-3.

In summary, our study has established a novel strategy to prioritize DAF-16 isoform-specific target genes that are involved in regulating life span and characterized a previously unknown role of ACS-3 for life span extension in reduced IIS background. Future studies will establish whether ACS-3 acts on specific fatty acid substrates through potentially conserved downstream mechanisms to contribute to life span extension.

# Materials and Methods

## *C. elegans* strains and maintenance

Animals were maintained at 15°C on nematode growth media NGM seeded with *Escherichia coli* strain OP50. Compound mutants were constructed using standard genetic techniques. Genotypes were confirmed by using PCR followed by restriction digestion to detect restriction fragment length polymorphisms or using three primer PCR assays to detect deletions based on product size. *acs-3(gk826522)* strain was outcrossed from VC40812 provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Amphid sheath reporter F16F6.9::dsRed strain was a gift from Dr. Shai Shaham. Percival incubators were used for maintenance and life span assays.

## Life span assays

Life span was assayed as previously described with minor modifications (Chen et al. 2015). Animals derived from a synchronized 6-hour egg lay were grown at 15°C until the L4 larval stage to prevent any dauer formation, transferred to 20°C during day 1 of adulthood, then placed on plates seeded with 20X OP50 containing 6.25 µg/ml 5'-fluoro-2'-deoxyuridine (FUDR; Sigma-Aldrich, MO, USA) to prevent progeny production and 0.001% nystatin (Sigma-Aldrich) to avoid fungal contamination. Plates harboring any males were discarded. Animals were scored as dead if they did not respond to being touched with a platinum wire pick, and animals with ruptured vulvas were censored. Statistical significance was assessed using the standard log-rank test in GraphPad Prism.

## Transgenic strain construction

Translational ACS-3::GFP construct was generated by fusing the ACS-3 coding sequence and a 4.6 kb *acs-3* promoter to GFP followed by *unc-54* 3'UTR in pPD95.75 as a vector backbone (Andrew Fire; Addgene plasmid # 1494 ; http://n2t.net/addgene:1494 ; RRID:Addgene_1494) using Gibson assembly kit (New England Biolabs, MA, USA). Animals were injected as previously described (Mello et al. 1991) using a Leica DMI300B microscope and Eppendorf FemtoJet pump. Extrachromosomal arrays were integrated as previously described (Mello et al. 1991) using a Stratalinker 1800 (Stratagene) at 325 μJ/cm$^2$ x 100. L4 animals with the ACS-3::GFP array were irradiated, then allowed to lay eggs until food was consumed. Transgenic animals were then singled and propagated. Transgenic animals whose progeny were 100% positive for the ACS-3::GFP reporter were backcrossed six times prior to analysis.

## Confocal microscopy

Animals were immobilized with 25 mM sodium azide. Animals were mounted on slides layered with a thin 3% agarose in $H_2O$ pad and images were captured by a Photometrics Prime 95B sCMOS monochrome camera attached to a Nikon TiE inverted Spinning Disk Confocal Microscope using NIS-element software (Nikon).

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# Chapter 3 Collagen COL-75 induces ER Unfolded Protein Response Cell Non-autonomously in *C. elegans*

## Abstract

We previously identified the conserved ER transmembrane translocon-associated protein-1 (TRAP-1) as a novel regulator of DAF-2/IGFR signaling and demonstrated TRAPα, the mammalian ortholog of TRAP-1, is required for preproinsulin translocation, proinsulin processing and secretion, and insulin biogenesis. In humans, TRAPα is expressed in multiple tissues in addition to pancreas, suggesting it is very likely to have other biological functions distinct from its role in insulin biogenesis. TRAPα knockdown activates eIF2α in the rat pancreatic beta cell line while *trap-1* mutant induces *hsp-4::GFP* ER UPR reporter expression in *C. elegans*. To investigate the role of *trap-1* in ER homeostasis, we conducted a mutagenesis screen for *m*odifiers of the *trap-1* mutant phenotype (*mtro* screen). The *mtro* screen identified several known ER UPR pathway components as well as a strain harboring a missense mutation *(N162K)* in the *col-75* collagen gene that causes constitutive intestinal *hsp-4::GFP* expression. A transcriptional *col-75* promoter dsRed fusion protein reveals COL-75 is expressed in limited tissues including amphid socket glia, excretory cells, phasmid sheath glia, and vulva. These expression patterns do not colocalize with the *hsp-4::GFP* expression induced by *col-75(N162K)*, suggesting the possibility that missense collagen mutant may induce ER UPR cell non-autonomously. Collagen missense variants associated with human disease induce ER stress.

Thus, understanding the role of COL-75 in ER UPR may yield insights into the therapeutic development for collagenopathies that currently lack effective disease mechanism-targeting therapies.

# Introduction

All proteins must fold properly in order to function and nearly one-third of cellular proteins are cotranslationally translocated into the endoplasmic reticulum (ER) during their biogenesis. Protein biogenesis can be an error-prone process where unfolded or misfolded ER proteins accumulation may lead to ER stress and perturb ER homeostasis. ER stress has been associated with many aging-related diseases including non-alcoholic fatty liver disease, atherosclerosis, cancer, diabetes, and neurodegenerative diseases such as Alzheimer, Parkinson, and Huntington's diseases (Gidalevitz et al. 2010, Tabas 2010, Lebeaupin et al. 2018). In response to ER stress, evolutionarily conserved machinery known as ER unfolded protein response (UPR) activates downstream transcriptional network to increase capacity for protein folding, degradation, and secretion while tuning down global translation to reduce ER workload. The main components of the mammalian ER UPR are conserved in *C.elegans*, including the unfolded protein sensors IRE-1, PEK-1/PERK, and ATF-6 as well as two HSPA5/BiP homologs HSP-3 and HSP-4. Under normal condition, BiP binds to all three unfolded protein sensors to inhibit their activity. In the event of ER stress, misfolded proteins in the ER lumen titrate BiP from the canonical ER stress sensors and subsequently activates three branches of the UPR pathway. After the dissociation of BiP, IRE-1 becomes activated via dimerization and trans-autophosphorylation. Activation of IRE-1 leads to noncanonical RNase splicing of the *xbp-1* mRNA and subsequently activates the *xbp-1*-dependent transcriptional programs that can enhance protein trafficking, quality control, and folding machineries as well as genes encoding

multiple ER chaperones, including *hsp-4*/BiP. When PEK-1/PERK is released from BiP, it goes through dimerization and autophosphorylation to become active. PEK-1/PERK activation then leads to phosphorylation of eukaryotic translation initiation factor-2 (eIF2α), which decreases the frequency of mRNA translation and reduces ER workload. In the absence of ER stress, BiP binds to ATF-6 and shields the Golgi localization sequence of ATF-6 to remain inactive on the ER membrane. ATF-6 is transported to Golgi for protease splicing after it is released from BiP, then the cleaved ATF-6 transcription factor domain becomes active and migrates to the nucleus to bind to ER-stress responsive promoter elements to upregulate transcription of UPR target genes. The structural and functional conservation of the ER UPR pathway in *C. elegans* as a genetically tractable model organism makes it a useful tool to investigate the molecular mechanisms of the canonical ER UPR pathways and can provide insights into ER homeostasis maintenance in other organisms.

## *trap-1* is a suppressor of *eak-7;akt-1* (*seak)* gene

We previously performed a genetic screen to identify novel regulators suppressing the daf-c phenotype of an *eak-7;akt-1* double mutant strain that has increased DAF-16/FoxO activity. We isolated a mutant strain *dpDf665* containing a roughly 3.5kb deletion that spanned the *trap-1* (*tr*anslocon-*a*ssociated *p*rotein-1) locus and 3 exons of the upstream gene *Y71F9AL.1* (Li et al. 2019). To determine if *trap-1* is the causal mutation in this mutant strain, three independent *trap-1* null alleles were generated and they phenocopied *dpDf665* deletion while a null mutation in *Y71F9AL.1* did not suppress the dauer phenotype in *eak-7;akt-1* mutant background (Li et al. 2019). This result suggests that *trap-1* is a *bono fide seak* gene that can suppress *eak-7;akt-1* dauer arrest.

## *trap-1* specifically influences dauer arrest phenotype regulated by IIS

In addition to the IIS, DAF-7/TGFβ and DAF-9/steroid hormone pathways can also regulate dauer arrest phenotype, where mutations in either of these two pathway components can promote animals to arrest as dauer (Hu 2007). In order to determine if TRAP-1 functions as a general dauer arrest regulator in other signaling contexts, double mutants of *trap-1;daf-1* and *trap-1;daf-9* reveal that *trap-1* mutation does not suppress dauer arrest phenotype in *daf-1*/TGFβ receptor mutant background and *daf-9*/steroid hormone mutant background. In contrast, *trap-1* mutation is able to suppresses the dauer arrest phenotype caused by reduction in DAF-2/IGFR activity, suggesting TRAP-1 specifically antagonizes IIS to promote dauer arrest (Li et al. 2019). We tested other *daf-2/IGFR* mutant alleles and found *trap-1* mutation can only suppress the *daf-2* alleles *e1368* and *m212* both of which encode receptors with missense mutations in the extracellular ligand-binding domain (Li et al. 2019). Although the nature of each *daf-2* allele mutant is not entirely understood, we found *trap-1* mutation is unable to suppress the dauer arrest phenotype of the *daf-2* mutant alleles that affect the cytoplasmic tyrosine kinase domain (Li et al. 2019). This suggests the possibility that *trap-1* may function upstream of DAF-2/IGFR to regulate dauer arrest. DAF-2/IGFR is regulated by agonistic and antagonistic insulin-like peptides (Pierce et al. 2001, Hung et al. 2014). In the presence of the agonistic insulin-like peptides, the DAF-2/IGFR is active and wild-type animals develop normally at 25°C because DAF-16/FoxO activity is suppressed. When the agonistic insulin-like peptides are mutated and the antagonistic insulin-like peptides remain functional such as the case of *ins-4 ins-6;daf-28* triple mutant, animals exhibit daf-c phenotype because the antagonistic insulin-like peptides now suppress DAF-2/IGFR to activate DAF-16/FoxO activities and therefore triple mutant animals arrest as dauers (Hung et al. 2014). We found that *trap-1* mutation can partially suppress the

dauer arrest phenotype of *ins-4 ins-6;daf-28* triple mutant which is consistent with a model whereby TRAP-1 promotes antagonist insulin-like peptide action to modulate dauer arrest (Li et al. 2019).

## TRAPα is required for insulin biogenesis

The biological function of mammalian TRAP-1 homolog TRAPα has not been established, previous study has demonstrated that TRAPα can interact with the preprolactin signal peptide during *in vitro* protein translocation (Wiedmann et al. 1987). Expression of human insulin in *C. elegans* suppresses DAF-2/InsR signaling (Pierce et al. 2001); therefore, we wanted to explore the possibility that TRAPα may be involved in promoting insulin biogenesis as we observed in *C elegans*. We generated rat INS 832/13 pancreatic beta cell line lacking TRAPα by CRISPR-Cas9-mediated genome editing to test this hypothesis. Immunoblotting assays against preproinsulin and pulse-chase experiments monitoring the processing and secretion of insulin in TRAPα knockout cells found defects in preproinsulin ER translocation, proinsulin maturation, and insulin secretion (Li et al. 2019). These findings implicate TRAPα plays a critical role in the biosynthesis of insulin.

## TRAP-1 is expressed in the ER and regulates ER UPR

To study the spatiotemporal expression pattern of TRAP-1, we generated a transgenic *C. elegans* strain expressing a functional single-copy TRAP-1::mCherry fusion protein (Li et al. 2019). TRAP-1::mCherry is widely expressed in embryo, larvae, and adult animals. Closer examination found TRAP-1::mCherry expression in the pharynx, intestine, hypodermis, and vulva. To define the subcellular localization of TRAP-1, we found TRAP-1::mCherry colocalizes with the ER protein peptidase fused to GFP, GFP::SP12, indicating the endogenous

TRAP-1 localizes to the ER. Similarly, immunostaining of rat insulinoma cells with anti-TRAPα antibodies reveals colocalization with ER proteins recognized by anti-KDEL antibodies (Li et al. 2019). Since TRAP-1 is expressed in multiple tissues in *C. elegans* and human TRAPα is widely expressed in several tissues as opposed to the limited expression of insulin in pancreas, we reasoned that TRAP-1 is very likely to participate in the biogenesis of other ER client proteins and may be a general regulator to maintain ER homeostasis. Prior work in mouse fibroblast found TRAPα physically associates with ER-associated degradation (ERAD) substrates, suggesting that TRAPα may play a role in reducing ER stress (Nagasawa et al. 2007). In *C. elegans*, ER stress can induce the transcription of *hsp-4*, which encodes a homolog of the human ER BiP; therefore, we utilized a transgenic strain expressing *hsp-4::GFP* reporter as a proxy for disrupted ER homeostasis to test our hypothesis (Calfon et al. 2002). While green fluorescence was not detected from *hsp-4::GFP* in wild-type background, *trap-1;hsp-4::GFP* animals readily exhibit GFP signal under the same culture condition, suggesting *trap-1* mutation can induce ER stress (Li et al. 2019). Although reduction of TRAPα activity in rat INS 832/13 cells did not induce IRE1-dependent splicing of *xbp-1* mRNA, TRAPα knockdown by siRNA increased the phosphorylation of eIF2α, which indicates mammalian TRAPα may also influence ER homeostasis (Li et al. 2019).

# Results

## A genetic screen for modifiers of *trap-1* mutant phenotype (*mtro* screen)

To further study the molecular basis for the role of TRAP-1 in promoting ER homeostasis, we performed a forward genetic screen for modifiers of *trap-1* mutant phenotype (*mtro* screen). We reasoned that a genetic screen for mutations which could affect the *hsp-*

*4::GFP* expression phenotype of *trap-1* mutant might identify novel regulators of ER UPR pathways. Therefore, we mutagenized *trap-1(null)* animals harboring an integrated *hsp-4::GFP* reporter with N-ethyl-N-nitrosourea (ENU) and screened rare F2 progeny for enhanced or suppressed GFP expression. After verifying the penetrance of the change in GFP in subsequent generations, nine independent mutants with enhanced GFP expression (*etro* – enhancer of *trap-1*) and three independent mutants with suppressed GFP expression (*stro* – suppressor of *trap-1*) were subjected to whole genome sequencing. For *etro* mutant strains with increased *hsp-4::GFP* expression, candidate causal mutations were separated from the parental *trap-1(null)* mutant background and tested for their ability to induce GFP expression in a wild-type *trap-1* background. For mutant strains exhibiting decreased *hsp-4::GFP* expression (*stro*), candidate causal mutations were verified by ascertaining retention of the decreased GFP phenotype in the *trap-1(null)* mutant background after separation of all linked ENU-induced mutations. Candidate causal mutations were identified based on known of hypothesized roles in ER homeostasis. For mutant strains without obvious candidate mutations, we performed single nucleotide polymorphism mapping to help us narrow down to a specific region of the chromosome that would allow us to individually test the candidate causal mutations.

## The *mtro* screen identifies genes that influence ER homeostasis

We have verified causal alleles in one mutant with reduced *hsp-4::GFP* expression and seven mutants with increased *hsp-4::GFP* expression (Table 3.1). The *mtro* screen identified known ER UPR pathway components such as the ER unfolded protein sensor *ire-1* and *C. elegans* HSPA5/BiP ortholog *hsp-3*. A missense mutant affecting the kinase domain of the ER unfolded protein sensor IRE-1 has been shown to suppress ER UPR triggered by tunicamycin treatment (Calfon et al. 2002). One *mtro* strain harboring *ire-1(G26E)* mutation was outcrossed

with wild-type animals to separate neighboring mutations that are also present in the same strain and tested for its ability to suppress *hsp-4::GFP* expression in *trap-1* mutant background. We found *trap-1;ire-1(G26E)* double mutant reduces the *hsp-4::GFP* expression caused by ER stress induced by *trap-1* mutant, suggesting *ire-1* is a causal *mtro* mutant (Figure 3.1).

**Table 3.1 Causal mutations in eight independent *mtro* mutants.**

| *hsp-4::GFP* expression | Mutated gene | Human ortholog | Causal mutation | Conserved residue? |
|---|---|---|---|---|
| suppressed (*stro*) | *ire-1* | IRE1 | G26E | No |
| Enhanced *(etro)* | *hsp-3* | HSPA5/BiP | T34A | Yes |
| | | | I66S | Yes |
| | | | deletion | N/A |
| | *pdi-2* | PDIA1/P4HB | K322M | Yes |
| | *pdi-6* | PDIA6 | W204R | Yes |
| | *smg-2* | UPF1 | W723* | N/A |
| | *col-75* | collagen | N162K | No |

The *mtro* screen identified three strains harboring mutations in *hsp-3* (Figure 3.2), which encodes one of the two human orthologs of HSPA5/BiP; the other is *hsp-4* (Kaufman 1999). Two missense alleles (*T34A* and *I66S*) affecting conserved amino acids (Figure 3.3 and 3.4) and one partial deletion (Figure 3.5) of *hsp-3* enhance *hsp-4::GFP* expression in *trap-1* mutant background. To verify *hsp-3* as a *bono fide mtro* mutant, we tested an independent *hsp-3(ok1083)* allele and found *trap-1;hsp-3(ok1083)* double mutant phenocopies the mutagenized *hsp-3 mtro* mutant strains (Figure 3.6). All three *hsp-3 mtro* mutants can induce *hsp-4::GFP* expression in wild-type background similar to the phenotype that has been previously observed in *hsp-3(ok1083)* (Kapulkin et al. 2005).

**Figure 3.1 *ire-1(G26E)* is a causal *mtro* mutant that can suppress *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;ire-1(G26E)*, *ire-1(G26E)*, *trap-1*, and wild-type. Scale bar: 500μm.

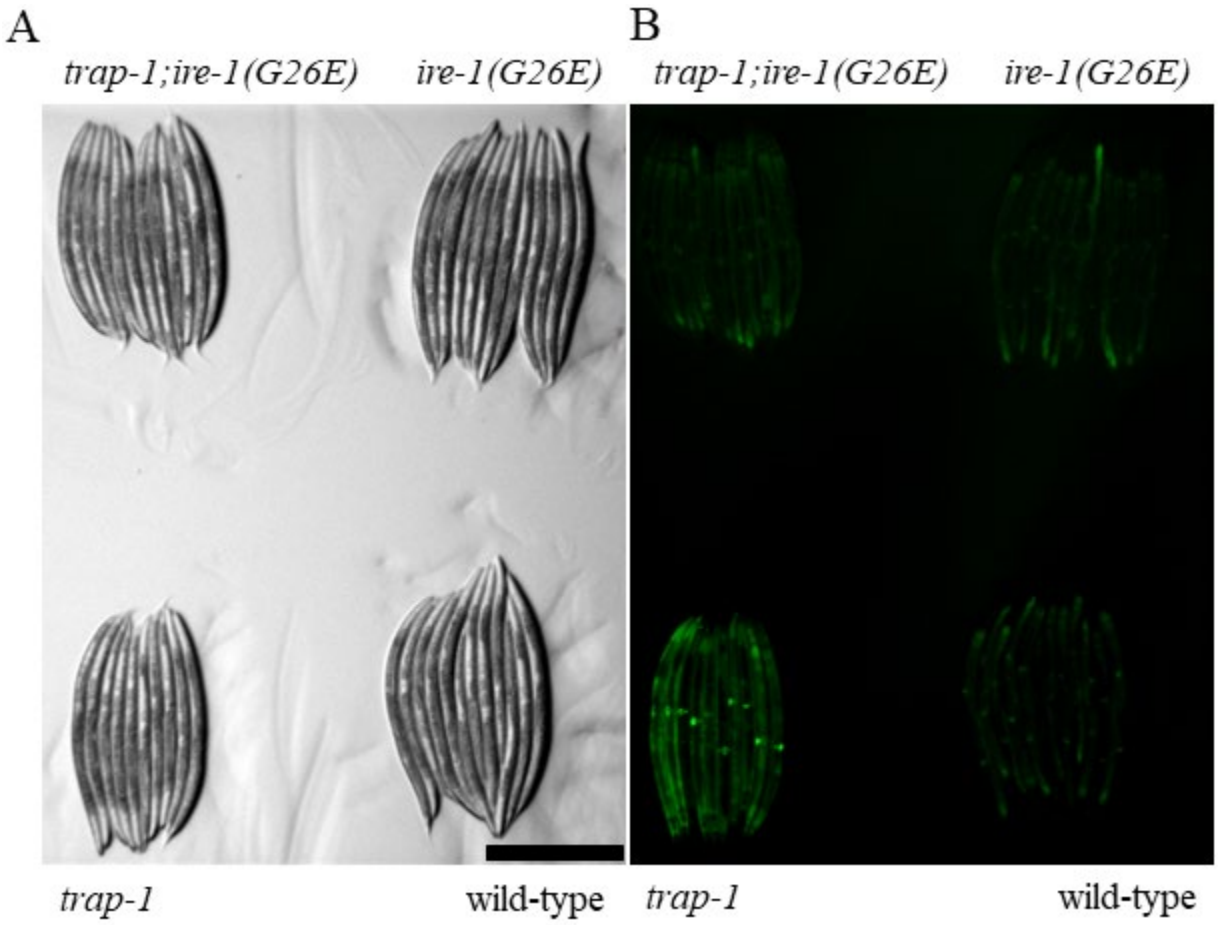


**Figure 3.2 Schematic of *hsp-3* genomic region.**
*mtro* screen identified three *hsp-3* mutant alleles (T34A, I66S, and deletion). An independent *ok1083* deletion allele was tested for its ability to enhance *hsp-4::GFP* expression in *trap-1* mutant background.

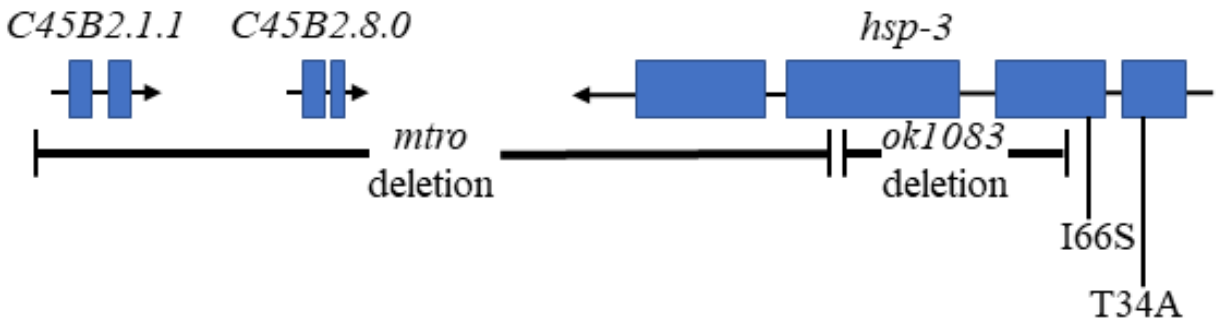


**Figure 3.3 *hsp-3(T34A)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;hsp-3(T34A)*, *hsp-3(T34A)*, *trap-1*, and wild-type. Scale bar: 500µm.

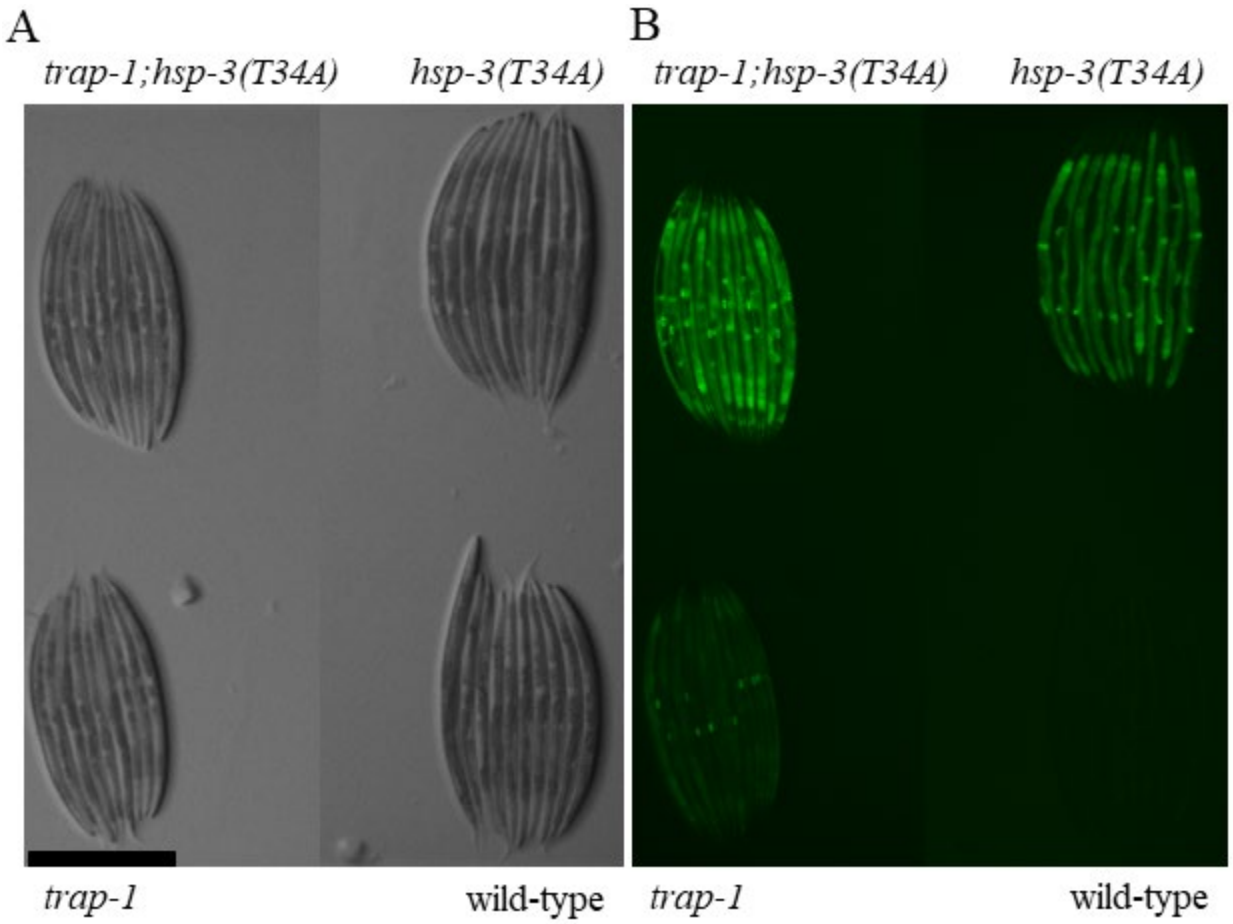


**Figure 3.4 *hsp-3(I66S)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;hsp-3(I66S)*, *hsp-3(I66S)*, *trap-1*, and wild-type. Scale bar: 500µm.

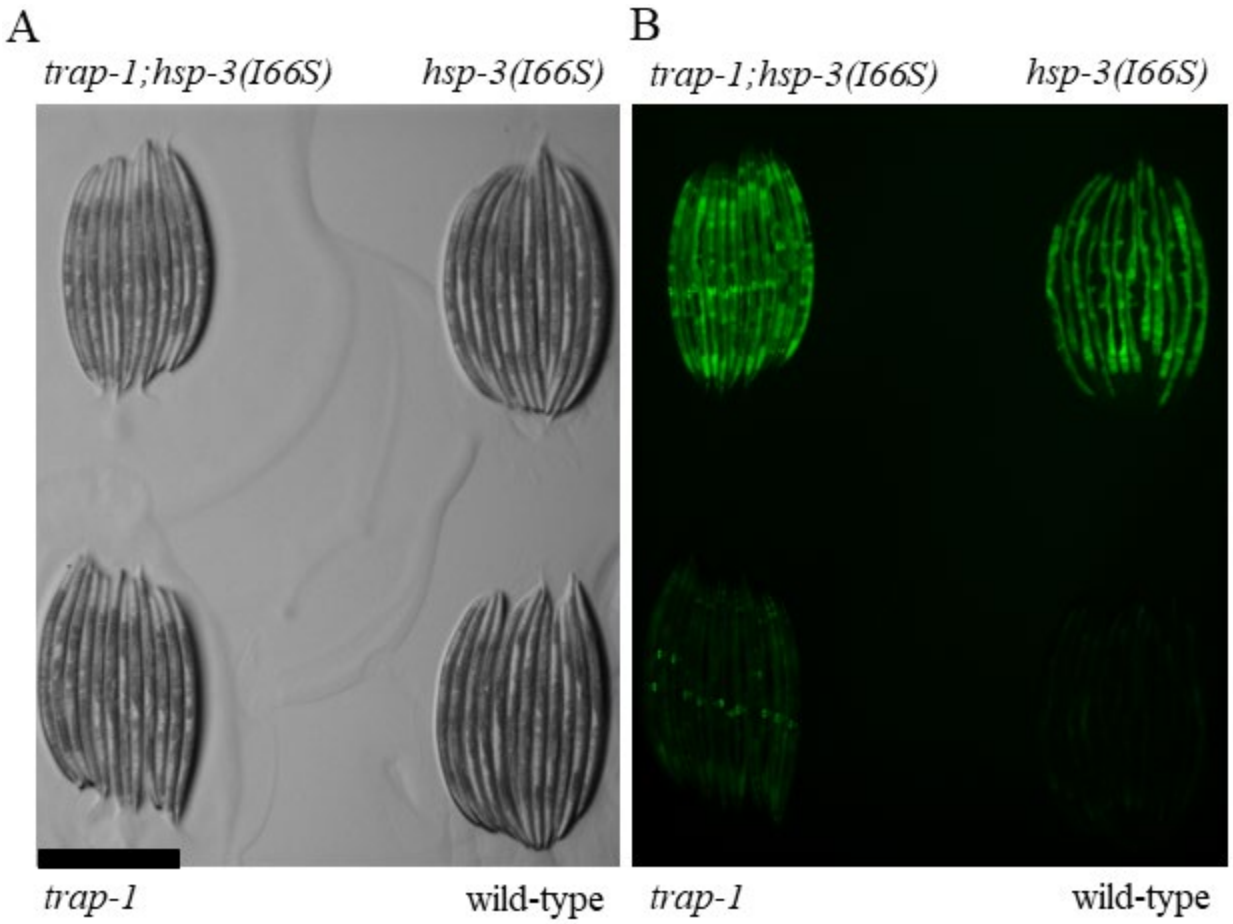


**Figure 3.5 *hsp-3(mtro deletion)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;hsp-3(mtro deletion)*, *hsp-3(mtro deletion)*, *trap-1*, and wild-type. Scale bar: 500µm.

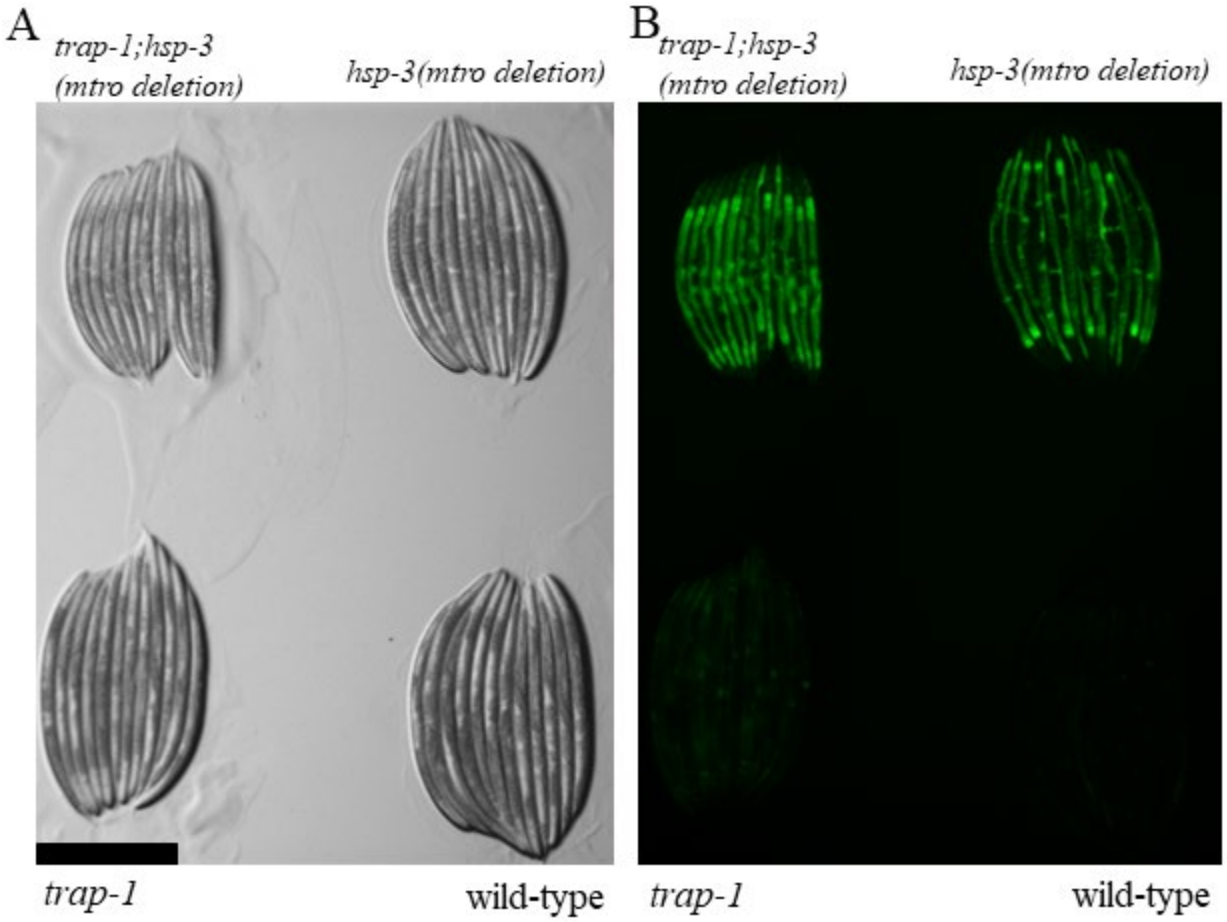
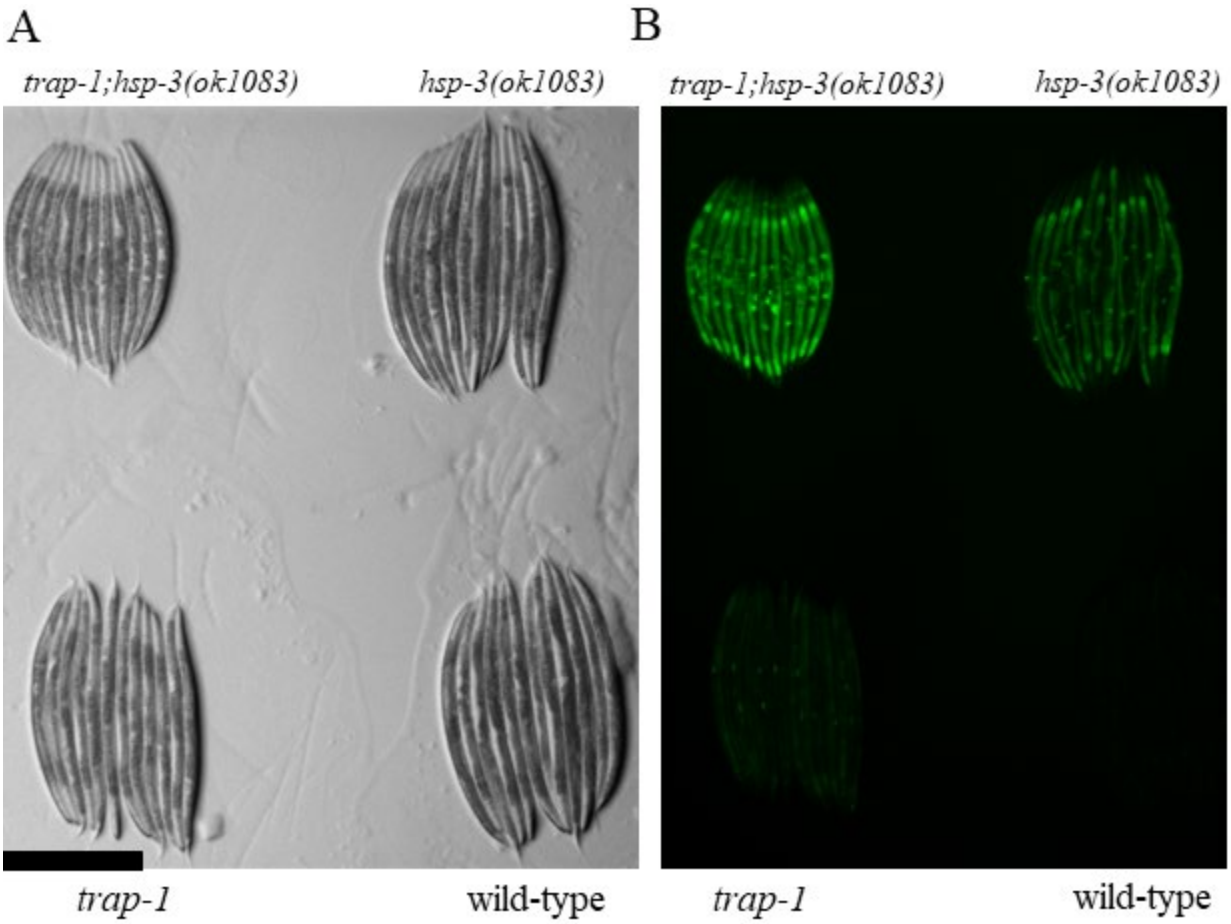


**Figure 3.6 An independent *hsp-3(ok1083)* allele phenocopies *hsp-3 mtro* mutants and enhances *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;hsp-3(ok1083)*, *hsp-3(ok1083)*, *trap-1*, and wild-type. Scale bar: 500µm.



In addition to *hsp-3*, we have identified other causal mutations in *pdi-2(K322M)*, *pdi-6(W204R)*, and *smg-2(W723*)*, all of which increases *hsp-4::GFP* expression and has known functions in maintaining ER homeostasis. The two missense *hsp-3/BiP* alleles and the *pdi-2* and *pdi-6* alleles all affect amino acids that are conserved in human BiP, PDIA1, and PDIA6. *pdi-2* and *pdi-6* encode protein disulfide isomerases that play a role in facilitating proper protein folding (Eletto et al. 2014, Jang et al. 2019). Unlike the missense *hsp-3* mutants identified in other *mtro* strains, both missense alleles of *pdi-2(K322M)* and *pdi-6(W204R)* mutants enhance *hsp-4::GFP* expression in *trap-1* mutant background (Figure 3.7 and 3.8). However, *pdi-2(K322M)* mutant does not induce *hsp-4::GFP* expression in wild-type background (Figure 3.7) whereas *pdi-6(W204R)* single mutant can mildly induce *hsp-4::GFP* expression (Figure 3.8). Previous study found *pdi-2* RNAi knockdown induces calreticulin expression, which is a calcium-binding chaperone located in the lumen of ER and plays a role in the folding of newly synthesized proteins (Lee et al. 2007). Recent study reported knockout of mammalian PDIA1 leads to glucose intolerance in mice fed with high fat diet and further analysis found PDIA1 regulates proinsulin disulfide maturation in the ER (Jang et al. 2019). RNAi knockdown of *pdi-6* has been shown to induce *hsp-4::GFP* expression and previous study also demonstrated PDI-6 facilitates the decay of active IRE-1 to attenuates the duration of IRE-1 activity by binding to the luminal domain of IRE-1 (Eletto et al. 2014). Similar to *hsp-3*, nonsense mutation in *smg-2(W723*)* can both enhance *hsp-4::GFP* expression in *trap-1* mutant background as well as induce *hsp-4::GFP* expression in wild-type background (Figure 3.9). *smg-2* encodes the *C elegans* homolog of the key nonsense mediated decay (NMD) component UPF1 in yeast and previous study has implicated the role of *smg-2* in ER UPR where loss-of-function *smg-2* mutation increases spliced *xbp-1* mRNA when compared to wild-type animal (Richardson et al.

2011). Taken together, these causal mutations indicate the *mtro* screen can effectively identify proteins that are critical for regulating ER homeostasis.

**Figure 3.7 *pdi-2(K322M)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;pdi-2(K322M)*, *pdi-2(K322M)*, *trap-1*, and wild-type. Scale bar: 500µm.

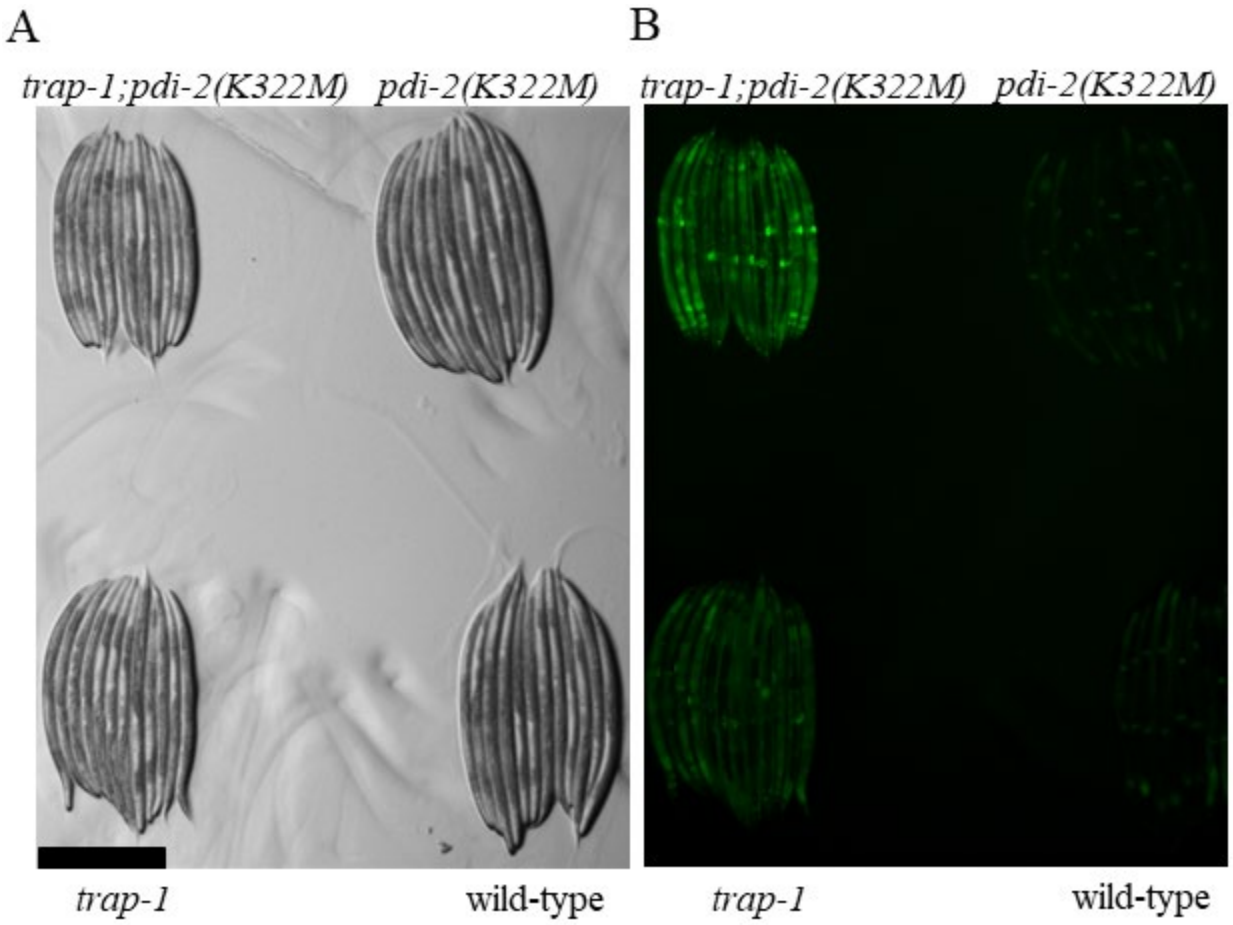


**Figure 3.8 *pdi-6(W204R)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;pdi-6(W204R)*, *pdi-6(W204R)*, *trap-1*, and wild-type. Scale bar: 500µm.

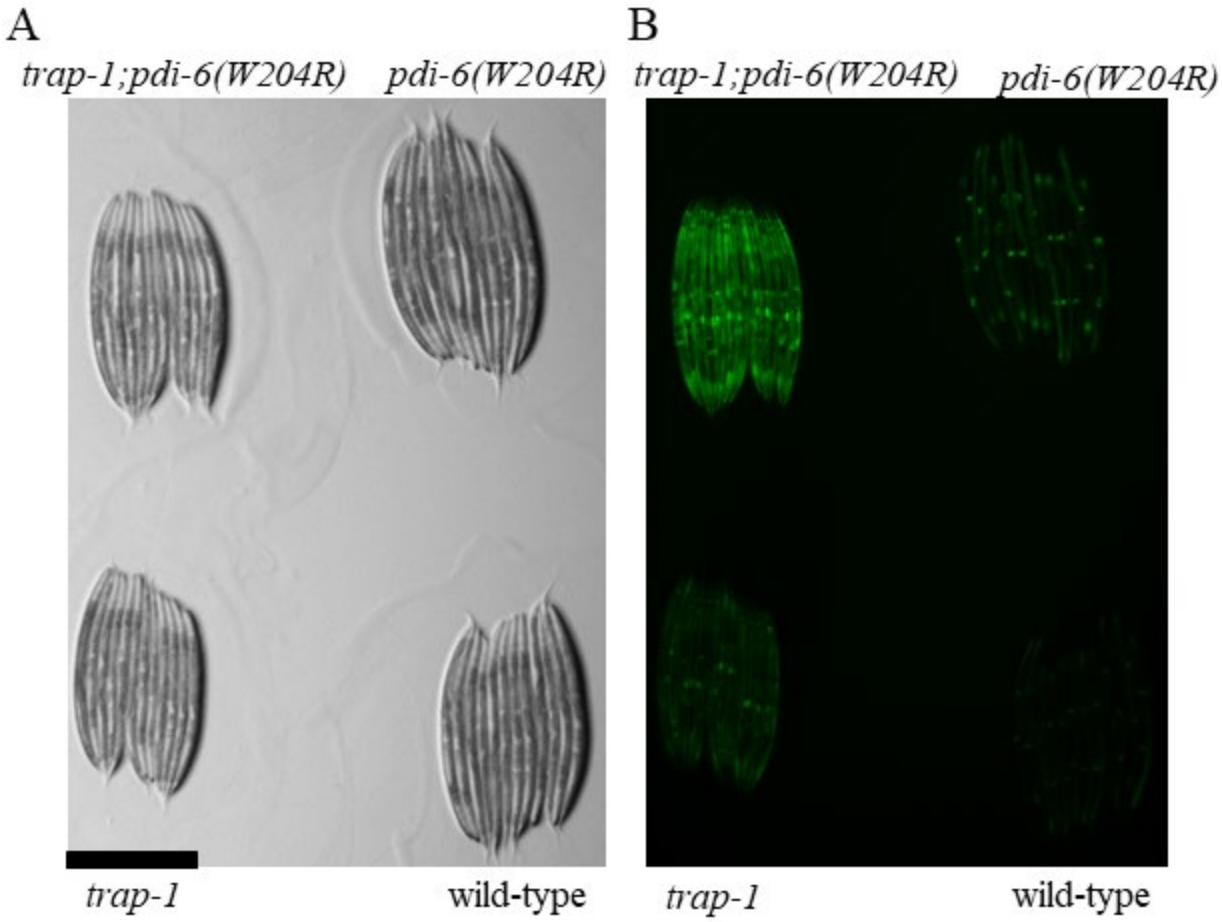
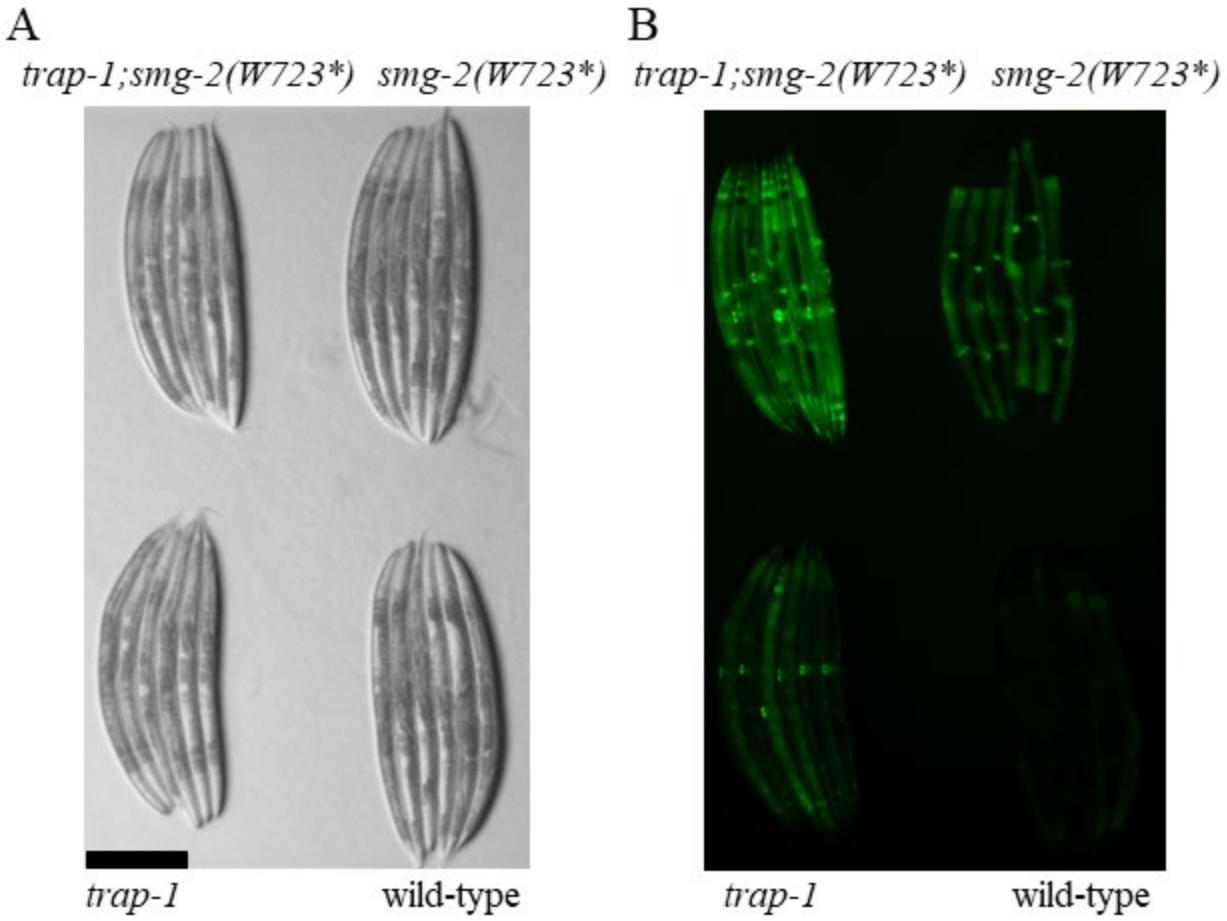


**Figure 3.9 *smg-2(W723*)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;smg-2(W723*)*, *smg-2(W723*)*, *trap-1*, and wild-type. Scale bar: 250µm.



## Neomorphic *col-75* alleles activate the ER UPR

One *mtro* strain did not have an obvious causal mutation candidate, however, single nucleotide polymorphism mapping suggests the increased *hsp-4::GFP* expression phenotype is closely linked to the center region of chromosome II (data not shown). Our sequencing data only has two nonsynonymous mutations in this particular region: *eas-1* and *col-75*. To determine if either one of these two mutations is the causal *mtro* mutation in this strain, we crossed wild-type animal with the mutagenized strain and generated one line that only has *col-75* mutation and another line that only has *eas-1* mutation. *col-75(N162K)* mutation enhances *hsp-4::GFP* expression in *trap-1* mutant background and induces *hsp-4::GFP* expression in wild-type background (Figure 3.10), whereas *trap-1;eas-1* double mutant only has similar *hsp-4::GFP* expression level as observed in *trap-1* mutant (Figure 3.11). These results suggest *col-75(N162K)* is the causal *mtro* mutation.

To test if the canonical IRE-1-XBP-1 ER unfolded protein pathway is involved in the induction of *hsp-4::GFP* caused by *col-75(N162K)*. We generated *col-75(N162K);xbp-1* double mutant and observed strong suppression of the *hsp-4::GFP* expression compared to that in *col-75(N162K)* single mutant animals (Figure 3.12). This suggests XBP-1 is required for the induction of *hsp-4::GFP* expression by *col-75(N162K)*.

*C. elegans col-75* encodes a predicted cuticle collagen with a domain structure characteristic of fibril-forming collagens (Figure 3.13) (Ricard-Blum 2011). To determine if *col-75* is the *bona fide* mutation that induces *hsp-4::GFP* expression, we assayed independent *col-75* mutant alleles to find out if they can phenocopy the mutagenized *col-75(N162K)*. We compared *hsp-4::GFP* expression in *col-75(N162K)* to that in three other *col-75* mutant backgrounds: the nonsense allele *Q224**, as well as the missense alleles *P258L*, and *G208E* (Figure 3.13). *hsp-*

*4::GFP* expression in *col-75(Q224*)* nonsense mutants is similar to that in a wild-type *col-75* background and significantly lower than that in *col-75(N162K)* (Figure 3.14A). *col-75(P258L)* induces *hsp-4::GFP* expression but to a lesser degree than *col-75(N162K)* (Figure 3.14B). Interestingly, *col-75(G154R)* induces *hsp-4::GFP* expression strongly and specifically in posterior intestinal cells (Figure 3.14C dashed box). These results suggest col-75(N162K) is a neomorphic allele and the increased *hsp-4::GFP* expression phenotype is not due to loss-of-function of COL-75. We observed heterozygous *col-75(N162K)* mutants do not express increased levels of *hsp-4::GFP* compared to wild-type *col-75(N162K)* siblings (data not shown), indicating that *col-75(N162K)* is recessive. In addition, COL-75 may have unknown roles other than being a structural molecule for extracellular matrix because different missense alleles can induce distinct *hsp-4::GFP* expression patterns or COL-75 may possess different functions depending on the tissue in which it is expressed.

**Figure 3.10 *col-75(N162K)* is a causal *mtro* mutant that can enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;col-75(N162K)*, *col-75(N162K)*, *trap-1*, and wild-type. Scale bar: 250µm.

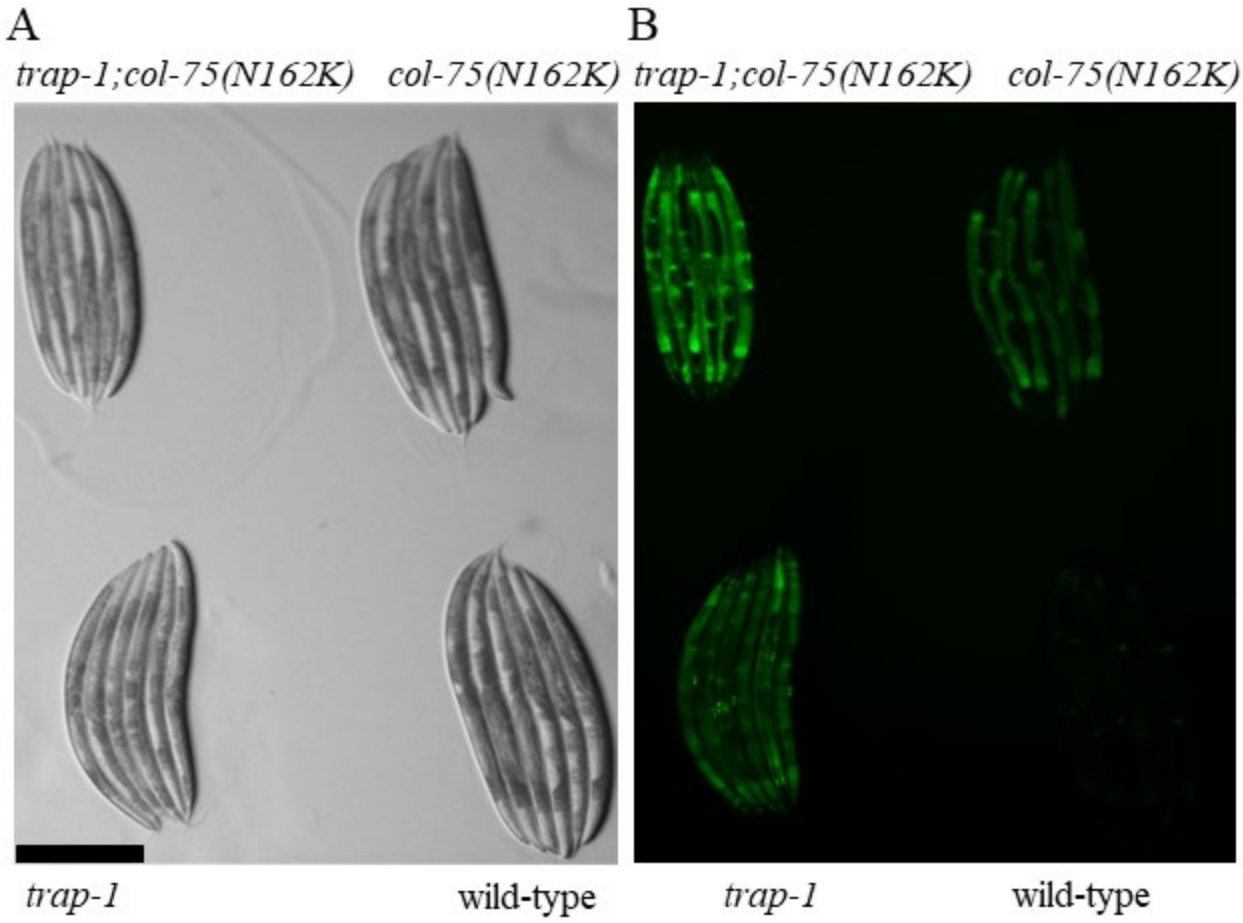


**Figure 3.11 *eas-1 mutant can not* enhance *hsp-4::GFP* expression in *trap-1* mutant background.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *trap-1;eas-1*, *eas-1*, *trap-1*, and wild-type. Scale bar: 500µm.

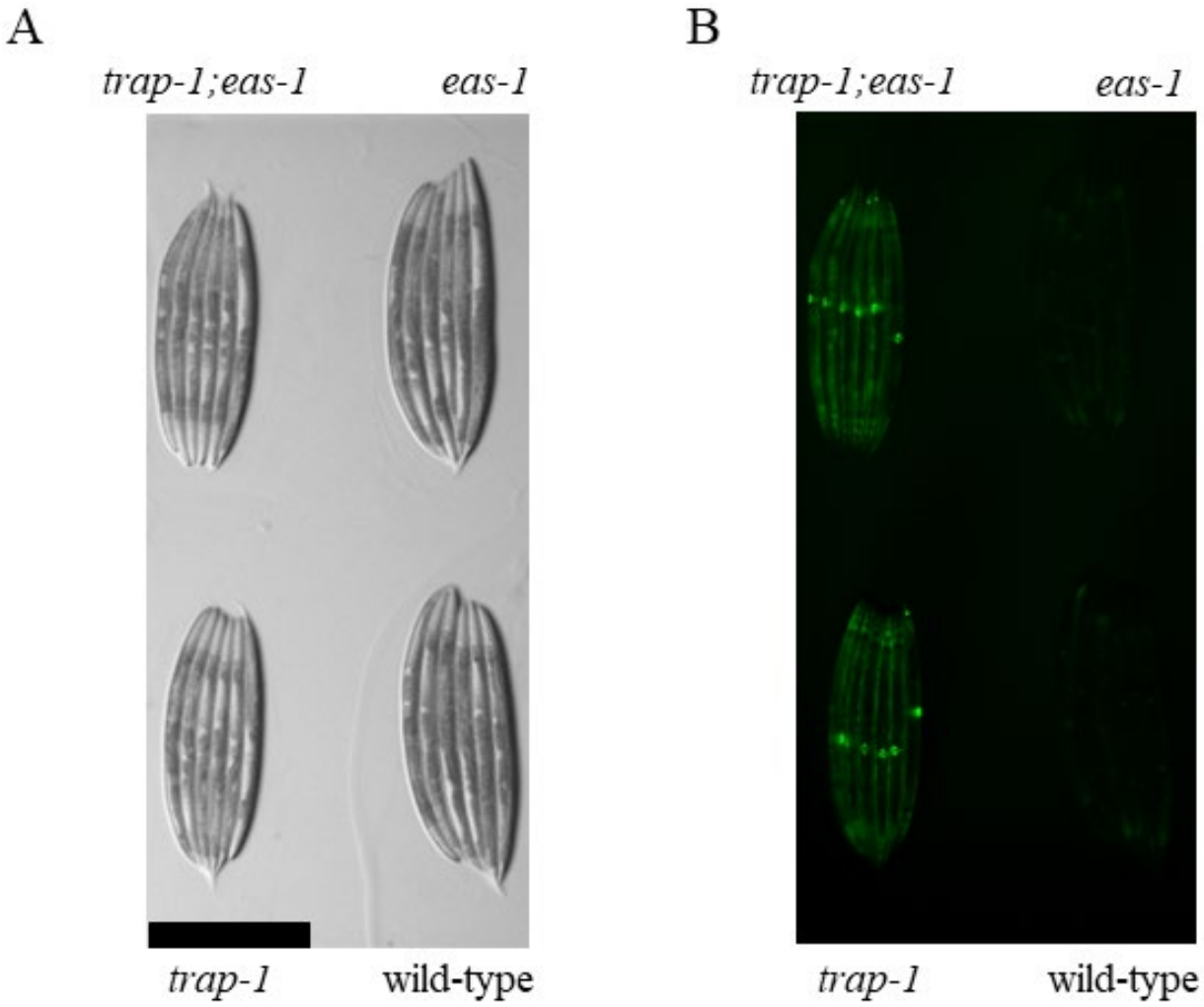


**Figure 3.12 XBP-1 is required for induction of *hsp-4::GFP* caused by *col-75(N162K)*.**
(A) Bright field and (B) fluorescence images of young adult *hsp-4::GFP* animals in *col-75(N162k);xbp-1*, *col-75(N162K)*, *xbp-1*, and wild-type. Scale bar: 250µm.

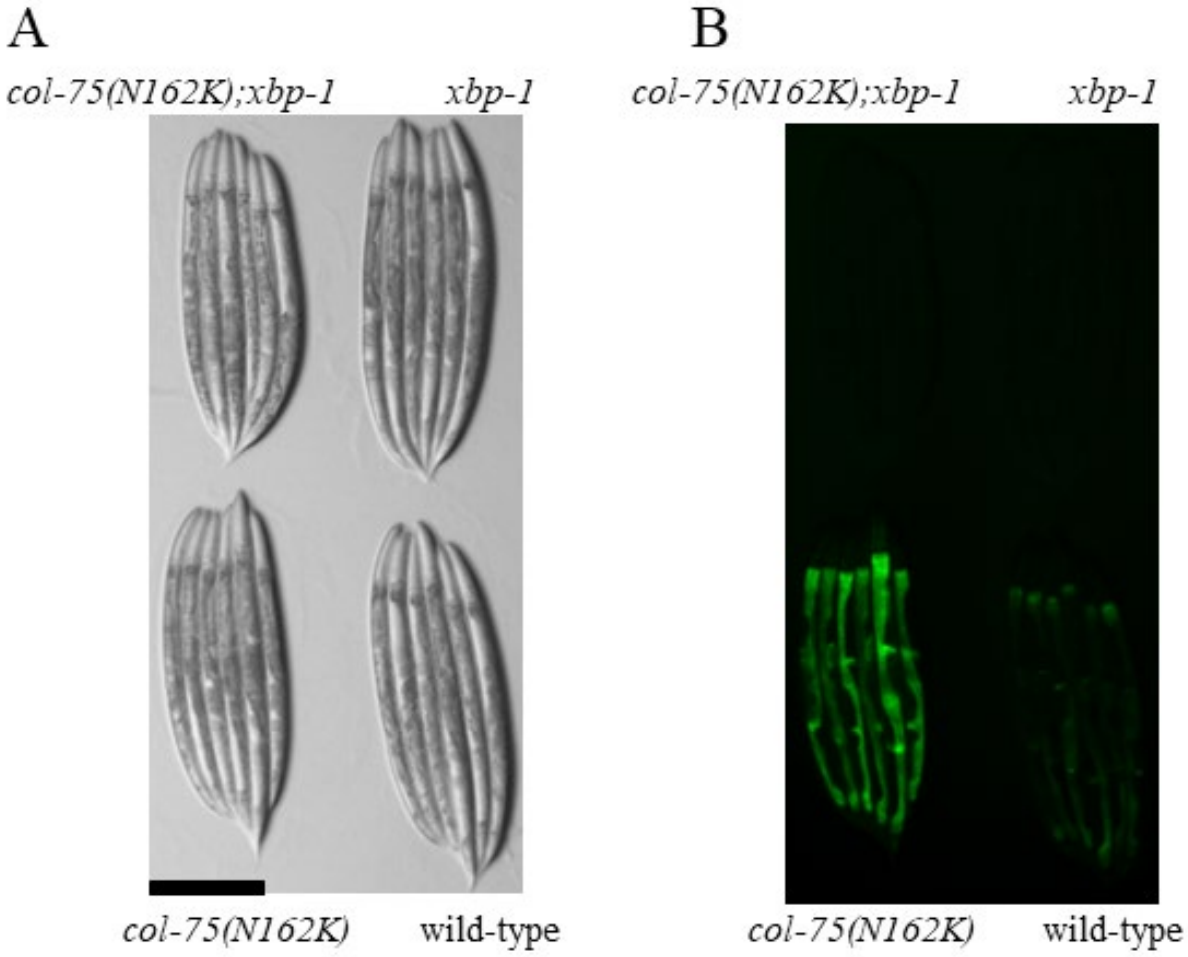



**Figure 3.13 Schematic of *C. elegans* COL-75.**
Domains are based on a sequence search of the Pfam database. The positions of missense alleles are shown.

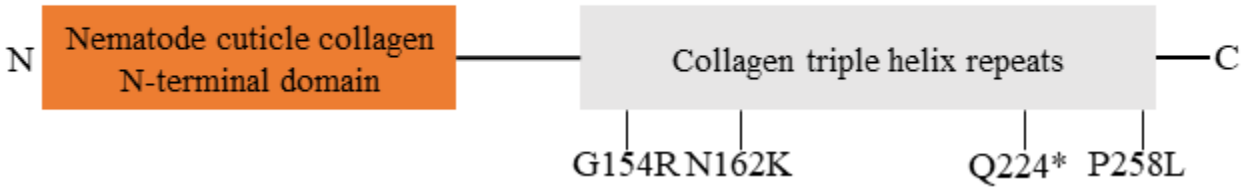
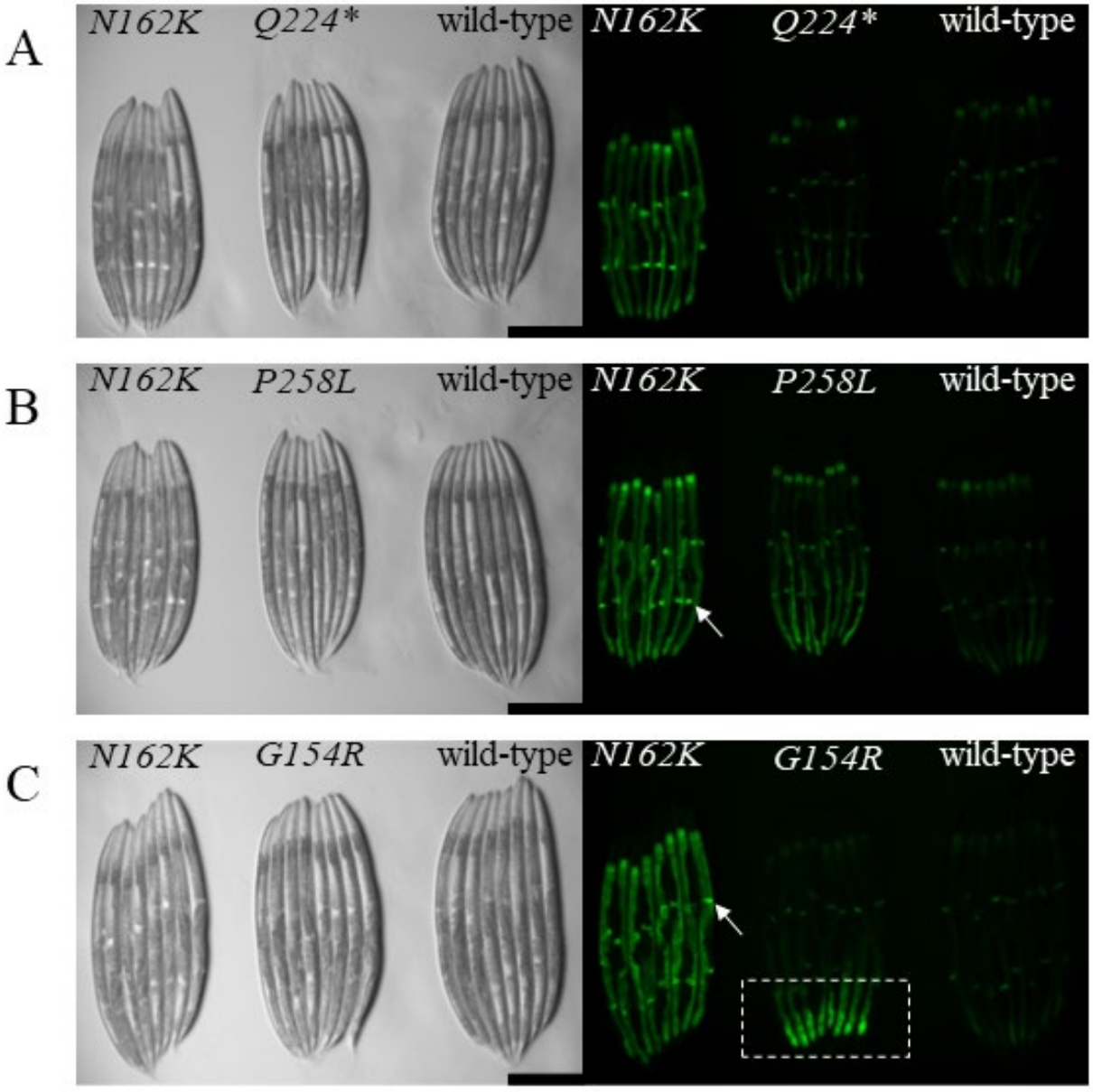


**Figure 3.14 Multiple missense *col-75* alleles induce *hsp-4::GFP* expression.**
Bright field (Left) and fluorescence (right) images of young adult *hsp-4::GFP* animals in (A) *col-75(Q224*)*, (B) *col-75(P258L)*, and (C) *col-75(G154R)* compared to wild-type and *col-75(N162K)* in the same field of view. Arrows denote spermathecae. Scale bar: 250µm.



## *col-75p::dsRed* is expressed in amphid socket cells, excretory cells, vulva, and phasmid sheath cells but not in the intestine

Single-cell RNA-seq of *C. elegans* reveals that *col-75* is expressed in a limited number of cells: the excretory cells, the socket glia, and pharyngeal neurons (Cao et al. 2017). However, *col-75* alleles induce *hsp-4::GFP* primarily in intestine and spermatheca (Figure 3.14). To experimentally determine the spatial expression pattern of COL-75, we generated transgenic animals expressing a transcriptional *col-75p::dsRed* fusion protein under the control of the endogenous *col-75* promoter and *unc-54* 3'UTR. Since COL-75 is predicted to be a secreted protein, a translational reporter would not be useful for identifying cells and tissues in which COL-75 is synthesized. We observed *col-75p::dsRed* expression in amphid socket cell and excretory cells (Figure 3.15A), phasmid sheath cells in the tail region (Figure 3.15B), and vulval muscle (Figure 3.15C).

## *col-75(N162K)* may induce *hsp-4::GFP* expression cell non-autonomously

*hsp-4/BiP* is widely expressed in many tissues in *C. elegans* whereas *col-75* is only expressed in limited tissues (Figure 3.14). To further examine the possibility that *col-75(N162K)* can induce ER stress cell autonomously, we generated *col-75(N162K);IS[hsp-4::GFP];Ex[col-75p::dsRed]* transgenic strain expressing integrated *hsp-4::GFP* and *col-75p::dsRed* extrachromosomal array to specifically image cells expressing *col-75* and look for *hsp-4::GFP* signals within those cell types. Surprisingly, green fluorescence was not detected in amphid socket glia and excretory cell (Figure 3.16A), phasmid sheath cell (Figure 3.16B), and vulva (Figure 3.16C). Taken together, these results implicate *col-75(N162K)* may induce *hsp-4::GFP* expression in the intestine cell non-autonomously.

**Figure 3.15 *col-75p::dsRed* expression pattern imaged by confocal microscopy.**
*col-75* transcript is detected in amphid socket glia (arrow head) and excretory cell (arrow) in (A) as well as phasmid sheath glia in (B) and vulva in (C). Scale bar: 25µm.

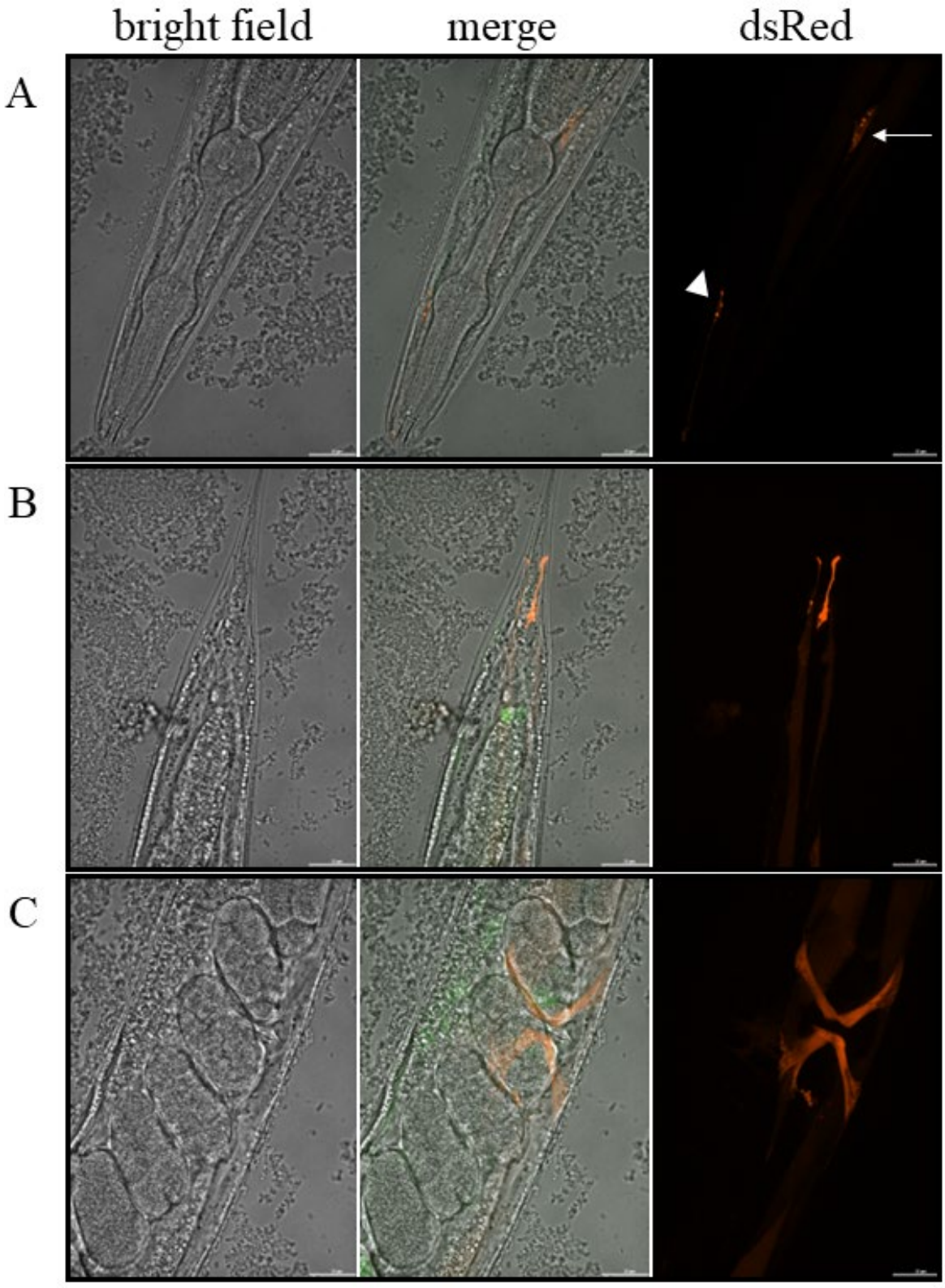
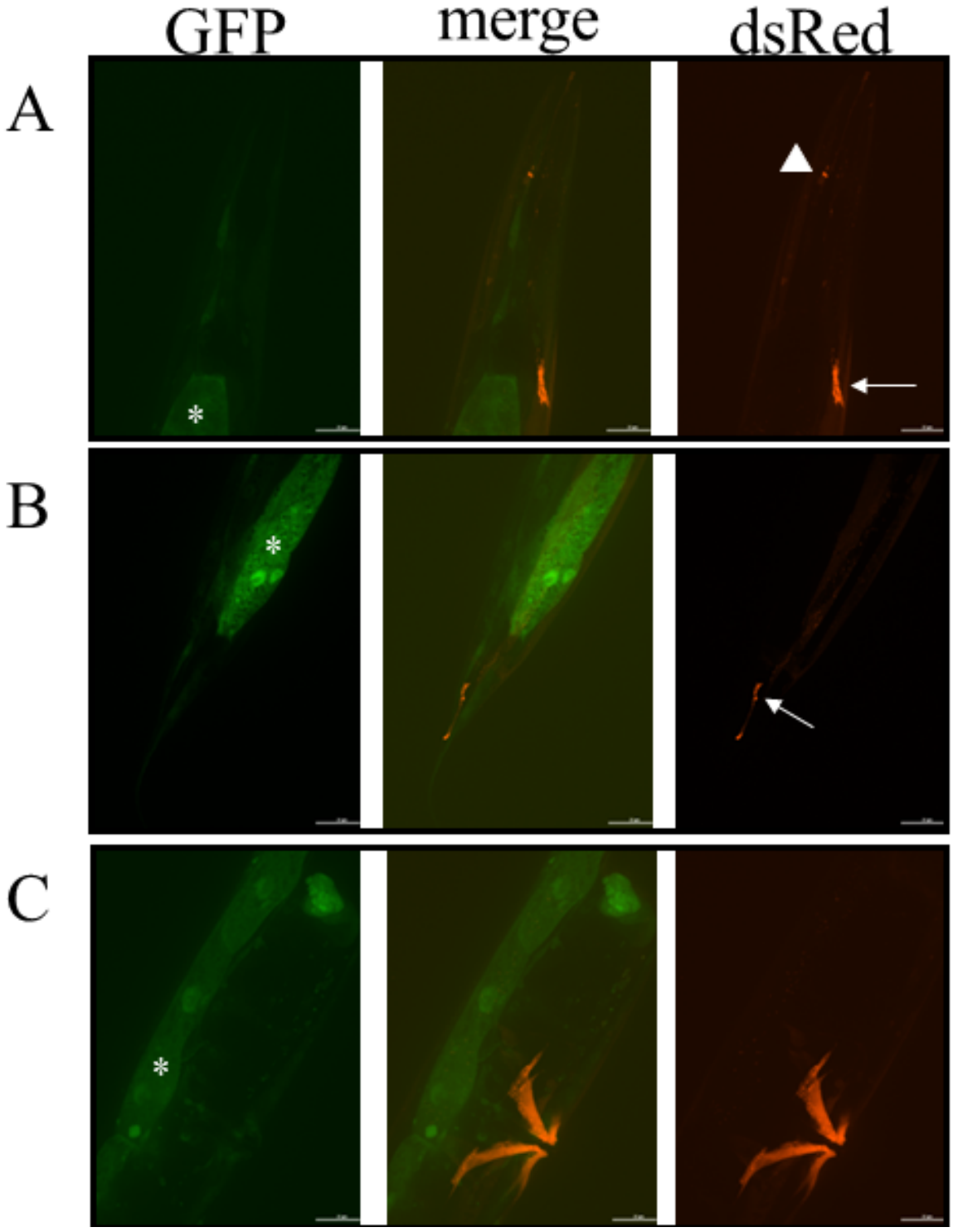


**Figure 3.16 *col-75p::dsRed* expression does not colocalize with *hsp-4::GFP* expression induced by *col-75(N162K)*.**
*col-75(N162K);IS[hsp-4::GFP];Ex[col-75p::dsRed]* transgenic animals express *col-75p::dsRed* in amphid socket glia (arrow head) and excretory cell (arrow) in (A) as well as phasmid sheath glia in (B) and vulva in (C), while *hsp-4::GFP* is primarily expressed in the intestine (denoted by *). Scale bar: 25µm.



## Discussion

Collagen is the most abundant proteins in mammals and it is defined by the presence of a triple helical motif comprised of three-amino-acid glycine repeats (-G-X-Y-), where X and Y can be any amino acid, but often are proline or hydroxyproline. The human genome contains 44 collagen genes encoding 28 types of trimeric proteins in the collagen superfamily (Salamito et al. 2021). Recent bioinformatic pipeline analysis has identified a total of 181 collagen genes in *C. elegans (Teuscher et al. 2019)*, and the majority of nematode collagens functions as the structural proteins for the two major extracellular matrices: the cuticle and basement membrane (Kramer 2005, Page and Johnstone 2007, Teuscher et al. 2019). In a genetic screen for mutations that modulate expression of *hsp-4::GFP* ER UPR reporter, we isolated a missense allele of the collagen gene *col-75* predicted to change asparagine 162 to lysine (*N162K*), which causes constitutive intestinal *hsp-4::GFP* expression. The function of COL-75 is unknown and none of the *col-75* alleles analyzed to date have obvious phenotypes such as blister (*bli*), dumpy (*dpy*), or roller (*rol*) phenotypes associated with many *C. elegans* collagen gene mutations that affect body morphology (Johnstone 2000). In contrast to a *col-75* nonsense mutant, which does not activate the ER UPR, three independent *col-75* missense alleles induce constitutive intestinal *hsp-4::GFP* expression in qualitatively and quantitatively distinct manners (Figure 3.14). This is consistent with the observation that insertion of mutant collagen into the cuticle usually leads to a more severe phenotype than null alleles or mutations that result in a loss or a reduction in the amount of collagen in the extracellular matrix (Johnstone 2000, Page 2001). Since *col-75(N162K)* is not a loss-of-function allele, its emergence from an unbiased ENU mutagenesis screen highlights the usefulness of classical genetics in revealing new biological insights through neomorphic alleles.

Such discovery would not have emerged from RNAi-based or CRISPR-based screens that are designed to study gene function through reduction of gene activity.

Collagen biosynthesis is an intricate and multistep process consist of both intracellular and extracellular events. As for all secreted proteins, collagens are synthesized as proα-chains by ribosomes on the ER membrane and are cotranslationally translocated into the lumen of ER to be assembled into triple helix molecules. Procollagen proα-chains need to undergo critical post-translational modifications involving cleavage of signal peptides, proline hydroxylation, lysine hydroxylation and glycosylation (Ricard-Blum 2011). After post-translational modification, collagen propeptides associate into either homotrimers or heterotrimers to form triple helix procollagen molecule with the assistance of ER chaperones including BiP and calreticulin as well as collagen modifying enzyme protein disulfide isomerases (Makareeva et al. 2011). The procollagen is then transported through the Golgi apparatus and secreted into the extracellular space where the N- and C- terminal propeptides are cleaved by extracellular matrix enzyme N- and C- proteinases (Makareeva et al. 2011, Ricard-Blum 2011, Salamito et al. 2021). Mature collagens can self-assemble spontaneously to form fibril structure which is strengthened by intra- and intermolecular covalent cross-linking of collagens (Ricard-Blum 2011, Salamito et al. 2021).

The importance of ER homeostasis in collagen biosynthesis is underscored by the fact that multiple human collagen missense mutations activate ER UPR in relatively rare heritable diseases (Wong and Shoulders 2019, Bateman et al. 2022). The triple helix repeat domains of *C. elegans* COL-75 are most similar to those in human COL21A1, whose function is still unknown. While mice do not have COL21A1 gene (Fitzgerald and Bateman 2004), a microarray-based genome-wide linkage analysis of the Malaysian population revealed low copy number of *COL21A1* is associated with nonsyndromic orofacial cleft, which is a birth deformity that affects

lip and/or palate (Mohamad Shah et al. 2019). Mutations in the fibrillar collagen that disrupt triple helix structure are most prevalent in the collagenopathies. These mutations usually replace glycine with another amino acid where nearly 80% of all *COL1A1* mutations that lead to osteogenesis imperfecta, also known as brittle bone disease characterized by fragile bones, are glycine substitutions (Bateman et al. 2022).

Several germline mutations in human *COL* genes analogous to *col-75(N162K)* are known to cause diseases. A N617K missense mutation in human *COL10A1* causes metaphyseal chondrodysplasia, Schmid type (MCDS) (Bonaventure et al. 1995), and a N1627K missense mutation in human *COL4A1* causes brain small vessel disease with ocular abnormalities (Rødahl et al. 2013). In addition, two similar missense mutations that lie within the triple helix repeat domains, a Q1150K mutation in human *COL4A2* and a Q455K mutation in human *COL8A2*, cause susceptibility to intracerebral hemorrhage and Fuchs endothelial corneal dystrophy, respectively (Biswas et al. 2001, Jeanne et al. 2012). Knock-in mice models for *COL10A1(N617K)* and *COL8A2(Q455K)* have been generated and further studies have demonstrated mutant collagen alleles can induce ER stress in relevant disease target cells/tissues: *COL10A1(N617K)* activates the ER UPR in chondrocytes (Rajpar et al. 2009), and *COL8A2(Q455K)* induces ER stress in corneal epithelium (Jun et al. 2012). These findings implicate a relationship between the induction of ER stress and disease pathogenesis in MCDS and Fuchs endothelial corneal dystrophy. However, ER UPR activation by mutant collagen often leads to accumulation of collagen molecules in the ER, which causes secondary loss of collagen function due to reduced collagen secretion (Wong and Shoulders 2019). It is important to also consider the possibility that mutant collagen may escape from ER quality control machinery and may alter extracellular matrix composition, structure, interactions, as wells as communication

between cell and extracellular matrix since all of which are likely to influence disease pathology (Bateman et al. 2022). Therefore, it is difficult to directly attribute ER UPR induction as the underlying mechanism of mutant collagen alleles to the pathogenesis of collagenopathies. To distinguish the pathogenic effects between the ER UPR induction and the unexpected consequences of mutant collagen, Rajpar and colleagues (2009) generated transgenic mice in which the *cog* thyroglobulin mutant that causes ER stress was expressed under the control of the *COL10A1* promoter in hypertrophic chondrocytes. These mice developed clinical and pathologic signs of MCDS due to the targeted UPR induction caused by the misfolded thyroglobulin, demonstrating that ER stress *per se* can contribute to MCDS pathogenesis (Rajpar et al. 2009). In another misfolding COL10A1 MCDS transgenic mouse model, treatment with eIF2α signaling cascade blocking agent, Integrated Stress Response Inhibitor (ISRIB), downregulates the PERK arm of the ER UPR pathway and alleviates the pathologies of chondrodysplasia, suggesting the crucial role of ER UPR in MCDS (Wang et al. 2018).

The thorough elucidation of the collagen biosynthesis and modification is essential for understanding the pathogenesis of collagenopathies and future development of therapeutic strategies that can benefit affected populations. One essential collagen modification that confers thermal stability to collagen triple helixes at human body temperature is the generation of 4-hydroxyproline residues catalyzed by proline 4-hydroxylase (P4H) in the ER lumen (Myllyharju 2008). Proline 4-hydroxylases are $\alpha_2\beta_2$ tetramers in which the β subunit is identical to the enzyme and chaperone protein disulfide isomerase (PDI) (Freedman et al. 1994). Germline proline 4-hydrxylase beta (P4HB) mutations cause Cole-Carpenter syndrome, which is an osteogenesis imperfecta-like illness (Rauch et al. 2015). The emergence of PDI-2, which is an ortholog of P4HB, from our *mtro* screen, further strengthening the importance of collagen and its

biosynthesis in the modulation of ER UPR. Taken together, these data strongly suggest that understanding how *col-75* mutant alleles activate the ER UPR in *C. elegans* will illuminate the pathogenesis of many genetic diseases caused by missense mutations in human *COL* genes.

Since *col-75(N162K)* does not exhibit any phenotype of other known collagen mutants, we wanted to examine the biological relevance of ER UPR induction as suggested by the widely-used *hsp-4::GFP* reporter. Preliminary results reveal that *col-75(N162K)* animals were as resistant to the ER stress inducer tunicamycin as wild-type (data not shown). This suggests the mild ER stress caused by *col-75* N to K missense mutation does not significantly impair organismal health as opposed to the detrimental health effect of ER UPR induced by N to K missense mutation in human collagen genes. Although the induction of *hsp-4::GFP* expression by *col-75(N162K)* requires *xbp-1* (Figure 3.12), we wanted to verify activation of endogenous XBP-1 by using RT-PCR to directly measure levels of spliced *xbp-1* (*xbp-1S*) (Taylor and Dillin 2013) induced by *col-75* mutant in the absence of the *hsp-4::GFP* reporter. Intriguingly, our preliminary data shows *col-75(N162K)* mutant minimally increases *xbp-1S* levels compared to total *xbp-1* (data not shown). Measuring *xbp-1* transcript levels from whole animals may not be sensitive enough to detect the increased levels of *xbp-1S* caused by mutant collagen variants because *col-75* is only expressed in very limited cell types. Alternatively, this finding suggests the possibility of signaling pathways acting parallel to *xbp-1S* may also induce *hsp-4::GFP* expression, either due to activation of PEK-1, ATF-6, other stress-responsive transcription factors that interface with the ER UPR such as SKN-1 (Glover-Cutter et al. 2013), or an undiscovered transcription factor that is activated by misfolded collagen. Together, these preliminary results underscore the importance of determining where does *xbp-1* function to induce intestinal *hsp-4::GFP* expression.

Identifying the sites of action of *col-75(N162K)* in ER UPR induction will provide important mechanistic insights into the unknown function of COL-75. While *col-75p::dsRed* is not expressed in the same cells/tissues where *hsp-4::GFP* expression is induced by *col-75(N162K)* (Figure 3.16), single-cell RNA-seq analysis indicates *col-75* is expressed at very low levels in the intestine (Cao et al. 2017). To determine whether low-level expression of *col-75(N162K)* in the intestine suffices to activate the ER UPR cell autonomously, we will generate transgenic animals expressing a *col-75(N162K)* cDNA under the control of promoters that drive intestine-specific expression at relatively modest levels. We will also test the ability of *col-75(N162K)* expression in the excretory cell, socket glia, phasmid sheath cells, and vulva to induce intestinal *hsp-4::GFP* expression. Since *col-75(N162K)* is recessive (data not shown), we will assay these constructs in a *col-75(Q224*);hsp-4::GFP* background to avoid potential confounding influences of wild-type COL-75. If *col-75(N162K)* acts cell autonomously to induce intestinal *hsp-4::GFP* expression, then only intestine-specific expression of intestine will induce intestinal *hsp-4::GFP* expression. Alternatively, the ability of other constructs to induce intestinal *hsp-4::GFP* expression would indicate that *col-75(N162K)* can act cell non-autonomously to induce *hsp-4::GFP* expression.

However, induction of intestinal *hsp-4::GFP* by forced expression of *col-75(N162K)* in the intestine does not establish that endogenous *col-75(N162K)* acts cell autonomously to activate *hsp-4::GFP*, since non-physiologic overexpression of mutant collagen in any tissue has the possibility to induce cell-autonomous ER UPR activation. We will test the ability of intestine-specific reduction of *col-75(N162K)* activity to abrogate *hsp-4::GFP* induction. To accomplish this, we will generate *col-75(N162K);hsp-4::GFP* animals in the RNAi-defective mutant background (Qadota et al. 2007) and restore RNAi sensitivity only in specific tissues by

injecting animals with tissue-specific rescuing constructs then subject animals to *col-75* RNAi. Abolition of *hsp-4::GFP* expression by *col-75* RNAi in the excretory cell, socket cells, or phasmid sheath cell will implicate those cells as an important site for cell non-autonomous model of *col-75(N162K)* action in intestinal *hsp-4::GFP* induction..

In conclusion, we undertook an unbiased genetic screen for mutations that modulate expression of *hsp-4::GFP* ER UPR reporter and identified a novel missense collagen mutant *col-75* that primarily induces intestinal ER UPR. Further characterizations suggest *col-75* may act cell non-autonomously to induce ER stress. While the exact function of COL-75 remains unclear, we propose future studies to determine the site of actions for *col-75* mutation in regulating ER UPR induction, which has the potential to establish the important role of collagen in ER proteostasis and facilitate development for new therapeutic targets to treat collagenopathies.

# Materials and Methods

## *C. elegans* strains and maintenance

Animals were maintained at 15°C on nematode growth media NGM seeded with *Escherichia coli* strain OP50. Compound mutants were constructed using standard genetic techniques. Genotypes were confirmed by using PCR followed by restriction digestion to detect restriction fragment length polymorphisms or using three primer PCR assays to detect deletions based on product size. *col-75(Q224*)* strain was outcrossed from VC40365, *col-75(P258L)* strain was outcrossed from VC30056, and *col-75(G154R)* strain was outcrossed from VC40573. All three strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

## Genetic screen for modifiers of *trap-1*

The *m*odifier of *trap-1* (*mtro*) screen was performed by mutagenizing *trap-1;hsp-4::GFP* mutants with N-ethyl-N-nitrosourea and screening for rare animals in the F2 generation with either enhanced or suppressed *hsp-4::GFP* expression levels as described. Genomic DNA isolated from *trap-1;hsp-4::GFP* modifier strains was sequenced and analyzed as described (Dumas et al. 2013).

## Transgenic strain construction

Transcriptional *col-75::dsRed* construct was generated by fusing a 1.5 kb *col-75* promoter to dsRed followed by *unc-54* 3'UTR in MC10-RFP as a vector backbone (Piali Sengupta; Addgene plasmid # 8938 ; http://n2t.net/addgene:8938 ; RRID:Addgene_8938) using Gibson assembly kit (New England Biolabs, MA, USA). Animals were injected as previously described (Mello et al. 1991) using a Leica DMI300B microscope and Eppendorf FemtoJet pump.

## Fluorescence microcopy

Animals were mounted on a NGM plate and immobilized in a drop of 25mM sodium azide. Detection of green fluorescence was imaged using a Leica M165FC stereomicroscope with a (Leica) DFC9000 GT camera and a Lumencor sola light engine fluorescence lamp.

## Confocal microscopy

Animals were immobilized with 25 mM sodium azide. Animals were mounted on slides layered with a thin 3% agarose in $H_2O$ pad and images were captured by a Photometrics Prime 95B sCMOS monochrome camera attached to a Nikon TiE inverted Spinning Disk Confocal Microscope using NIS-element software (Nikon).

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# Chapter 4 Conclusion and Future Directions

## Overview

This dissertation built upon our previous studies to further investigate the critical downstream target genes of DAF-16/FoxO transcription factor and the molecular basis for the role of a conserved ER transmembrane protein TRAP-1 (*tr*anslocon-*a*ssociated *p*rotein-1) in promoting ER homeostasis. In chapter two, we used an unbiased transcriptome profiling approach and DAF-16 isoform-specific prioritization strategy to identify DAF-16/FoxO target genes that are associated with longevity. We than followed up with a genetic characterization of the acyl-coA synthase gene, *acs-3*, that contributes to life span regulation in the context of reduced insulin-like signaling. In chapter 3, we performed an unbiased forward genetic screen to identify *m*odifiers of the *trap-1* mutant phenotype (*mtro* screen). We identified several known ER UPR pathway components and a missense mutant allele of collagen *col-75* gene. Further characterization suggests that *col-75* may act cell non-autonomously to induce ER stress. In the final chapter, we summarize our findings and propose experiments for future studies.

## ACS-3 acts downstream of DAF-16/FoxO to promote longevity

To identify downstream mechanisms through which DAF-16 acts to regulate longevity, we profiled the transcriptomes of long-lived *daf-2/IGFR* mutant animals in the context of wild-type *daf-16/FoxO* and isoform-specific *daf-16/FoxO* mutant alleles (Chen et al. 2015). Since both DAF-16A and F isoforms are the major isoforms in regulating life span (Kwon et al. 2010, Chen et al. 2015), we employed a prioritization strategy based on the hierarchical action of DAF-16 isoforms in longevity control and focused our attention to target genes that are primarily regulated by DAF-16A isoform and the target genes that are redundantly regulated by both DAF-16A and F isoforms.

*acs-3* is redundantly upregulated by both DAF-16A and F isoforms. We demonstrated that ACS-3 is required for full life span extension in animals with reduced DAF-2/IGFR signaling since a nonsense mutation in *acs-3* suppresses the life span extension of *daf-2/IGFR* mutant. Overexpression of ACS-3::GFP can rescue the shortened life span phenotype of *daf-2;acs-3* double mutant, however, it is not sufficient to extend life span in the wild-type background or further extend the longevity of a *daf-2* mutant.

The *in vivo* biological functions of *acs-3*, especially in the context of aging, have yet to be determined. Expression of an *acs-3* cDNA construct in seam cell, which has not been implicated in the regulation of lipid metabolism, rescues the elevated fat storage phenotype of a missense *acs-3* allele (Mullaney et al. 2010). This suggests *acs-3* may act cell non-autonomously to influence organismal physiology as well as longevity. Therefore, it is of particular interest to determine the site of action for ACS-3 in promoting life span extension. We propose tissue-specific RNAi knockdown of *acs-3* in *daf-2* mutant background to identify the tissue requirement in life span control. In a complementary approach, we could reconstitute *acs-3*

cDNA driven by promoters specifically expressed in the amphid sheath cell, seam cell, and vulva of *daf-2;acs-3(null)* animals and test their ability to reverse the shortened life span phenotype.

Another strategy to elucidate the biological function of ACS-3 might be to determine whether ACS-3 acts in pathway with other downstream longevity regulators using genetic epistasis and molecular experiments. The predicted biochemical function of ACS-3 is to activate fatty acid for lipid metabolism. Published studies suggest the life-extending function of lipases depends on the generation of lipid signals that promote autophagy and downstream transcriptional events (Lapierre et al. 2011, O'Rourke et al. 2013, Folick et al. 2015). ACS-3 may play an important role in channeling or partitioning specific fatty acid species to promote autophagy. We could use reporter strains to assess the role of *acs-3* in autophagy. We could also attempt to identify ACS-3 substrates using unbiased metabolomic approaches and look for metabolites or fatty acids that are altered in a *daf-16* and *acs-3* dependent manner in a *daf-2* mutant background. In a candidate ligand approach, we could test the requirement of ACS-3 activity in animals supplemented with either oleic acid or palmitoleic acid, which have been associated with different long-lived *C. elegans* models (Han et al. 2017, Imanikia et al. 2019). Recent study has shown that lysosomal lipid chaperone LBP-8, which is necessary for the pro-longevity effect of lysosomal lipase LIPL-4, binds to oleic acid (Folick et al. 2015, Tillman et al. 2019). Interestingly, both oleic acid and palmitoleic acid have been found to induce the expression of GFP::LGG-1 autophagosome marker (Niso-Santano et al. 2015). Detailed molecular characterization in *acs-3* has the potential to elucidate the critical role of fat metabolism in longevity and offers new therapeutic targets to treat aging-associated diseases and promote healthy aging in human.

## COL-75 may induce ER UPR cell non-autonomously

In the course of studying the role of the conserved ER transmembrane protein TRAP-1 in the biogenesis of insulin-like peptides, we discovered that TRAP-1 and its mammalian ortholog TRAPα have conserved roles in promoting ER homeostasis that are independent of their function in insulin-like peptide biosynthesis (Li et al. 2019). To gain insight into this function of TRAP-1, we performed a forward genetic *mtro* screen for mutants that modify the *hsp-4::GFP* ER UPR reporter expression phenotype caused by *trap-1* mutant. We have pinpointed causal alleles in one *mtro* mutant with suppressed *hsp-4::GFP* expression and 8 *mtro* mutants with enhanced *hsp-4::GFP* expression as many of which are known components of the ER UPR pathway. In one strain with increased *hsp-4::GFP* expression, we identified a causal missense allele of the collagen gene *col-75* predicted to change asparagine 162 to lysine (N162K) in the triple helix domain. Independent *col-75* missense mutant alleles phenocopy the increased *hsp-4::GFP* expression phenotype of *col-75(N162K)*, suggesting the predicted misfolded COL-75 is likely biologically relevant in perturbing ER homeostasis. *col-75;xbp-1* double mutant suppresses the *hsp-4::GFP* expression caused by *col-75(N162K)*, suggesting the misfolded COL-75 variant can activate the canonical IRE-1-XBP-1 arm of the ER UPR pathway. Transcriptional *col-75p::dsRed* transgenic reporter reveals the expression pattern of *col-75* is distinct from the tissues with the strongest *hsp-4::GFP* expression in *col-75(N162K)* mutant background, indicating the possibility that *col-75* may induce ER UPR cell non-autonomously.

How does misfolded collagen variant induce ER UPR is still unknown. Our preliminary finding reveals *col-75(N162K)* minimally induces endogenous level of spliced *xbp-1* (*XBP-1S*) mRNA, suggesting the possibility of *xbp-1S*-independeent induction of *hsp-4::GFP* expression. We could assess the activation of PEK-1 or ATF-6 to detect the involvement of the other ER

UPR branches biochemically. The fact that *col-75(N162K)* mutant does not exhibit any common phenotypes observed in other collagen mutant strains hints to the compensatory effect of ERAD and/or autophagy since they are major quality control mechanisms in the degradation of misfolded ER proteins (Qi et al. 2017, Ferro-Novick et al. 2021). We could test the hypothesis that *col-75(N162K)* animals are able to maintain organismal health in spite of constitutive ER UPR activation through upregulation of ERAD and/or autophagy, both of which can be assayed by using fluorescent report strains. Identifying the site of action of *col-75(N162K)* in ER UPR is particularly important to distinguish between the mode of cell autonomous or non-autonomous induction of intestinal *hsp-4::GFP* expression. To accomplish this, we could use two approaches of driving expression of misfolded COL-75 variants and RNAi knockdown of *col-75(N162K)* activity in a tissue-specific manner as described previously in the discussion of chapter 3.

Establishing the identities of genes that influence the induction of ER UPR by misfolded collagen variant is critical to understand the mechanistic underpinning mediated by *col-75(N162K)*. We will perform an unbiased forward genetic screen to identify genes that modify the *hsp-4::GFP* expression caused by *col-75(N162K)*. As we did for the *mtro* screen that yielded *col-75(N162K)*. We expect to identify mutants whose gene products normally function to promote COL-75 folding, trimerization, posttranslational modification and trafficking, as well as those that play general roles in maintaining ER homeostasis to enhance *hsp-4::GFP* expression. For mutants with decreased *hsp-4::GFP* expression, we anticipate to identify alleles of *ire-1* and *xbp-1*, and gene products that normally function to convey information about COL-75 misfolding to *hsp-4*. One potentially exciting class of mutants with reduced GFP expression would be those that are involved in communication cross tissues and are capable of transmitting signals from the excretory or amphid socket cells, or phasmid sheath cells to the intestine to

increase *hsp-4::GFP* expression [if *col-75(N162K)* acts non-autonomously to induce intestinal *hsp-4::GFP* expression].

# Conclusion

This dissertation sought to understand the function of *acs-3* in regulating DAF-16-mediated longevity and the molecular role of *col-75* in ER UPR. While we have not yet reached definitive answers to both inquiries, we have identified a new DAF-16/FoxO target gene with interesting and potentially conserved effects on fatty acid metabolism as well as longevity and demonstrated the presence of mutant collagen variant in specific tissues may lead to ER UPR to influence organismal health. We hope that the characterization described here will go on to facilitate studies which further elucidate the mechanisms of life span extension and develop new interventions to treat or prevent diseases associated with elder population and ER homeostasis.

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