Identifying a role for nonessential eIF3 subunits *eif-3.K* and *eif-3.L* in the regulation of endoplasmic reticulum homeostasis and longevity in *Caenorhabditis elegans*

by

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ABSTRACT

The eukaryotic initiation factor 3 (eIF3) is a protein complex composed of 13 subunits in mammals, and is an essential scaffold of the molecular interactions required for the formation of the 43S preinitiation complex (PIC). While these 13 subunits are broadly conserved within the eukaryotic phylogeny, both biochemical and evolutionary evidence suggests that translation initiation can proceed with a vastly reduced number of eIF3 subunits, with as few as six subunits in the yeast species *Saccharomyces cerevisiae.* In this study, I report that homologs of eIF3 subunits eIF3k and eIF3l are nonessential in *Caenorhabditis elegans*, and that in their absence there is no defect in bulk protein translation. Surprisingly, mutants lacking these subunits exhibit both enhanced endoplasmic reticulum homeostasis and increased longevity, which implicates a potential regulatory role for these subunits in the maintenance of organismal physiology.

Thesis Supervisor: Dennis H. Kim
Title: Ivan R. Cottrell Professor of Immunology

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Chapter One

Introduction

In this thesis I will describe the genetic characterization of two genes that encode proteins that reside within the eukaryotic initiation factor complex eIF3. One of these genes emerged from a forward genetic screen in *C. elegans* for mutations that confer resistance to endoplasmic reticulum (ER) stress. We observe that these mutant animals are additionally long-lived, which we observe is via a distinct genetic pathway from that of resistance to endoplasmic reticulum stress. For this reason, the introduction will proceed with a description of these three relevant topics: ER homeostasis and the unfolded protein response, the genetic study of longevity in *C. elegans*, and the translation initiation factor eIF3, which will be synthesized in Chapter 2.

Overview

The production of thousands of distinct protein products presents eukaryotic cells with a significant physiological burden, and on several fronts. First, nascent proteins often require the co- or post-translational assistance of folding chaperones to achieve their native conformation, and the rapid acquisition of native states is critical in preventing aggregation in the highly concentrated protein environment of the cell. These chaperones hydrolyze ATP, thereby placing immense energetic demands on the cell. Second, many proteins must acquire post-translational modifications in order to achieve their proper conformation and function. Third, this process is highly dynamic, as both internal and external signals exert varying forces on translational output, thereby requiring physiological mechanisms to couple folding requirements to folding capacity. And fourth, any protein destined for the endocytic or secretory pathway, such as secreted proteins, transmembrane proteins, and proteins destined for lysosomes and

other endocytic compartments, must be co-translationally trafficked across a lipid bilayer into the endoplasmic reticulum (ER), where it is folded and maintained within a topologically distinct compartment.

The Endoplasmic Reticulum

The endoplasmic reticulum (ER) is the largest organelle within most eukaryotic cells, and is comprised of a continuous and interconnected web of tubes and sheet-like cisternae that both define the nuclear envelope and permeate the rest of the cellular volume (Shibata et al., 2006). This compartment is topologically distinct from the cytoplasm and is enclosed by as single lipid bilayer. Consistent with its relative size within the cell, this organelle possesses a host of responsibilities. In addition to being a vital store of intracellular calcium and the site of phospholipid biosynthesis, it serves as the entry point of proteins into the secretory pathway and therefore participates in the folding, maturation, and sorting of client proteins within the ER lumen prior to export to the Golgi. Proteins shuttled through the ER encounter an oxidizing redox environment, in contrast to the reducing environment of the cytosol, and this redox state facilitates the formation of disulfide bonds that assist in maintaining the native state of secreted proteins within the oxidizing environment of the extracellular environment.

As the folding needs of the ER outstrip the ability of the organelle to fold and process these proteins, a phenomenon referred to as ER stress, an adaptive homeostatic mechanism is engaged that adjusts cellular physiology in order to bring the flux in and out of the compartment into alignment, that is collectively called the Unfolded Protein Response (UPR).

The Unfolded Protein Response

It was first observed over 30 years ago that the accumulation of misfolded protein in the ER dramatically upregulates the expression of a family of ER- resident glucose-regulated proteins (GRPs) (Kozutsumi et al., 1988), later identified as ER chaperones (Dorner et al., 1992), and this study was an early indicator of the fundamental homeostatic logic of the UPR—namely that protective pathways become upregulated in response to proteostatic challenges to the ER. That these proteins are upregulated in response to glucose was attributed to the disruption of glycosylation in the presence of high glucose, and proper glycosylation is critical to the proper processing and sorting of ER-resident proteins. Genetic and biochemical study in the following decade identified the mediators of this response as well as the mechanisms underlying their function.

In order to maintain a productive folding environment within the ER, eukaryotic cells must first overcome the challenge of detecting the accumulation of unfolded protein, and then they must convey this signal across the lipid bilayer where, in metazoans, it is transduced into both transcriptional and translational changes that remediate the folding environment of the ER. In metazoans this is mediated through three branches, defined by the integral membrane protein sensors IRE-1, PERK, and ATF6 (Figure 1). The most ancient and conserved of these pathways, mediated through IRE1, is conserved among all eukaryotes and remains the most well-characterized branch of the UPR, while PERK and ATF6 likely arose later in evolution and are only present in metazoans.

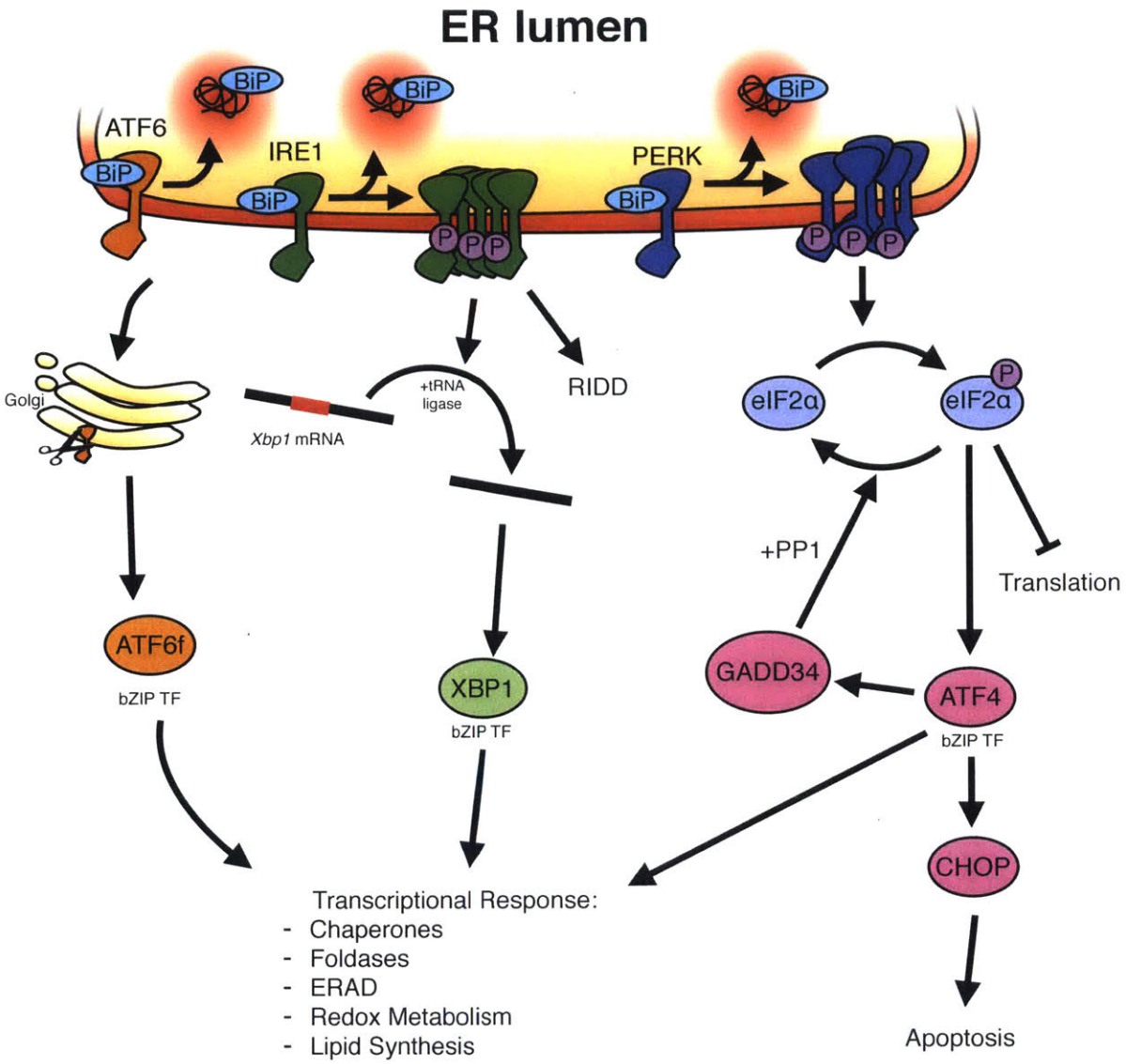


Figure 1. Mechanism of Unfolded Protein Response (UPR) activation and signal transduction. Integral membrane protein sensors ATF6, IRE1, and PERK are activated by dissociation of the ER-resident chaperone BiP. IRE1 undergoes oligomerization and trans-autophosphorylation to activate an endoribonuclease domain that cleaves an intron from the *Xbp1* mRNA with the assistance of a tRNA ligase. Expression from this spliced transcript produces an active bZIP transcription factor XBP1 that activates a transcriptional response that promotes ER homeostasis. PERK is similarly activated, though in addition to trans-autophosphorylation it serves as a kinase for the initiation factor eIF2α. Phosphorylation of eIF2α both inhibits general protein translation as well as promotes translational upregulation from uORF-containing transcripts, including that of the bZIP transcription factor ATF4. ATF4 both promotes the UPR transcriptional response but additionally promotes expression of GADD34, which in conjunction with Protein Phosphatase 1 (PP1), dephosphorylates eIF2α, thereby serving as a negative feedback regulator of this pathway. The apoptosis-promoting transcription factor CHOP

is also synthesized. Dissociation of ATF6 relaxes its retention within the ER. Transport to the Golgi results in its cleavage by the proteases S1P and S2P, yielding a bZIP transcription factor, ATF6f, which in addition to the other two branches of the UPR regulates a concerted transcriptional response.

Components and mechanism of the Unfolded Protein Response

IRE1

IRE1 (inositol requiring enzyme 1) was co-discovered in two separate yeast screens designed to identify mutants that do not induce ER-chaperone expression following induction of ER stress by the inhibition of N-linked glycosylation (Cox et al., 1993; Mori et al., 1993). This protein consists of an N-terminal sensor domain within the ER lumen and a CDK2-like serine/threonine kinase domain fused to a unique ribonuclease domain within the cytosol (Sidrauski and Walter, 1997). This protein is maintained in its inactive form through its binding with the ER folding chaperone BiP within the ER lumen (Carrara et al., 2015), and as unfolded protein intermediates compete for BiP binding, Ire1 is released and then assembles into activated, higher-order oligomers (Bertolotti et al., 2000). An alternative model of activation suggests that, at least in yeast, unfolded proteins themselves are the activating ligands responsible for Ire1 activation (Credle et al., 2005; Gardner and Walter, 2011). While kinase activity is not required for Ire1 function (Rubio et al., 2011), trans-autophosphorylation within the Ire1 oligomer is required for its cooperative assembly and disassembly (Chawla et al., 2011). Upon activation, the ribonuclease domain of Ire1 participates in the noncanonical cleavage of a short intron from the mRNA transcript of *HAC1* in yeast (Sidrauski and Walter, 1997) or *XBP1* in metazoans (Calfon et al., 2002). Ligation of the spliced exons in both yeast and metazoans is then mediated by a tRNA ligase (Jurkin et al., 2014; Lu et al., 2014; Sidrauski et al., 1996), and *HAC1/XBP1* is the only substrate known to participate in this unusual splicing reaction. Proper splicing of *HAC1* or *XBP1* yields an

active bZIP transcription factor, and the transcriptional targets of HAC1/XBP1 include ER chaperones, components of the ER biosynthesis pathway, genes of the secretory pathway, as well as genes required for ER-associated degradation (ERAD) system (Lee et al., 2003; Shaffer et al., 2004; Sriburi et al., 2007), which is a mechanism that participates in the retrotranslocation and degradation of misfolded proteins (reviewed in (Smith et al., 2011)). Each component of this concerted transcriptional program assists in the restoration of ER homeostasis.

In addition to the transcriptional response mediated through HAC1/XBP1, the ribonuclease of IRE1 has also been found to mediate cleavage from a subset of additional mRNAs that are associated with the ER membrane, through a mechanism called IRE1-dependent decay (RIDD) (Hollien and Weissman, 2006). This additional function of IRE1 has been observed in fission yeast, plants, flies, and mammals (Hollien et al., 2009; Kimmig et al., 2012; Mishiba et al., 2013), and it is thought to transiently reduce the translational burden from ER-localized proteins while liberating translation and translocation components for the incoming wave of UPR-regulated gene products. Surprisingly, RIDD does not require IRE1 oligomerization (Tam et al., 2014), indicating that the allosteric changes associated with oligomerization are only essential to permit cleavage of *HAC1/XBP1*.

While the yeast genome contains a single isoform of Ire1p, two different homologs of Ire1 are present within mammalian cells: IRE1α, which is expressed ubiquitously, and IRE1β, which is present only in the gut epithelium (Bertolotti et al., 2001). Surprisingly, IRE1β is unable to associate with BiP and therefore might have

evolved towards the detection of specific substrates within the intestinal ER lumen (Oikawa et al., 2012).

ATF6

A second branch of the UPR is mediated through the type-II transmembrane protein Activating Transcription Factor 6 (ATF6). Unlike the other branches of the UPR, activation of ATF6 results in its transport to the Golgi (Chen et al., 2002), most likely initiated by loss of ER-retention by BiP release, where the Golgi-resident site 1 protease (S1P) and site 2 protease (S2P) cleave the luminal and cytosolic domains from the transmembrane portion of this gene (Ye et al., 2000). The cytosolic domain of ATF6 contains a bZIP transcription factor, which when liberated translocates to the nucleus to activate gene expression (Haze et al., 1999).

While *C. elegans* produce only one isoform of this gene, which is surprisingly involved in the constitutive rather than ER-stress-inducible expression of its targets (Shen et al., 2005), the mammalian genome contains two ATF6 isoforms: ATF6α and ATF6β. These two isoforms exhibit differential stability and gene activation potential, and so different ratios of these isoforms is thought to regulate the strength and duration of the resulting transcriptional response (Thuerauf et al., 2007). ATF6α is also able to form heterodimers with XBP1, which in mammalian cells has a greater affinity for its binding motif than either transcription factor alone (Yamamoto et al., 2007).

PERK

The third and last branch of the UPR is mediated by an additional type-I transmembrane protein called double-stranded RNA-activated protein kinase (PKR)-like

ER kinase, or PERK. Activation of PERK occurs similarly to IRE1: dissociation of BiP results in its oligomerization and auto-phosphorylation (Bertolotti et al., 2000). In addition to its own auto-phosphorylation, PERK also phosphorylates the eukaryotic initiation factor eIF2α at serine-51, which when phosphorylated becomes a competitive inhibitor for its own guanine nucleotide exchange factor (GEF), eIF2B. As a result, bulk protein translation is attenuated and the flux of protein into the ER is slowed. PERK is one of four eIF2α kinases present in mammalian cells, each of which phosphorylates this same serine-51 residue in response to different cellular stresses: GCN2 responds to amino acid starvation, PKR responds to viral infection, and HRI responds to heme deficiency. Collectively these four kinases function in a pathway called the Integrated Stress Response (ISR) due to their common transcriptional and translational response (Harding et al., 2002).

Although bulk protein translation is inhibited by eIF2α phosphorylation, some transcripts which contain upstream open reading frames (uORFs) within their 5' UTR are enhanced for translation following eIF2α phosphorylation, due to leaky scanning produced by low levels of the eIF2-GTP-Met-tRNA$_i$ ternary complex (reviewed in (Hinnebusch, 2005)). One such gene is the bZIP transcription factor Activating Transcription Factor 4 (ATF4). Microarray studies comparing wild-type with ATF4$^{-/-}$ and PERK$^{-/-}$ mouse fibroblast cells indicate that this pathway is responsible for the proper induction of many genes involved in the UPR transcriptional response (Harding et al., 2003). Many genes induced by ATF4 involve secretory processes between the ER and

Golgi, which provides another mechanism for remediating ER stress as well as enhancing ATF6 transport and activation (Teske et al., 2011).

Two other important downstream targets of ATF4 are the growth arrest and DNA damage-inducible 34 (GADD34) and the transcription factor C/EBP homologous protein (CHOP). GADD34 is a largely unstructured protein, and serves as an adapter for the serine/threonine protein phosphatase 1 (PP1) (Choy et al., 2015). This protein complex then promotes eIF2α dephosphorylation, thereby serving as a negative feedback loop which restores normal protein synthesis (Novoa et al., 2001). If ER homeostasis cannot be restored, activation of the pro-apoptotic transcription factor CHOP induces apoptosis (Zinszner et al., 1998).

Physiological Consequences of UPR deficiency

The greater complexity of the UPR in multicellular metazoans likely arose from the challenge posed by maintaining multiple cell types, including professional secretory cells. Some cells must cope with radical changes to their secretory program, such as plasma cells of the mammalian immune system, which undergo a radical expansion of their ER in order to secrete large amounts of immunoglobulin (Ig) proteins, and unsurprisingly XBP-1 is required for this process (Iwakoshi et al., 2003). Cells within the myeloid lineage such as erythrocytes, on the other hand, have almost no need for a protein secretory system. Therefore, while the UPR may have evolved in single-celled eukaryotes to cope with periodic environmental stressors, it is required in higher metazoans such as mammals for the development and maintenance of secretory cell types.

Animals that are deficient in UPR function exhibit a range of developmental and secretory impairments, indicating the increased diversity of UPR effector pathways in metazoans arose to combat specific physiological, rather than environmental, challenges. IRE1$\alpha^{-/-}$ mice, for example, are embryonicly lethal (Urano et al., 2000) and XBP1$^{-/-}$ mice, also embryonic lethal, show impaired liver development (Reimold et al., 2000). ATF6$\alpha^{-/-}$ and ATF6$\beta^{-/-}$ mice both viable, but pups homozygous for both mutations also inviable (Yamamoto et al., 2007). Lastly PERK$^{-/-}$ mice are viable, but post embryonically they exhibit signs of diabetes mellitus (manifested as decreased serum insulin and increased serum glucose as compared to WT) and exocrine pancreatic insufficiency (Harding et al., 2001).

Contributions from our lab have provided yet another example of a physiological requirement for the unfolded protein response. We observe that *C. elegans* mutants deficient in *xbp-1*, and not *pek-1* or *atf-6*, fail to develop to adulthood when exposed to the pathogen *Pseudomonas aeruginosa* (Richardson et al., 2010). This developmental requirement for *xbp-1* upon *P. aeruginosa* exposure is alleviated in a double mutant in which the p38 MAPK and innate immunity regulator *pmk-1* is additionally removed. Together these data indicate that *xbp-1* serves an essential function in the host protection against the secretory insult that accompanies innate immune activation. This physiological requirement for *xbp-1* in the tolerance of innate immune activation will serve as the basis for a forward genetic screen presented in Chapter 2.

The genetic study of longevity in *Caenorhabditis elegans*

With a lifespan of only two-three weeks, genetic research into the determinants of longevity in *C. elegans* has proven incredibly fruitful. Many genetic pathways that influence longevity were first identified in *C. elegans* and later found to influence aging in diverse species, from yeast to mouse. A common mechanism of these genetic perturbations is the coopting of cellular processes that contribute to cellular maintenance. While numerous mechansisms have been identified that modulate the longevity of *C. elegans*, including inhibition of mTOR and decreased mitochondrial respiration, I will focus on two which are of particular relevance to this study.

The Insulin/IGF-1-like signaling (IIS) pathway

The best characterized pathway that contributes to longevity in *C. elegans* is that of the insulin/IGF-1-like signaling (IIS) pathway (Figure 2), whose influence on lifespan was reported almost 25 years ago (Kenyon et al., 1993) with the description of a temperature-sensitive mutation in the insulin-like receptor *daf-2* that extends lifespan over two-fold. This influence on longevity was found to require the downstream FOXO family transcription factor *daf-16*, which was already known to be required for the entry into the developmentally arrested dauer larval stage (Vowels and Thomas, 1992). *daf-2* is the only insulin/IGF-1 receptor expressed in worms, and receives signals from nearly 40 insulin-like insulin peptides which can serve as agonists or antagonists to the DAF-2 receptor (Pierce et al., 2001). Signaling from this receptor activates the downstream phosphoinositide 3-kinase (PI3K) AGE-1, a gene in which mutation is also observed to extended lifespan (Lithgow et al., 1995). PI3K activation results in indirect DAF-16

phosphorylation through downstream kinases PDK-1, AKT-1, AKT-2, and SGK-1 (Hertweck et al., 2004), thereby promoting its exclusion from the nucleus.

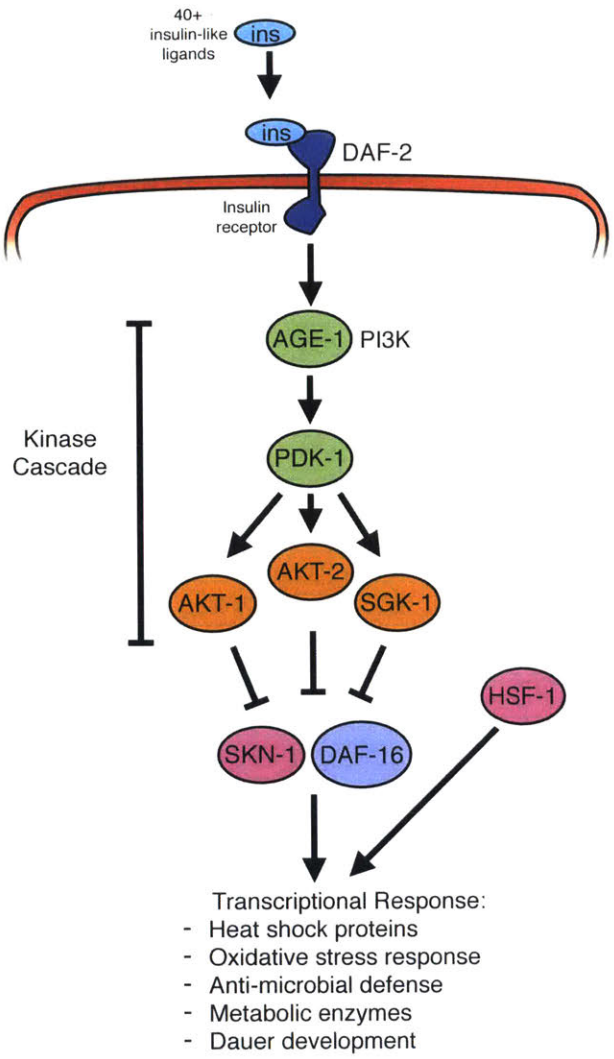


Figure 2. Genetic regulators of the Insulin/IGF-1-like signaling (IIS) pathway in *C. elegans*. 40 or more insulin-like peptides function as agonists and antagonists to a single insulin receptor in *C. elegans*, DAF-2. DAF-2 activates a kinase cascade that ultimately promotes nuclear exclusion of DAF-16 and SKN-1 through phosphorylation. Together with co-regulators SKN-1 and HSF-1, DAF-16 is able to activate a pro-longevity transcriptional response that is also essential for formation of the long-lived and stress-resistant dauar larval stage.

Bioinformatic analysis of insulin-signalling mutants have identified many target genes of DAF-16, and genes which are upregulated include those involved in cellular stress responses, antimicrobial defense, proteostasis, and metabolism (Halaschek-Wiener et al., 2005; McElwee et al., 2003; Murphy et al., 2003). Gene regulation induced by reduced insulin signaling closely resembles the gene expression changes associated with the formation of stress-resistant dauer larvae, which is consistent with the requirement for this transcription factor in dauer formation (McElwee et al., 2004). In addition to lifespan extension, IIS mutants share additional phenotypes with dauer larvae, including heat and oxidative stress resistance (Lithgow et al., 1995).

Though the transcription factor DAF-16 asserts pro-longevity signals, it does not function alone and requires the function of additional signaling molecules in order to confer lifespan extension. Another key effector of the IIS pathway is the transcription factor heat shock factor 1 (HSF-1), which is required both for lifespan extension in *daf-2* mutants (Hsu et al., 2003), as well as the induction of heat shock proteins (hsps) when IIS is diminished. A second effector of IIS-mediated longevity is the Nrf (NF-E2-related factor) transcription factor SKN-1. SKN-1 is required for the phase 2 detoxification response and guards against oxidative stress through the induction of detoxification and antioxidant enzymes (McMahon et al., 2001). This gene is regulated similarly do DAF-16, wherein kinases downstream of DAF-2 and AGE-1, namely AKT-1, -2, and SGK-1, phosphorylate and inactivate SKN-1. Under conditions of reduced insulin signaling, hypophosphorylation of SKN-1 induces its accumulation in nuclei and results in target gene expression (Tullet et al., 2008). Though critical for the oxidative stress response

downstream of IIS, SKN-1 can additionally be activated by both the p38 MAPK pathway (Inoue et al., 2005) as well as the UPR (Glover-Cutter et al., 2013; Hourihan et al., 2016), and therefore serves as an important integrator of diverse cellular stressors.

Several studies have attempted to clarify the tissues in which IIS regulates longevity. Tissue-specific rescue of a *daf-16* transgene into short-lived *daf-16(-)* or *daf-16(-);daf-2(-)* mutants found that DAF-16 activity in the intestine and no other tissue significantly increases lifespan, indicating that this is the likely tissue that mediates lifespan extension when IIS is reduced (Libina et al., 2003). This tissue-specific requirement for DAF-16 is consistent with another mechanism known to enhance lifespan in *C. elegans*, germline ablation (Kenyon and Hsin, 1999), which is accompanied by DAF-16::GFP accumulation in intestinal nuclei (Lin et al., 2001). Tissue specific rescue of the *daf-2* insulin receptor into the long-lived *daf-2(-)* mutant background indicates that neuronal expression is able to most strongly suppress lifespan extension, though a modest lifespan suppression is still observed when *daf-2* is reintroduced into the intestine, consistent with IIS function within this tissue to regulate longevity (Larsen, 1993; Wolkow et al., 2000).

Rates of protein synthesis

Reducing rates of protein synthesis through deletion and knockdown of factors in the translational apparatus has, in the last decade, emerged as an additional method for improving longevity in *C. elegans*. It should be noted that several additional modes of lifespan extension, such as reduced IIS and mTOR signaling, also result in reduced rates of translation, though genetic epistasis of translation inhibition with one or both of these pathways suggests that many of these genetic perturbations involve independent mechanisms of lifespan extension. By initiating RNAi feeding targeting essential genes in adult worms, many new classes of genes involving translational processes were identified that induce lifespan extension, most likely through the downregulation of protein translation. These include aminoacyl-tRNA synthetases, ribosomal proteins, and initiation factors (Chen et al., 2007; Curran and Ruvkun, 2007; Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). Knockdown of many of these genes was observed to additionally extend lifespan in the *daf-16(-)* mutant background, indicating that they do not extend lifespan by modulating insulin signaling (Curran et al., 2009), though there are conflicting results in the literature likely resulting from differences in experimental methodology (Hansen et al., 2007; Syntichaki et al., 2007; Wang et al., 2010). Knockdown of eIF4G homolog *ifg-1* was additionally found to be additive with both caloric restriction and TOR inhibition (Hansen et al., 2007).

Complicating the mechanistic understanding of these genetic knockdowns on longevity is the fact that knockdown of these different genes undoubtedly results in quantitative and qualitative differences in the resulting translational output. RNAi

knockdown of a tRNA-synthetase, for example, likely has a radically different translational profile from RNAi knockdown of an initiation factor. Furthermore, decreasing the kinetics of a step of translation initiation would not result in a proportional decrement in the production of every protein, as the rate of translation reflects both the abundance of an mRNA species as well as its translational efficiency, and the impairment of a step of translation initiation or elongation would not be expected to affect translational efficiency of different transcripts uniformly. A notable example of this was observed following knockdown of the eIF4G homolog *ifg-1* in *C.* elegans, which resulted in an overall decrease in rates of protein synthesis but an increased relative expression from stress response genes, which is based in part on their transcript length (Rogers et al., 2011). Decreasing rates of translation would also reduce competition of nascent or newly synthesized proteins with the translocation machinery of the ER or the mitochondria, as well as the proteostasis machinery required for protein folding and degradation (Taylor and Dillin, 2011). Consistent with this hypothesis, dietary restriction in *Drosophila* results in a relative increase in the translation and translocation of mitochondrial genes. The resulting enhanced mitochondrial activity promotes lifespan extension, and is dependent on the general translational repressor 4EBP1 (Zid et al., 2009).

The role of the eIF3 complex in translation initiation

Overview of eIF3 structure and function

Eukaryotic translation initiation is a highly regulated and ordered process that must ultimately bring the small ribosomal subunit, a closed-loop mRNA complex, and

Met-tRNA^{Met} into a complex at the proper start codon prior to large ribosomal subunit joining and, ultimately, protein synthesis (reviewed in Hinnebusch, 2014). This process is choreographed through the sequential binding and scaffolding interactions of dozens of proteins that either individually as eukaryotic initiation factors (eIFs) or as eIF protein complexes direct the steps of this process (Sonenberg and Hinnebusch, 2009). An early step in this process is the formation of the 43S preinitiation complex (PIC) (Figure 3), in which the eIF2:GTP:Met-tRNA$_i$ ternary complex is loaded onto the small (40S) ribosomal subunit with the assistance of eIF1, eIF1A, eIF3, and eIF5. The PIC then binds to the capped 5' end of an activated closed-loop mRNA complex via the cap-binding complex eIF4F (composed of the cap-binding protein eIF4E and its cofactors, eIF4A and eIF4G). Scanning then proceeds until an AUG codon is identified in the P-site, triggering arrest of scanning and the irreversible hydrolysis of GTP bound to eIF2. GTP hydrolysis promotes the release of eIF2:GDP as well as other initiation factors, and the large (60S) ribosomal joins and the elongation phase of translation commences.

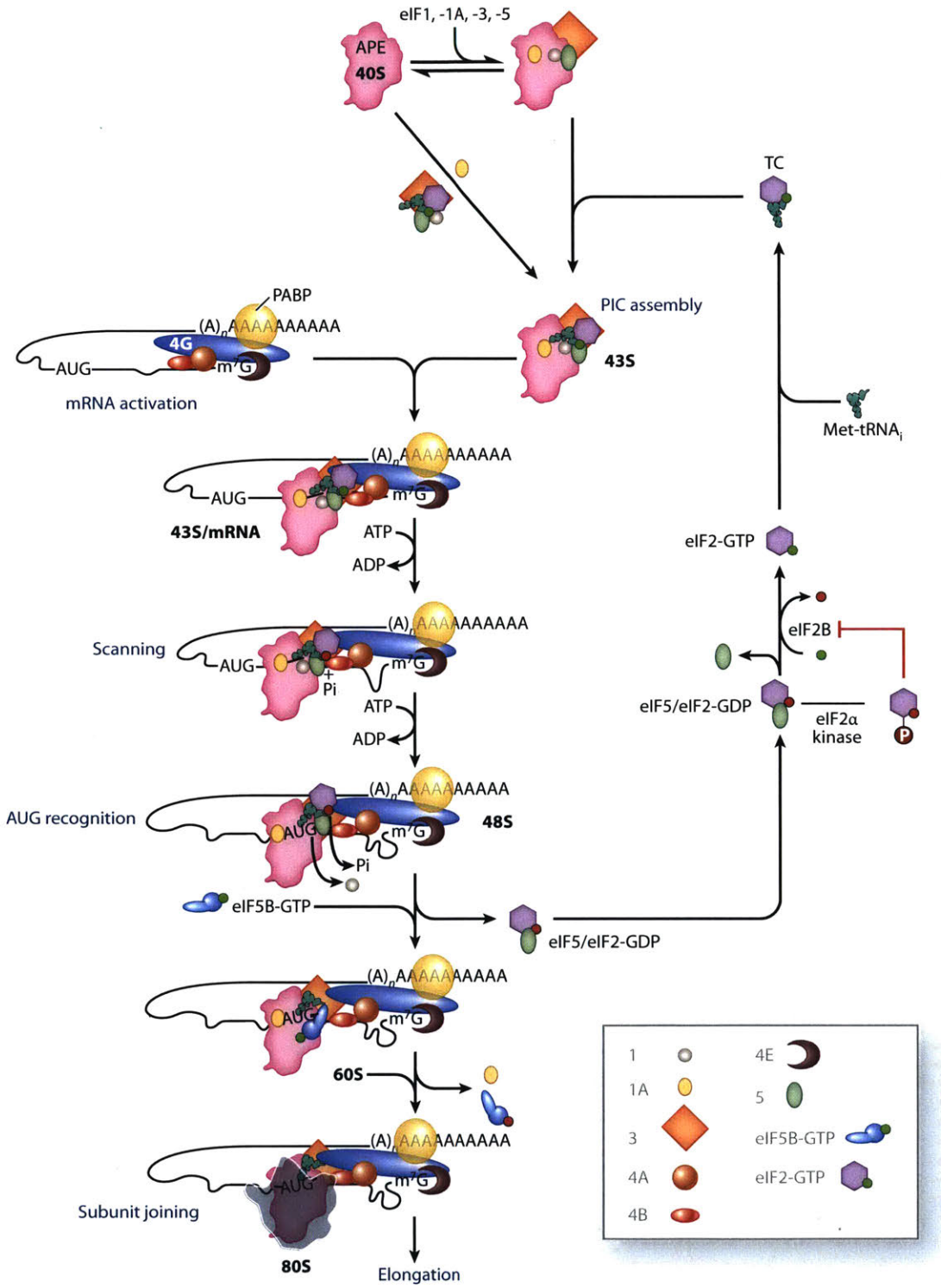


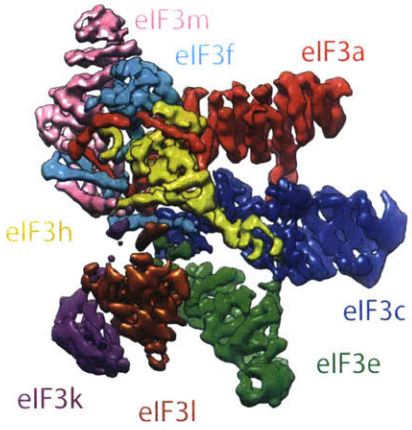
Figure 3. An overview of translation initiation. Adapted from (Hinnebusch, 2014).

The eIF3 complex, at ~800 kDa, is by far the largest and most structurally complex of the eukaryotic initiation factors, and is roughly half the size of the small ribosomal subunit. The mammalian eIF3 complex is composed of thirteen non-identical subunits named from eIF3a-m, with the substoichiometric eIF3j subunit enhancing the interaction between the 40S ribosomal subunit and the rest of the holocomplex (Fraser et al., 2004). While only six of these subunits are present in the budding yeast *Saccharomyces cerevisiae*, an eIF3 complex bearing all 13 subunits can be found within metazoan, plant, and fungal clades (Smith et al., 2013), indicating that the ancestral eukaryote most likely possessed all of the subunits found within the mammalian complex, and that the evolutionary pressure towards a compact genome may have resulted in a reduced repertoire of subunits being maintained in budding yeast as well as other eukaryotes.

Structural studies first by mass spectrometry (Zhou et al., 2008) and later by cryo-electron microscopy (Erzberger et al., 2014; Georges et al., 2015; Hashem et al., 2013; Simonetti et al., 2016) have revealed the molecular organization of this complex both in isolation and in the context of the 43S PIC. In both contexts this complex is found to organize around an 8-subunit "core" which is localized to the cytosolic face of the 40S subunit, with the five remaining subunits attached peripherally (Figure 4). The structural core of this complex is composed of six subunits (a, c, e, k, l, m) which contain a so-called PCI domain (named after the three protein complexes that are rich in these domains: the proteosome lid, the COP-9 signalosome, and the eIF3 protein complex), as well as two subunits (f and h) which contain an MPN (Mpr1/Pad 1 N-

terminal) domain. This core is stabilized by an alpha-helical bundle in which an alpha-helix is contributed from each subunit. Assembly of this complex requires the sequential assembly of multiple eIF3 sub-complexes (Smith et al., 2016). Following start-codon recognition, three of the peripheral subunits rearrange to the 40S intersubunit face (Figure 4B) (Simonetti et al., 2016).

A



B

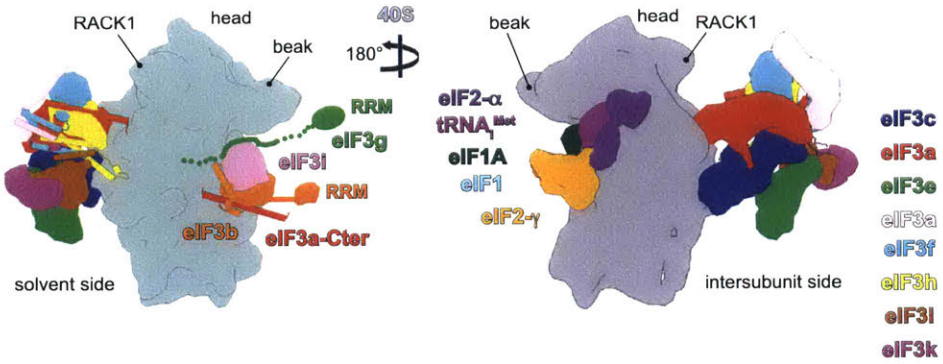


Figure 4. Assembly of eIF3 core and its association with the 40S ribosomal subunit. (A) Cryo-em structure of the eIF3 core. Adapted from des Georges et al., 2015. (B) Location of eIF3 subunits and various cofactors within the 43S Preinitiation Complex (PIC). Adapted from Simonetti et al., 2016.

Interestingly, this structural organization is additionally present in two other large eukaryotic macromolecular complexes—the COP9 signalosome (CSN) and the 26S proteasome lid—with an apparent one-to-one correspondence between subunits as well as remarkable topologic similarity, having the appearance of a five-lobed "hand" (Enchev et al., 2010; Pick et al., 2009). The CSN has been implicated in cellular functions as diverse as cell cycle progression (Doronkin et al., 2003), DNA repair (Doronkin et al., 2002), and gene regulation (Chamovitz, 2009), though its best-characterized molecular function is in the removal of Nedd8—an ubiquitin-like protein—from a family of E3 ubiquitin ligases, thereby enhancing their function (Lyapina et al., 2001). The last of these structural orthologs, the 26S proteasome lid, functions as part of the regulatory particle of the 26S proteasome, and is responsible for cleaving polyubiquitin chains from proteasome-targeted proteins prior to their degradation by the 26S proteasome (Dambacher et al., 2016; Finley, 2009). Consistent with a possible shared evolutionary origin among these complexes, eIF3 subunit eIF3f also has been identified as having deubiquitinase (DUB) activity, which has been associated with the regulation of Notch activation in mammalian cells (Moretti et al., 2010). To date this is the only known enzymatic function associated with eIF3, with its other functions thought to arise predominantly from its use as a molecular scaffold. Surprisingly, a single gene in *C. elegans*, *cif-1*, serves as the only homolog of CSN subunit Csn7 as well as eIF3m, and this subunit is required for both translational initiation and CSN function, indicating that this gene is a shared subunit of both protein complexes (Luke-Glaser et al., 2007).

These data collectively implicate cellular connections between protein synthesis and degradation.

As mentioned, the eIF3 complex in *S. cerevisiae* contains only six subunits: a, b, c, g, i, and j. This suggests that the main functionality of the complex can be performed by a vastly reduced repertoire of subunits. Surprisingly, evaluation of the functional core of the mammalian complex using an *in vitro* ribosome binding-toeprinting assay identified a partially overlapping set of subunits required for *in vitro* eIF3 function: a, b, c, e, f, and h (Masutani et al., 2007). This suggests that in addition to the general translational functions mediated by the eIF3 complex, the additional nonessential subunits may confer additional scaffolding and regulatory functions to this complex. Some additional translational functions of eIF3 have already been defined, including its participation in the recycling of posttermination ribosomal complexes (Pisarev et al., 2007). In support of this view, dysregulation of eIF3 subunit expression has been reported in the malignant transformation of many cancers (Akiyoshi et al., 2001; Hershey, 2015; Shi et al., 2006; Zhang et al., 2007). Biochemical and genetic study of these additional functions particularly within the last decade has yielded key insights into the ancillary functions of this important protein complex.

Translational regulation by eIF3

The earliest insight into specialized translational functions of the eIF3 complex came with the observation that two eIF3 complexes predominate in the fission yeast *S. pombe*, defined by the mutual exclusion of either the eIF3e or eIF3m subunits (Zhou et al., 2005). The eIF3m-containing complex appears to support general translation

initiation, while the eIF3e-defined complex translates only a small subset of cellular mRNAs. The basis of this discrimination has not been identified. Somewhat later, the deletion of eIF3h in fission yeast was found to induce defects in meiosis and sporulation, despite having only a marginal impact (10-15%) on global synthesis rates (Ray et al., 2008). The specificity of these pleitoropies suggests that deficiency of this subunit induces relative changes to the translational program at the level of individual mRNAs rather than to absolute rates of protein synthesis. Further study of this subunit in zebrafish identified two isoforms of eIF3h, one of which is exclusively expressed in the nervous system and facilitates development of these tissues through the selective translation of genes required for proper neural development (Choudhuri et al., 2010; 2013).

Additional mechanisms of translational regulation by eIF3 have recently been identified in mammalian cells. Meyer and colleagues have identified a mechanism by which eIF3 can bind a modified ribonucleotide, N^6-methyladenosine (m^6A), within the 5' UTR of some transcripts in order to initiate translation through an unknown scanning-dependent cap-independent mechanism (Meyer et al., 2015). It had previously been observed that m^6A deposition within the 5' UTR of mRNAs is dynamic and responsive to diverse cellular stresses, an example being the increased m6A deposition within the 5' UTR of the heat shock protein HSP70 in response to heat shock (Dominissini et al., 2012), and the cap-independent translation initiation conferred by eIF3 likely allows for continued function of stress-response pathways when general cap-dependent translation is inhibited. Although this modified nucleotide is additionally present in the

mRNA of plants (Zhong et al., 2008), yeast (Clancy et al., 2002), and flies (Hongay and Orr-Weaver, 2011), it is absent in *C. elegans* due to the lack of a methyltransferase METT3 homolog (Greer et al., 2015).

Concurrent with this discovery, Lee and colleagues identified yet another mechanism of translational regulation by eIF3. By performing photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP), they found that several eIF3 subunits: a, b, d, and g, are able to bind directly to mRNAs via stem-loop structures within the 5' UTR of these transcripts (Lee et al., 2015), and that this eIF3-bound gene set is enriched for those associated with cancer-associated growth pathways including apoptosis, cell cycle progression, and differentiation. This interaction with eIF3 can be either stimulatory or inhibitory to translation from these transcripts. Recently, this specialized translational mechanism was found to be mediated by the cap-binding activity of eIF3d, which can mediate cap-dependent translation from these select transcripts when the canonical cap-binding protein eIF4E is inactivated (Lee et al., 2016).

In addition to direct effects on transcript binding and protein synthesis, eIF3 can also serve as a platform upon which other proteins can exert translational control. A notable example of this is the nutrient sensor mTOR and its downstream effector, the kinase S6K1. Under basal conditions, the translation-promoting S6K1is bound to eIF3 in its unphosphorylated inactive state. Following exposure to growth promoting factors such as insulin, 12-myristate 13-acetate (PMA), and epidermal growth factor (EGF), mTOR binds eIF3, activates S6K1 through a phosphorylation reaction, and releases

S6K1-P, which is then able to promote translation through its phosphorylation of proximally associated proteins of the PIC, such as the initiation factor eIF4B (Holz et al., 2005). The binding of mTOR to eIF3 is also found to increase the affinity of eIF3 for eIF4G, which is a subunit of the cap-binding complex eIF4F, thereby providing additional enhancement to translation initiation (Harris et al., 2006). In contrast, the upregulation of the p56 family of mammalian proteins, which are induced in virus-infected cells, has been shown to bind eIF3 and inhibit general protein translation through at least two distinct mechanisms: either by blocking eIF3 interaction with the eIF2-GTP-Met-tRNA$_i$ (Hui et al., 2003), or by inhibiting the interaction between eIF3 and eIF4F (Hui et al., 2005). Taken together, these examples highlight the many ways in which eIF3 can exert translational control over both general process of translation initiation as well as the specific translational output from individual mRNAs.

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Chapter Two

Mutations in Nonessential eIF3k and eIF3l Genes Confer Lifespan Extension and Enhanced Resistance to ER Stress in *C. elegans*

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CER and KCR isolated and characterized the original screen isolate *qd213*
ENC performed RT-PCR experiment in Figure 5D and development assay in Figure S1
RD performed electron microscopy experiments
DJC performed all other experiments
MKT assisted in ribosome profiling and with computational analysis
DJC and DHK prepared the manuscript
DJC WVG and DHK reviewed and edited the manuscript

Introduction

The genetic study of longevity of *C. elegans* has established how single mutations in conserved signaling pathways may have dramatic effects on animal lifespan (Antebi, 2007; Kenyon, 2010). In addition, reduction-of-function mutations or RNAi-mediated knockdown of genes encoding components required for mRNA translation, which reduces levels of protein synthesis and reduces rates of growth and development, have also been shown to extend lifespan of *C. elegans* (Chen et al., 2007; Curran and Ruvkun, 2007; Hansen et al., 2007; Pan et al., 2007; Syntichaki et al., 2007). Alterations in mRNA translation can also influence the expression of genes that may contribute to changes in lifespan (McColl et al., 2010; Rogers et al., 2011), suggesting that lifespan extension is not simply a consequence of diminished levels of bulk mRNA translation when translation initiation is perturbed.

The regulation of mRNA translation is pivotal in a number of diverse responses to cellular stress. In particular, the accumulation of misfolded proteins in the endoplasmic reticulum (ER) activates a conserved compensatory response, the Unfolded Protein Response (UPR), which results in the increased expression of ER chaperones, components of ER-associated protein degradation, and attenuated translation through the phosphorylation of eIF2α (Walter and Ron, 2011). The UPR was initially characterized with toxins that cause misfolded protein accumulation in the ER, but physiological roles of the UPR are now well established in the development of secretory cell types and in the pathogenesis of disease (Walter and Ron, 2011; Wang and Kaufman, 2012). In *C. elegans*, the activation of innate immunity induces the UPR,

which is required for survival in the presence of pathogenic bacteria (Richardson et al., 2010). A number of studies suggest that ER homeostasis and UPR activation may have both cell-autonomous and cell-non-autonomous effects on organismal stress physiology and longevity (Denzel et al., 2014; Henis-Korenblit et al., 2010; Kulalert and Kim, 2013; Taylor and Dillin, 2013). In addition, the NRF2-type transcription factor SKN-1 in *C. elegans*, an established regulator of longevity and stress resistance (An and Blackwell, 2003; Oliveira et al., 2009; Tullet et al., 2008), is a focal point of key reciprocal regulatory interactions with UPR signaling pathways (Glover-Cutter et al., 2013; McColl et al., 2010). These data suggest that the maintenance of ER homeostasis is an important determinant of organismal stress response and longevity.

Here, we report the genetic characterization of subunits of the translation initiation factor eIF3, a 13-subunit complex that coordinates the assembly of the 43S pre-initiation complex that is competent for mRNA recruitment and translation initiation in eukaryotes (Hinnebusch, 2006; Walter and Ron, 2011). Mapping of eIF3 subunit interactions by mass spectrometry (Zhou et al., 2008) and recent cryo-electron microscopy structures (Georges et al., 2015; Simonetti et al., 2016) of the eIF3 complex have defined the configuration of eIF3 protein subunits and their interaction with the 40S ribosomal subunit. Whereas the 13 subunits of eIF3 are conserved from *C. elegans* to humans (Smith et al., 2013), eIF3 of *Saccharomyces cerevisiae* has only six subunits, and reconstitution of human eIF3 subunits in ribosome-toeprinting assays suggest that some eIF3 subunits, including eIF3k and eIF3l, may be dispensable for initiation of mRNA translation (Masutani et al., 2007). Of note, altered expression of eIF3 subunits

have been observed to be associated with malignant transformation of mammalian cells (Hershey, 2015; Shi et al., 2006). Moreover, recent work utilizing RNA crosslinking and immunoprecipitation methods has shown that some mammalian eIF3 subunits associate with distinct mRNA transcripts involved in cellular proliferation (Lee et al., 2015). Taken together, these prior studies of eIF3 suggest that the eIF3 complex may have dual roles—an essential functional core complex of eIF3 that is required for ribosome recruitment and initiation of mRNA translation, and an additional regulatory role that may modulate the differential translation of specific mRNAs or perhaps function outside the context of translation initiation.

Here, we report our studies that establish that eIF3 subunits *eif-3.K* and *eif-3.L* are nonessential in *C. elegans*, and that their loss does not affect rates of bulk protein synthesis. We find that loss of either subunit confers a 40% increase in lifespan and enhanced resistance to ER stress. Our data suggest that the evolutionarily conserved but nonessential *eif-3.K* and *eif-3.L* subunits of eIF3 function in the regulation of cellular ER homeostasis and organismal longevity.

Results

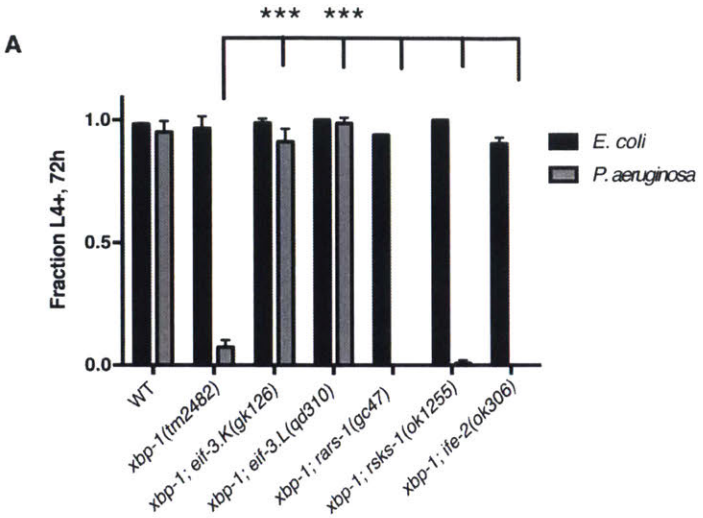
Mutations in *eif-3.K* and *eif-3.L* confer enhanced resistance to ER stress, independent of XBP-1

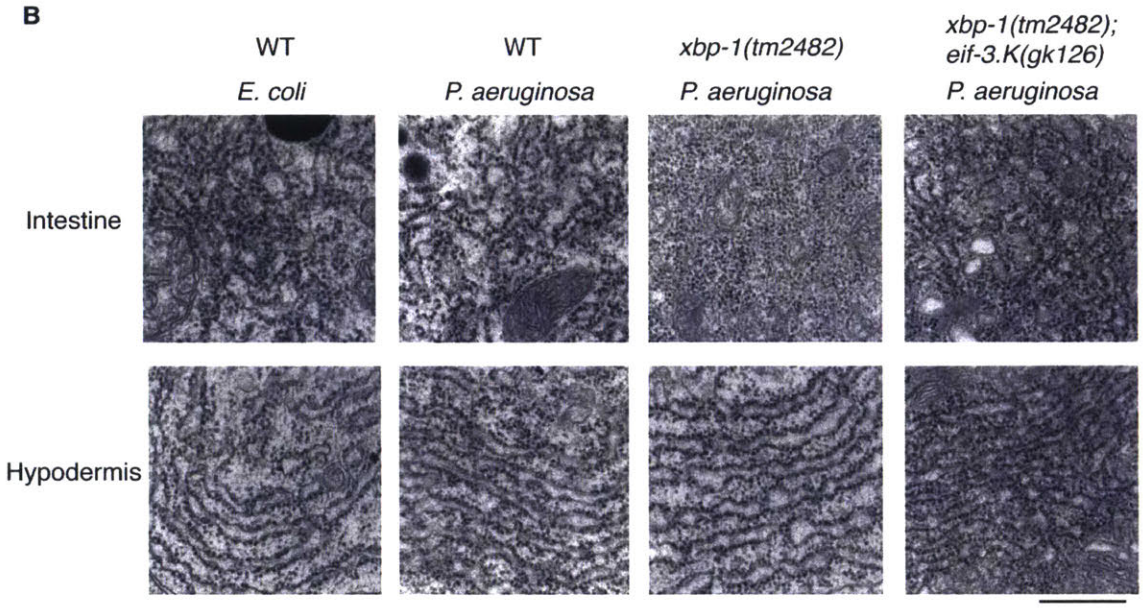
Previously, we demonstrated that the UPR is induced in the intestine of *C. elegans* in response to the activation of innate immunity following infection by pathogenic *P. aeruginosa* PA14 (Richardson et al., 2010). Activity of the UPR regulator XBP-1 was found to be essential for larval development on pathogenic *P. aeruginosa* but not on non-pathogenic *E. coli*, indicating an essential function for the UPR in the physiological tolerance of innate immune activation. In the current study, we performed a forward genetic screen to isolate mutations that could suppress the larval lethality of *xbp-1* mutant animals grown in the presence of *P. aeruginosa.* We identified one such suppressor mutation in the gene encoding the translation initiation factor subunit *eif-3.K*, *qd213*, which causes an early nonsense mutation in this gene (Fig S1). We confirmed that another allele of *eif-3.K*, *gk126*, which contains a deletion that eliminates the start codon and is a putative null allele, also suppressed the larval lethality of the *xbp-1* mutant animals in the presence of *P. aeruginosa* (Fig 1A).

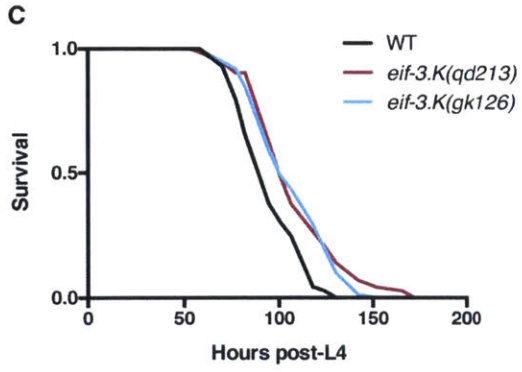
Fig 1. Loss of *eif-3.K* or *eif-3.L* suppresses larval lethality of *xbp-1* mutants on *P. aeruginosa*.

(A) Development assay monitoring the growth and viability of the indicated genotypes on *E. coli* or *P. aeruginosa* at 25°C. 50-100 eggs were laid on each plate and following 72h the fraction reaching the L4 larval stage or older were counted. Error bars reflect the S.D. of 3 plates. A Student's *t*-test was used to assess significance: **P<0.01, ***P < 0.001. (B) Transmission electron microscopy of L3 larvae cultivated on *E. coli* or *P. aeruginosa* at 60,000x magnification visualizing intestinal or hypodermal ER morphology. Scale bar, 500nm. (C) Survival curves of L4 larvae of the indicated genotypes at 25°C following transfer to plates containing *P. aeruginosa*. Two biological replicates were performed with similar results.

Figure 1


A
*** ***
Fraction L4+, 72h
1.0
0.5
0.0
E. coli
P. aeruginosa
WT
xbp-1(tm2482)
xbp-1; eif-3.K(gk126)
xbp-1; eif-3.L(qd310)
xbp-1; rars-1(gc47)
xbp-1; rsks-1(ok1255)
xbp-1; ife-2(ok306)


B
WT
E. coli
WT
P. aeruginosa
xbp-1(tm2482)
P. aeruginosa
xbp-1(tm2482); eif-3.K(gk126)
P. aeruginosa
Intestine
Hypodermis


C
WT
eif-3.K(qd213)
eif-3.K(gk126)
Survival
1.0
0.5
0.0
0
50
100
150
200
Hours post-L4

We previously noted that *xbp-1* mutants exposed to pathogenic *P. aeruginosa* exhibit changes in ER morphology in intestinal cells—in particular, the loss of normal sheet and tubular architecture with dilated luminal spaces consistent with chronic unmitigated ER stress, as visualized by transmission electron microscopy (Richardson et al., 2010). By contrast, no such changes in ER morphology were evident in neighboring hypodermal cells in *xbp-1* mutant animals in the presence of *P. aeruginosa* (Figure 1B). We observed that mutation of *eif-3.K* partially suppressed the aberrant rough-ER morphology of intestinal cells, suggesting that eif-3.K deficiency suppresses lethality by protecting against intestinal ER toxicity of the *xbp-1* mutant animals on *P. aeruginosa*.

We considered that mutations that eliminate *eif-3.K* function might suppress the larval lethality of *xbp-1* mutant animals grown on *P. aeruginosa* either by enhancing resistance to ER stress, or by diminishing the innate immune response, as we had previously observed with mutations in the *pmk-1* gene encoding the innate immune regulator p38 mitogen-activated protein kinase (MAPK) (Richardson et al., 2010). If a mutation in *eif-3.K* attenuated the innate immune response, then we would anticipate that the *eif-3.K* mutant would exhibit enhanced susceptibility to killing by *P. aeruginosa*. However, we found that *eif-3.K* mutant animals were in fact slightly resistant to *P. aeruginosa* infection (Fig 1C).

eif-3.K* and *eif-3.L* are not essential for viability or bulk protein synthesis in *C. elegans

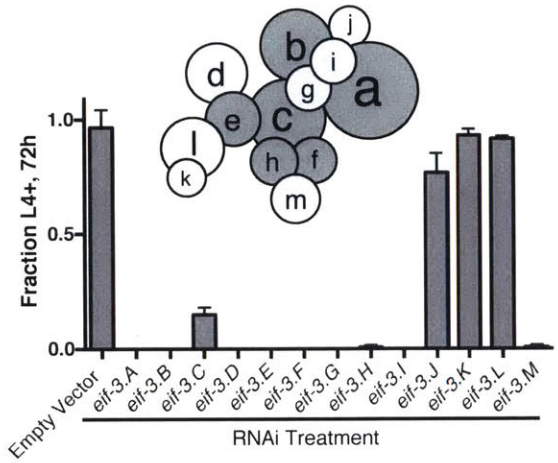
We were surprised to observe that *eif-3.K* is dispensable in *C. elegans*, as an ortholog of this eIF3 subunit is present in the genome of many metazoans, plants, and fungi within the eukaryotic phylogeny (Smith et al., 2013), suggesting an important function for this subunit. In order to understand the requirement for the other eIF3 subunits *in vivo*, we systematically knocked down each subunit by RNAi feeding and determined that almost all were required for normal growth and viability (Fig 2A), which corroborates prior genome-wide RNAi-based studies (Kamath et al., 2003; Sönnichsen et al., 2005). Knockdown of *eif-3.J* by RNAi was well-tolerated, but we generated a loss-of-function mutation in this gene, *eif-3.J (qd311)*, and observed that hermaphrodites homozygous for this mutation were sterile (Fig 2B). Knockdown of subunits *eif-3.K* and *eif-3.L*, by contrast, had no such effects on viability and fertility, and mutants carrying loss-of-function mutations in these genes are viable. This indicates that *eif-3.K* and *eif-3.L* are the only nonessential subunits of the eIF3 complex in *C. elegans*.

Fig 2. *eif-3.K* and *eif-3.L* are the only nonessential subunits in *C. elegans*, and mutants lacking these subunits do not have phenotypes associated with attenuated protein translation.

(A) Individual eIF3 subunits were knocked down for one generation by RNAi feeding at 20°C. Eggs were then laid on the normal laboratory food source *E. coli* OP50 and evaluated for their ability to reach the larval stage L4 or older after 72h at 20°C. Inset: Interaction diagram of eIF3 subunits based on mass spectrometry of Zhou et al. [22]. Gray subunits represent the functional core of the mammalian complex sufficient to allow for initiation *in vitro* by Masutani et al. [25]. (B) Progeny of *eif-3.J* heterozygotes were assayed for sterility and then genotyped. Number of worms scored for each genotype is indicated in parentheses. (C) Developmental time course of the indicated genotypes. Populations were synchronized by egg lay and monitored over time for their development to the L4 larval stage or older. (D) Brood size of the indicated genotypes at 20°C. Error bars represent the S.D. of 10 animals. (E) Egg-laying rate was determined by transferring worms to new *E. coli* plates in 12h periods, and progeny were counted following 24h incubation at 20°C. Error bars represent the S.D. of 10 animals.

Figure 2

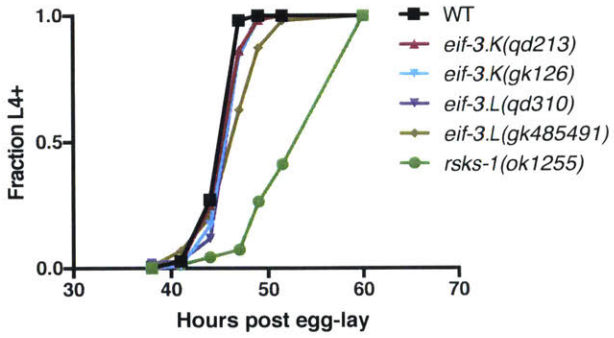
A



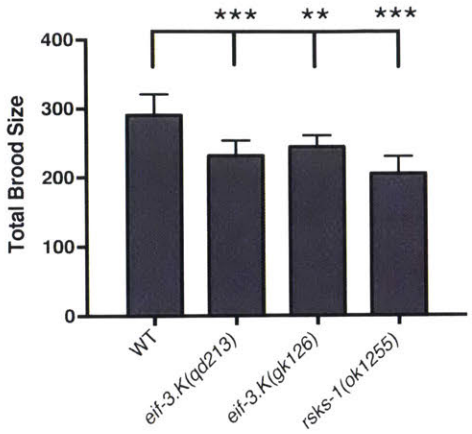
B

Genotype	Fraction Sterile
+ / +	0.0% (19)
eif-3.J / +	0.0% (56)
eif-3.J / *eif-3.J*	100.0% (22)

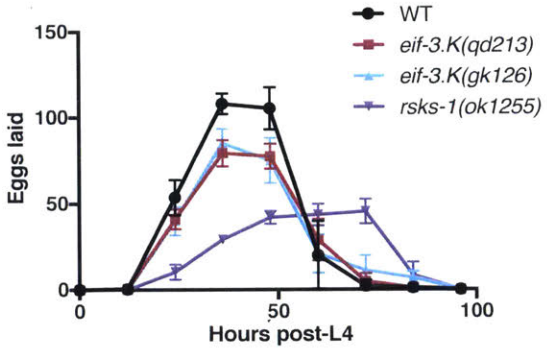
C



D



E



Biochemical analysis of the eIF3 complex in the filamentous fungus *Neurospora crassa* has shown that eIF3k and eIF3l form a dimer that then assembles with the rest of the complex (Smith et al., 2013). This is consistent with the aforementioned structural studies of eIF3 in which these two subunits are physically associated with each other on the periphery of the complex (Georges et al., 2015; Zhou et al., 2008), with eIF3k making almost all of its molecular contacts with eIF3 through the subunit eIF3l (Fig 2A, inset). These data suggest that any perturbation to the eIF3 complex in *C. elegans* generated by the absence of *eif-3.K* will be recapitulated by loss of *eif-3.L*, and phenotypes common to these two mutants likely reflect the function of these two genes within the context of the eIF3 complex. Consistent with this expectation, we observed that loss-of-function mutations in *eif-3.L* were able to suppress the larval lethality of the *xbp-1* mutant when exposed to *P. aeruginosa* (Fig 1A, Fig S1).

We initially hypothesized that loss of these eIF3 subunits might promote resistance to ER stress by attenuating bulk protein synthesis, which would diminish the secretory load to the ER. However, we observed that loss-of-function mutations in each of three genes required for normal rates of protein synthesis, *rars-1*, *rsks-1*, and *ife-2* (Hansen et al., 2007; Pan et al., 2007; Scott et al., 2013), were insufficient to suppress the larval lethality of *xbp-1* on *P. aeruginosa* (Fig 1A). Additionally, normal growth and larval developmental rate was observed in animals carrying loss-of-function mutations in *eif-3.K* and *eif-3.L* (Fig 2C), in contrast to the slowed growth rate of a mutant lacking the ribosomal protein S6 kinase *rsks-1*, which is known to have a decreased growth rate caused by attenuated protein synthesis (Hansen et al., 2007). We observed that the

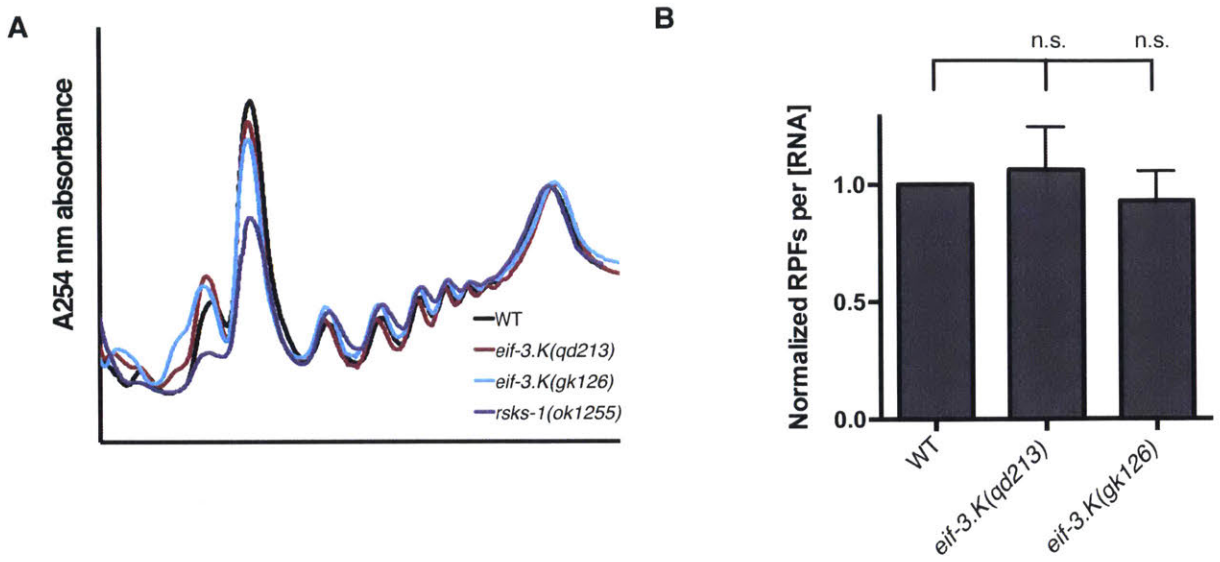
total brood size of *eif-3.K* mutants was diminished to ~ 80% of the wild-type brood size (Fig 2D). However, the age of peak egg-laying rate and the reproductive period of the *eif-3.K* mutant is identical to that of wildtype (Fig 2E), in marked contrast to that of the slow-growing *rsks-1* mutant.

We observe that polysome profiles of *eif-3.K* mutants were superimposable on the corresponding profiles of wildtype animals, with a similar fraction of RNA sedimenting in the 60S, monosome, and polysome fractions (Fig 3A), suggesting that rates of bulk translation initiation are not diminished in these mutants lacking EIF-3.K or EIF-3.L. By contrast, the polysome of the *rsks*-1 mutant is skewed towards the polysome fraction and away from the 40S/60S/monosome fraction, consistent with a defect in this mutant in translational elongation (Wang et al., 2001).

Fig 3. *eif-3.K* mutants do not have attenuated bulk translation.

(A) Polysome profiles of the indicated genotypes, harvested at the L4 stage. Identical quantities of total RNA were loaded onto sucrose gradients, and absorbance was measured at 254 nm. (B) Relative translation assayed by ribosome profiling, using whole yeast lysates as an internal standard. Whole lysates from *S. cerevisiae* were added to whole *C. elegans* lysates at a ratio of 1:20 based on total RNA concentration. Following ribosome profiling, the relative abundance of ribosome protected fragments (RPF) was determined by dividing the total number of reads that map unambiguously to the *C. elegans* genome by those that map unambiguously to the *S. cerevisiae* genome. Raw counts are presented in Table S1. Statistical significance was assessed by the Student's *t*-test.

Figure 3


A
A254 nm absorbance
WT
eif-3.K(qd213)
eif-3.K(gk126)
rsks-1(ok1255)
B
n.s.
n.s.
Normalized RPFs per [RNA]
1.0
0.5
0.0
WT
eif-3.K(qd213)
eif-3.K(gk126)

In order to assess rates of bulk protein synthesis on a quantitative basis, we performed ribosome profiling of wildtype and two alleles of *eif-3.K*, including an internal standard for normalization. To enable relative quantitation, a known quantity of whole yeast lysate was added to whole worm lysate, and following ribosome protected fragment (RPF) isolation and sequencing, this internal standard allowed us to determine relative rates of protein translation among genotypes by counting the total number of footprints that map unambiguously to the *C. elegans* genome and normalizing by the number of footprints that map unambiguously to the *S. cerevisiae* genome. We observe that by this quantitative biochemical method there is no attenuation of bulk protein synthesis in the *eif-3.K* mutant (Fig 3B). We performed this experiment with the intent of identifying genes whose translational efficiency (TE) is suppressed or enhanced in the *eif-3.K* and *eif-3.L* mutant backgrounds, but with the exception of the genes *eif-3.K* and *eif-3.L* themselves, were unable to identify statistically significant and reproducible deviations in footprint and total mRNA abundance, though we cannot exclude the possibility of translational changes to lowly expressed genes.

Mutations in *eif-3.K* and *eif-3.L* confer lifespan extension that is suppressed by a mutation in DAF-16

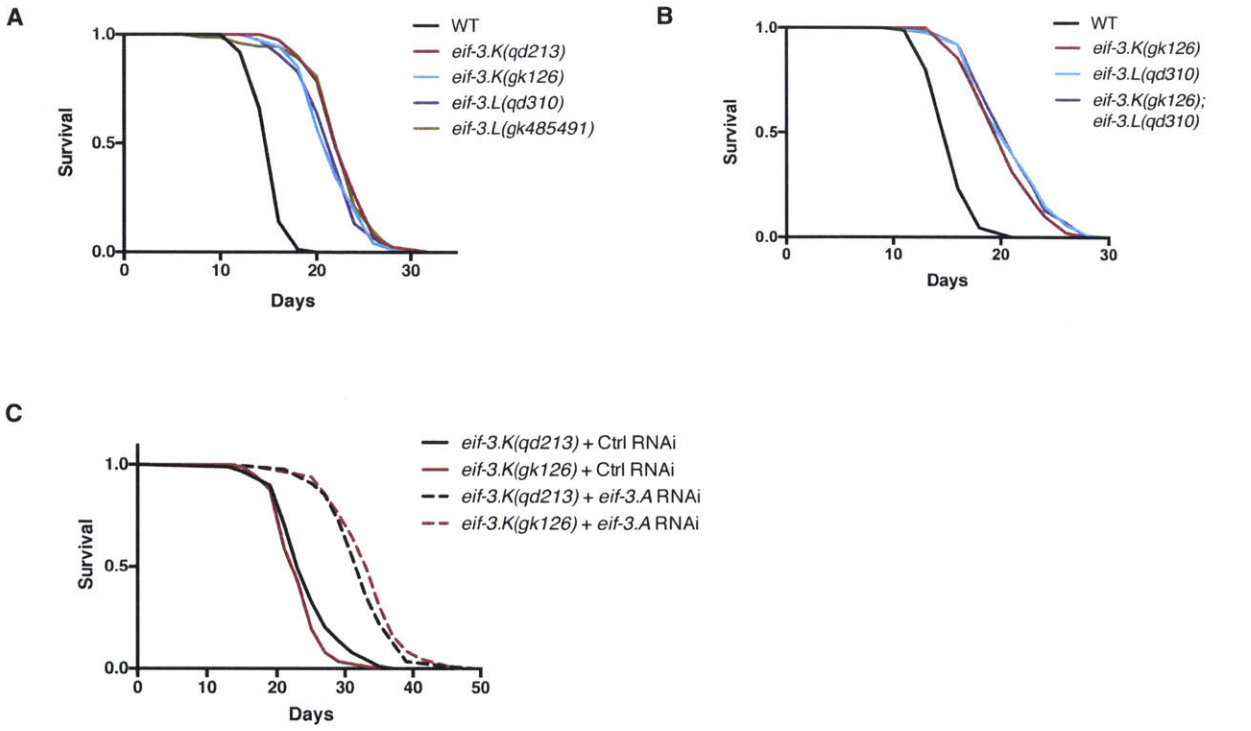
Strikingly, we observed a 40% increase in longevity among mutants lacking the nonessential eIF3 subunits *eif-3.K* and *eif-3.L* (Fig 4A). Prior studies have shown that molecular and genetic reduction of the levels of proteins required for mRNA translation, such as ribosomal proteins or initiation factors, is sufficient to extend lifespan (Chen et

al., 2007; Chiocchetti et al., 2007; Curran and Ruvkun, 2007; Hansen et al., 2007; Pan et al., 2007; Rogers et al., 2011; Steffen et al., 2008; Syntichaki et al., 2007; Tohyama et al., 2008), but in such instances bulk translation is diminished and the organism has a correspondingly slowed rate of growth and development. We did not observe a synergistic increase in longevity in a double mutant strain carrying mutations in both *eif-3.K* and *eif-3.L* (Figure 4B), indicating that in the wild-type background these two genes cooperate for their normal biological function.

Fig 4. Loss of *eif-3.K* or *eif-3.L* confers lifespan extension in *C. elegans* in a manner distinct from depletion of essential eIF3 subunits.

(**A-C**) Survival curves of the indicated genotypes at 25°C. Times indicated are days post-L4 stage. Lifespan statistics and replicate data is presented in Supplementary Table 2. For RNAi lifespan experiments, survival curves of the indicated genotypes transferred to control (*gfp*) or *eif-3.A* RNAi bacteria as L4 larvae. Worms were grown at 25°C for 3 days and shifted to 20°C for the remainder of the experiment.

Figure 4

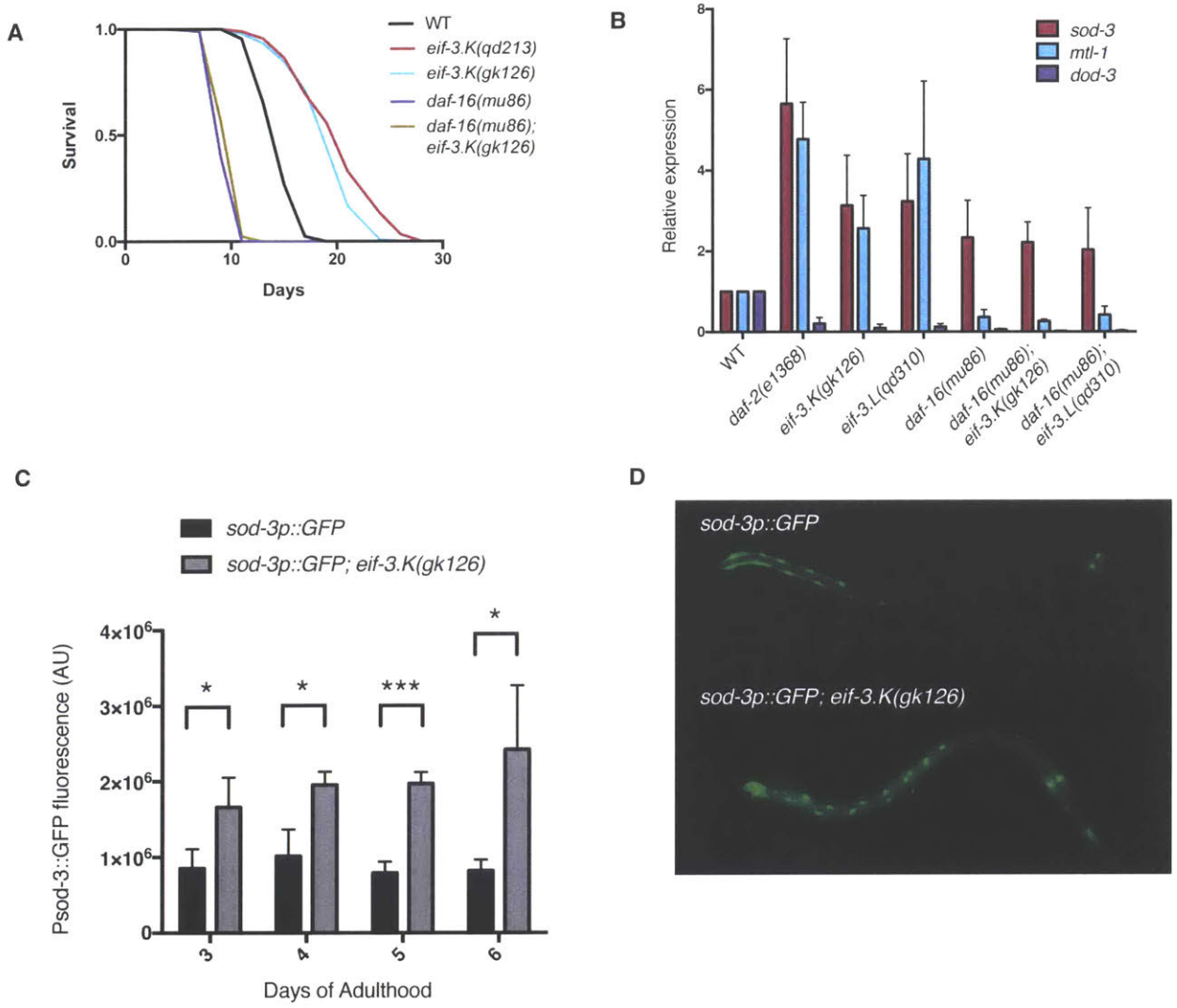

A
1.0
0.5
0.0
Survival
0
10
20
30
Days
WT
eif-3.K(qd213)
eif-3.K(gk126)
eif-3.L(qd310)
eif-3.L(gk485491)
B
1.0
0.5
0.0
Survival
0
10
20
30
Days
WT
eif-3.K(gk126)
eif-3.L(qd310)
eif-3.K(gk126);
eif-3.L(qd310)
C
1.0
0.5
0.0
Survival
0
10
20
30
40
50
Days
eif-3.K(qd213) + Ctrl RNAi
eif-3.K(gk126) + Ctrl RNAi
eif-3.K(qd213) + eif-3.A RNAi
eif-3.K(gk126) + eif-3.A RNAi

RNAi-mediated depletion of essential eIF3 subunits *eif-3.A (egl-45), eif-3.B,* and *eif-3.F* have previously been identified as a means of extending lifespan in *C. elegans* through the attenuation of protein synthesis (Curran and Ruvkun, 2007). We observed that RNAi of the essential eIF3 subunit *eif-3.A (egl-45)* could further increase the lifespan of the *eif-3.K* mutants (Fig 4C), consistent with the idea that loss of EIF-3.K and EIF-3.L subunits promotes longevity in a manner that is distinct from mechanisms caused by depletion of essential eIF3 subunits. The additive nature of these interventions on longevity suggests that loss of nonessential eIF3 subunits and depletion of essential eIF3 subunits contribute to lifespan extension through independent mechanisms.

Fig 5. Loss of *eif-3.K* or *eif-3.L* hyper-activates the DAF-16 transcription factor, which is required for lifespan extension.

(A) Survival curves of the indicated genotypes at 25°C. Times indicated are days post-L4 stage. Lifespan statistics and replicate data is presented in Supplementary Table 2. (B) Relative expression of *sod-3*, *mtl-1*, and *dod-3* in the indicated genetic backgrounds as assayed by qRT-PCR of synchronous L4 larvae. Error bars represent the S.D. of three replicates. (C) Total fluorescence of the *sod-3p::GFP* transgene in worms of the indicated genotypes was quantitated at several adult timepoints. Error bars represent the S.D. of 10 worms. (D) Composite image depicting representative images of Day 3 adult worms of each indicated genotype expressing the transgene *sod-3p::GFP*.

Figure 5


A
Survival
1.0
0.5
0.0
0
10
20
30
Days
WT
eif-3.K(qd213)
eif-3.K(gk126)
daf-16(mu86)
daf-16(mu86); eif-3.K(gk126)
B
Relative expression
8
6
4
2
0
sod-3
mtl-1
dod-3
WT
daf-2(e1368)
eif-3.K(gk126)
eif-3.L(qd310)
daf-16(mu86)
daf-16(mu86); eif-3.K(gk126)
daf-16(mu86); eif-3.L(qd310)
C
sod-3p::GFP
sod-3p::GFP; eif-3.K(gk126)
Psod-3::GFP fluorescence (AU)
4x10^6
3x10^6
2x10^6
1x10^6
0
*
*
***
*
3
4
5
6
Days of Adulthood
D
sod-3p::GFP
sod-3p::GFP; eif-3.K(gk126)

To gain insight into the downstream mechanisms involved in lifespan extension conferred by loss of EIF-3.K, we carried out genetic epistasis analysis with the *eif-3.K* mutant. We determined that a mutation in the Forkhead transcription factor DAF-16 completely suppressed the lifespan extension conferred by mutation of *eif-3.K* (Fig 5A), which suggests that DAF-16 functions downstream of, or in parallel to, the loss of *eif-3.K* or *eif-3.L* in modulating organismal longevity of *C. elegans*. In order to determine if activity of the DAF-16 transcription factor is modulated in the *eif-3.K/L* mutant backgrounds, we performed qRT-PCR on well-characterized DAF-16 targets, including two known to be upregulated by mutation in *daf-2* (*sod-3 and mtl-1*), as well as one known to be downregulated in *daf-2* mutants (*dod-3*) (Murphy et al., 2003). We found that the relative expression of these genes in the *daf-2* mutant background was recapitulated by loss of *eif-3.K* or *eif-3.L* (Fig 5B), indicative of increased DAF-16 activity in the absence of *eif-3.K* or *eif-3.L*. Expression of these DAF-16 target genes in the *daf-16; eif-3.K* and *daf-16; eif-3.L* double mutants was not appreciably different from that of the *daf-16* single mutant, indicating that the changes in expression of these genes in the *eif-3.K* and *eif-3.L* mutants is dependent on DAF-16. We also observed that fluorescence from a *sod-3p::GFP* transgene was two-fold higher in the *eif-3.K* mutant background, and that this increased fluorescence remains steady throughout early adulthood (Fig 5C). Fluorescence from this transgene was particularly increased within intestinal cells (Fig 5D), where DAF-16 activity has been shown to be particularly important in promoting lifespan extension (Libina et al., 2003). Together these data suggest that increased activity of DAF-16 contributes to the lifespan extension observed

in *eif-3.K* and *eif-3.L* mutant animals, consistent with our observed epistasis data (Fig 5A).

Mutations in *eif-3.K* and *eif-3.L* confer resistance to tunicamycin

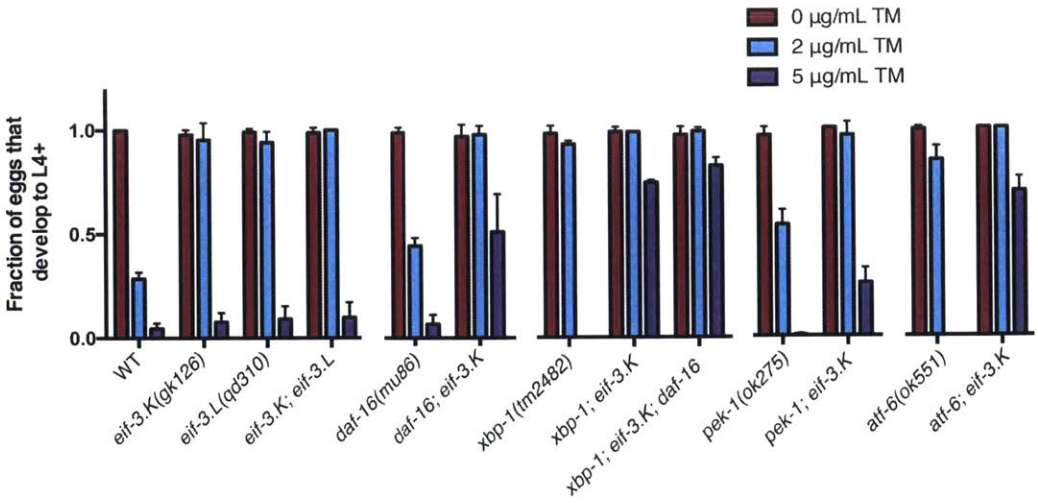
The observation that the loss of *eif-3.K* or *eif-3.L* could suppress the larval lethality and corresponding disruption of ER morphology of *xbp-1* mutant animals exposed to *P. aeruginosa* suggested that *eif-3.K* or *eif-3.L* mutants might exhibit enhanced resistance to ER stress. We assayed *eif-3.K* and *eif-3.L* mutants for their sensitivity to the ER-toxic drug tunicamycin, which inhibits protein glycosylation and results in protein accumulation in the ER. At a concentration of tunicamycin (2 µg/mL) at which the majority of wild type animals arrest and die during larval development, *eif-3.K* and *eif-3.L* mutants were resistant to this treatment (Fig 6A). Furthermore, we observed that *daf-16* was not required for resistance to tunicamycin, as the *daf-16; eif-3.K* double mutant was more resistant to tunicamycin than the *daf-16* single mutant. We wondered whether the resistance of the *eif-3.K* and *eif-3.L* mutants might involve some compensatory function from the other branches of the unfolded protein response, yet we find that none of the UPR regulators – *xbp-1*, *pek-1*, or *atf-6* – are required for the tunicamycin resistance of the *eif-3.K* mutant. These data indicate that, unlike lifespan extension, the improved ER homeostasis in these mutants is DAF-16 independent, and is additionally independent of the UPR.

Fig 6. Mutants lacking *eif-3.K* or *eif-3.L* are resistant to tunicamycin, independent of *daf-16* and regulators of the Unfolded Protein Response.

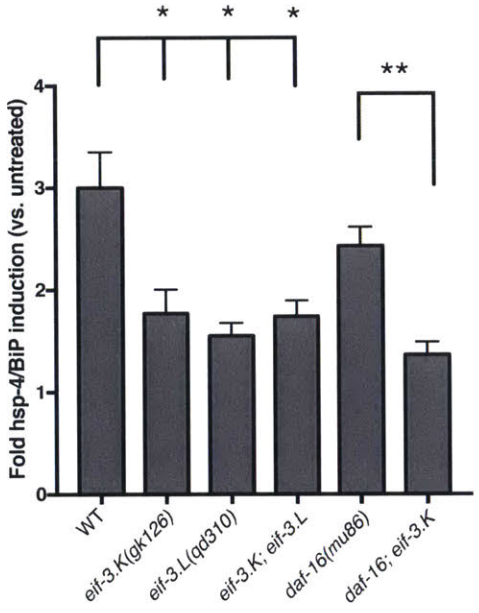
(A) Animals were scored for their ability to reach the L4 stage or older 72h after 50-100 eggs were laid on plates containing 0, 2, or 5 µg/mL tunicamycin. Error bars reflect the S.D. of three plates. Two biological replicates were performed with similar results. (B) *hsp-4* induction was measured by qRT-PCR after L4 larvae were transferred to plates containing 10 µg/mL tunicamycin for 4h. Error bars reflect the SEM of three replicates. A Student's *t*-test was used to assess significance: $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 6

A


0 µg/mL TM
2 µg/mL TM
5 µg/mL TM
Fraction of eggs that develop to L4+
1.0
0.5
0.0
WT
eif-3.K(gk126)
eif-3.L(qd310)
eif-3.K; eif-3.L
daf-16(mu86)
daf-16; eif-3.K
xbp-1(tm2482)
xbp-1; eif-3.K
xbp-1; eif-3.K; daf-16
pek-1(ok275)
pek-1; eif-3.K
atf-6(ok551)
atf-6; eif-3.K

B


*
*
*
**
Fold hsp-4/BiP induction (vs. untreated)
4
3
2
1
0
WT
eif-3.K(gk126)
eif-3.L(qd310)
eif-3.K; eif-3.L
daf-16(mu86)
daf-16; eif-3.K

We next assayed the induction of the ER-resident folding chaperone *hsp-4/BiP* upon acute tunicamycin treatment, which is a reporter of unfolded protein response (UPR) activation. We observed that *eif-3.K* and *eif-3.L* mutants exhibited reduced induction of *hsp-4/BiP* mRNA at high concentrations of tunicamycin (Fig 6B), and that this trend was also observed in the absence of *daf-16*. At first glance, this might appear somewhat paradoxical, as the wild type strain that has more susceptibility to tunicamycin has an increased induction of protective chaperone expression, but we suggest that these data reflect an enhancement to the ER folding capacity in the *eif-3.K* mutant that is capable of remediating stress produced by acute tunicamycin exposure. This phenomenon of improved tunicamycin resistance despite decreased *hsp-4* induction has previously been observed in *daf-2* mutants, but in a context that is *daf-16*-dependent (Henis-Korenblit et al., 2010).

EIF-3.K is expressed ubiquitously in *C. elegans*

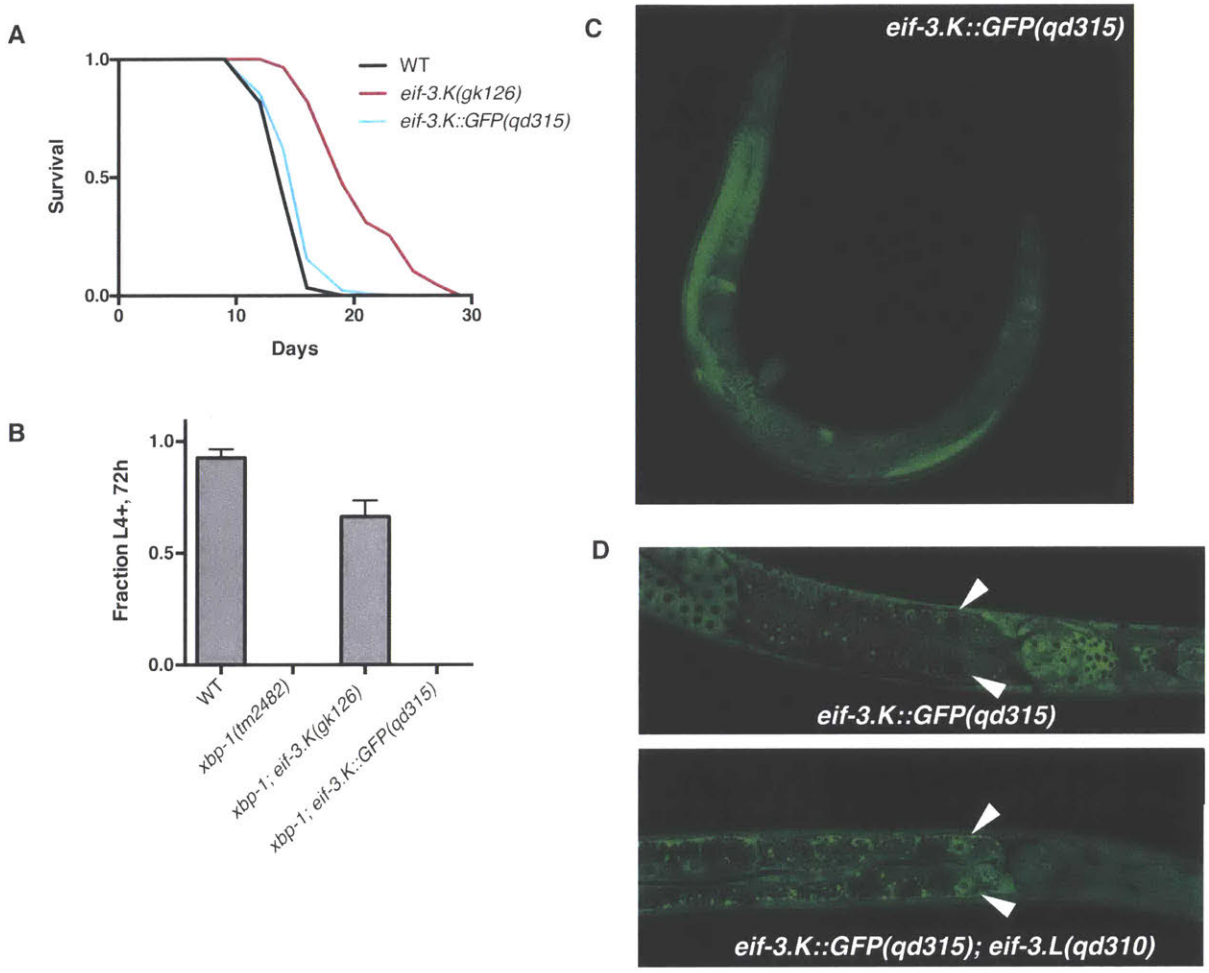
Given that subunits *eif-3.K* and *eif-3.L* are nonessential, and given the apparent bias towards retention of these two genes in multicellular eukaryotes (Smith et al., 2013), we wondered whether expression of these genes might be restricted to specific tissues. To this end, we engineered a C-terminal GFP tag onto the endogenous locus of *eif-3.K* using CRISPR/Cas-9, and validated function of this gene by phenotypic analysis. The fluorescently-tagged allele was neither long-lived (Fig 7A) nor able to suppress the larval lethality of the *xbp*-1 mutant grown on *P. aeruginosa* (Fig 7B), suggesting that the fusion-protein retained wild-type function. We find that EIF-3.K::GFP is expressed in all

tissues, with especially bright fluorescence in the *C. elegans* germline (Fig 7C). As expected, expression is restricted to the cytosol, the site of translation initiation (Fig 7D).

Fig 7. EIF-3.K::GFP is ubiquitously expressed in *C. elegans* and is localized to the cytosol.

(A) Lifespan curves of wild-type, the *eif-3.K* mutant, and a strain containing a fluorescently-tagged allele of *eif-3.K*. (B) Developmental assay monitoring the growth and viability of the indicated genotypes on *P. aeruginosa* at 25°C. Error bars reflect the S.D. of 3 plates. (C) Fluorescent micrograph of EIF-3.K::GFP at 10x magnification. (D) Fluorescent confocal image depicting intestinal nuclei of L4 larvae of the indicated genotypes at 20x magnification. Arrows indicate intestinal nuclei.

Figure 7


A
1.0
0.5
0.0
Survival
0
10
20
30
Days
WT
eif-3.K(gk126)
eif-3.K::GFP(qd315)
B
1.0
0.5
0.0
Fraction L4+, 72h
WT
xbp-1(tm2482)
xbp-1; eif-3.K(gk126)
xbp-1; eif-3.K::GFP(qd315)
C
eif-3.K::GFP(qd315)
D
eif-3.K::GFP(qd315)
eif-3.K::GFP(qd315); eif-3.L(qd310)

We observed that mutation of *eif-3.L* in the strain carrying the EIF-3.K::GFP permits the diffusion of EIF-3.K::GFP into the nucleus, though nucleolar exclusion is maintained (Fig 7D). This observation is consistent with the crystallographic and mass spectrometric evidence that *eif-3.L* serves as a bridge between *eif-3.K* and the rest of the eIF3 complex.

We performed tissue-specific rescue of the *eif-3.K* mutant in an effort to determine the specific tissues in which *eif-3.K* activity influences lifespan and ER stress resistance. We observed partial rescue of the lifespan extension phenotype of the *eif-3.K* mutant when *eif-3.K* was expressed under the control of multiple different tissue-specific promoters, including those directing expression in the muscle, intestine, and nervous system (Fig S2A), suggesting that the downstream consequence of EIF-3.K function in multiple tissues contributes to the modulation of longevity. We also performed tissue-specific rescue of *eif-3.K* in the *xbp-1; eif-3.K* mutant and evaluated these transgenic animals for their ability to develop on *P. aeruginosa* (Fig S2B). We anticipated that *eif-3.K* functions cell autonomously in the intestine to regulate ER homeostasis, and consistent with this expectation, we observed that intestinal expression of *eif-3.K* could rescue larval lethality in *xbp-1; eif-3.K* animals. Unexpectedly, we observed that neuronal expression of *eif-3.K* in *xbp-1; eif-3.K* animals was also able to restore larval lethality. However, we note that an important caveat in the interpretation of these experiments involving the heterologous overexpression of *eif-3.K* in specific tissues is potential toxicity that might diminish survival in lifespan and larval development assays.

DISCUSSION

Whereas multiple studies have established connections between the knockdown or loss of translation initiation factors and ribosome-associated proteins with improved longevity, our data demonstrate that loss of two conserved subunits of the eIF3 complex, EIF-3.K and EIF-3.L, confers extension in lifespan without effects on bulk translation and corresponding effects on rates of growth and development. We have also determined that loss of EIF-3.K or EIF-3.L also confers enhanced resistance to ER stress, both in growth and development on tunicamycin, as well as in *xbp-1* animals exposed to *P. aeruginosa*. Our genetic analysis suggests that lifespan extension is dependent on DAF-16, whereas loss of EIF-3.K and EIF-3.L confers enhanced resistance to ER stress independent of DAF-16, thus suggesting that distinct mechanisms are involved in conferring lifespan extension and ER stress resistance in *eif-3.K* and *eif-3.L* mutant animals. Moreover, our data suggest that EIF-3.K and EIF-3.L promote enhanced resistance to pharmacological and physiological ER stress independent of the three arms of the Unfolded Protein Response mediated by IRE-1-XBP-1, PEK-1, and ATF-6.

A number of studies point to roles for the eIF3 complex in the post-transcriptional regulation of gene expression. *Schizosaccharomyces pombe*, for example, possess two distinct eIF3 complexes, which are distinguished by the presence of either the eIF3e or eIF3m subunits, and the eIF3e-containing complex translates only a very limited set of mRNAs (Zhou et al., 2005). In zebrafish, a novel isoform of eIF3h is expressed only in the eyes and nervous system and guides development of these tissues through the

translational regulation of a subset of mRNAs (Choudhuri et al., 2010; 2013). Most recently, the mammalian eIF3 complex was found to bind N6-methyladenosine residues within the 5' UTR of some mRNAs in order to enhance their translation, including the heat shock protein HSP70, through a cap-independent mechanism (Meyer et al., 2015). We have also not excluded an alternative hypothesis in which EIF-3.K and EIF-3.L might regulate cellular physiology though pathways outside of the eIF3 complex and mRNA translation. For example, the eIF3k subunit itself has been implicated in a caspase-dependent apoptosis-promoting function (Huang et al., 2012; Lin et al., 2008). Nevertheless, our favored model, in view of the privileged position of EIF-3.K and EIF-3.L in close proximity to the translational apparatus, is that these nonessential but conserved accessary subunits of eIF3 may influence the physiology of aging and ER homeostasis through interactions with the eIF3 complex that modulate the differential translation of mRNAs. While in the current study we were unable to identify reproducible changes to the translational efficiency of individual mRNAs in mutants lacking these subunits by analyzing whole animals at basal conditions, we suspect that translational changes may still be occurring in perhaps a subset of tissues, possibly in response to a stressor or condition which has not yet been experimentally tested.

Materials and Methods

Strains and genetics:

C. elegans were cultured on OP50 as described (Brenner, 1974). The following strains were generated in the lab through mutagenesis or obtained from the *Caenorhabditis Genetics Center*: N2 (Bristol), ZD891 *eif-3.K(qd213)*, ZD892 *eif-3.K(gk126)*, ZD1258 *eif-3.L[C17G10.9(qd310)]*, ZD1098 *eif-3.L[C17G10.9(gk485491))*, ZD1828 *eif-3.K(gk126); eif-3.L(qd310)*, ZD1364 *rsks-1(ok1255)*, ZD1022 *daf-16(mu86)*, ZD1036 *daf-16(mu86); eif-3.K(gk126)*, ZD418 *xbp-1(tm2482)*, ZD613 *xbp-1(tm2482); agIs219 [T24B8.5:GFP:unc-54-3' UTR]; eif-3.K(qd213)*, ZD893 *xbp-1(tm2482); eif-3.K(gk126)*, ZD1085 *xbp-1(tm2482); rars-1(gc47)*, ZD990 *xbp-1(tm2482); rsks1(ok1255)*, ZD988 *xbp-1(tm2482); ife-2(ok306)*, RB772 *atf-6(ok551)*, ZD1252 *atf-6(ok551); eif-3.K(gk126)*, MC366 *pek-1(ok275)*, ZD1253 *pek-1(ok275); eif-3.K(gk126)*, ZD1829 *xbp-1(tm2482); eif-3.K(gk126); daf-16(mu86)*, ZD1292 *hIn1 unc-54(h1040) / eif-3.J[Y40B1B.5(qd311)] I*, ZD1421 *eif-3.K(qd315[eif-3.K:2xTY1:GFP:3xFLAG])*, ZD1422 *eif-3.K(qd315[eif-3.K:2xTY1:GFP:3xFLAG]); eif-3.L[C17G10.9(qd310)]*.

Mutation of *eif-3.J* by CRISPR/Cas9:

A loss-of-function allele of *eif-3.J (Y40B1B.5)* was generated as previously described (Arribere et al., 2014; Farboud and Meyer, 2015). The gRNA was constructed using the pRB1017 backbone, targeting the sequence agccgctccaacattcgccatgg, which occurs within the first 160bp of the CDS. This gRNA was injected at a concentration of 45 ng/μL, along with the co-injection marker pCFJ90 at 2.5 ng/μL, eft-3p::Cas9::NLS::tbb-1 3'UTR at 50 ng/μL. Transgenic F1s were screened for deletion by Sanger sequencing,

and an early nonsense allele was identified, designated *qd311*. As homozygotes for this mutation are sterile, this allele was balanced with the hIn1 LGI balanced chromosome.

***P. aeruginosa* development assay:**

Worms of the indicated genotypes were egg-layed onto either 6 cm NGM plates seeded with *E. coli* OP50 or 3.5 cm Slow Kill Assay (SKA) plates seeded with *P. aeruginosa* PA14 as described (Tan et al., 1999). For SKA plates, 7μL of overnight cultures of PA14 in LB were seeded onto the center of a 3.5 cm SKA plate. These plates were incubated at 37°C for 24h, and room temperature for 24h prior to use. Following egg-lay onto OP50 or PA14, plates were transferred to 25°C for 72h and then worms were scored based on their development to the L4 larval stage or older. Plates contained 50-100 eggs and three plates were averaged within each experiment.

Transmission electron microscopy:

Worms of the indicated genotypes were synchronized by hypochlorite treatment and grown on *E. coli* or *P. aeruginosa* at 25°C until the L3 larval stage, for about 23h. Worms were then fixed and imaged as described (Hall et al., 2012), using Standard Immersion Fixation. Images were acquired at 60,000x.

***P. aeruginosa* survival assay:**

SKA plates were prepared as described above, but with the addition of 50 $\mu g\,ml^{-1}$ 5-fluoro-2'-deoxyuridine (FUDR) in order to suppress progeny production. 30 L4 worms were transferred to each SKA plate, incubated at 25°C, and scored every 12 hours for survival. Three plates were scored and combined per genotype in order to generate survival curves.

RNAi knockdown of eIF3 subunits:

Approximately 20 L4 worms were transferred to RNAi plates that were seeded with HT115 *E. coli* containing plasmids targeting the genes of interest as collected from the Ahringer RNAi library (Kamath et al., 2003). After 2 days at 16°C, gravid worms were transferred to OP50 plates and allowed to lay 50-100 eggs, in triplicate. Following 72h at 20°C, the fraction of worms reaching the L4 stage or older were counted. RNAi clones not present in the library (*eif-3.C* and *eif-3.M(cif-1)*), were constructed by ligating ~1kB of the genomic coding region into the empty vector L4440 followed by transformation into the *E. coli* strain HT115. RNAi plates consisted of NGM supplemented with 2mM isopropyl b-D-1 thiogalactopyranoside (IPTG) and 25 μg/mL carbenicillin.

Developmental time-course:

Worms of the indicated genotype were synchronized by egg laying, and assessed periodically for their development to the L4 stage or older. This assay was carried out at 20°C.

Brood Size Assay:

Worms were synchronized by egg-laying. Following 24h at 20°C, worms were transferred singly to plates containing *E. coli* OP50 every 12h for the duration of the egg-laying period. Following 24h, the progeny were counted. 10 worms were scored per genotype.

Polysome profiling:

Polysome profiling was carried out essentially as described (Arribere et al., 2011; Pan et

al., 2007), but with the following changes. Roughly ~100,000 worms were synchronized by bleaching and grown to the L4 stage. Worms were washed once in M9, and again in M9 + 0.1mg/mL cyclohexamide, before being flash frozen in liquid N_2. Worm pellets along with 1mL lysis buffer (10 mM Tris-HCl, pH 7.4, 5mM MgCl2, 100mM KCl, 2mM dithiothreitol, 100 $\mu g\,ml^{-1}$ cycloheximide, 1% Triton X-100, 500 $U\,ml^{-1}$ RNasin Plus, and protease inhibitor (1x complete, EDTA-free, Roche)), were lysed using 40 strokes on a dounce homogenizer. Lysates were cleared of debris by centrifugation (15 mins at 20,000g), and 25 OD_{260} units of lysate was loaded onto 10-50% sucrose gradients. Samples were spun for 2.75h at 35,000 rpm in a Beckman SW41 rotor, and profiles were generated using a BioComp gradient master.

Ribosome profiling:

Worm lysates were prepared as described above. Yeast lysates were prepared by growing *S. cerevisiae* strain FY2 to exponential growth phase (O.D. 600 of ~1.0) in 50 mL YPD medium at 30°C, pelleted at 2,000x*g* for 2 minutes, and resuspended in 3mL lysis buffer . Approximately 0.5g of 0.5mm glass beads was added to resuspended yeast and vortexted at maximum speed for 2 minutes. Lysates were then cleared of debris by centrifugation at top speed for 10 minutes at 4°C. Lysates were flash-frozen in liquid N_2 until ready for use. To enable quantitation following ribosome profiling, 25 OD_{260} units of worm lysate was mixed with 1.25 OD_{260} units of yeast lysate, and ribosome footprinting was performed as described (Ingolia et al., 2012). Following sequencing, libraries were aligned to both the *C. elegans* and *S. cerevisiae* genomes and total reads aligning to each genome were tabulated.

Lifespan analysis:

Lifespan assays were carried out as previously described (Youngman et al., 2011). Briefly, 30 L4 worms were transferred to NGM plates containing 50 $\mu g\,ml^{-1}$ 5-fluoro-2'-deoxyuridine (FUDR) in triplicate and the assay was carried out at 25°C. Worms were scored every 1-2 days for survival. For lifespan on RNAi bacteria, worms were transferred to RNAi plates containing 50 $\mu g\,ml^{-1}$ FUDR, and plates were shifted to 20°C after 3d in order to minimize explosion.

qRT-PCR of DAF-16 targets:

Approximately 2,000 synchronized L4 worms of the indicated genotypes were harvested in M9 buffer, washed in M9 to purge the intestine of bacteria, and flash frozen. RNA was isolated and qRT-PCR was performed as described (Richardson et al., 2010). Genes were normalized to the housekeeping gene *act-1*, and relative expression was calculated using the $\Delta\Delta$Ct method (Livak and Schmittgen, 2001).

Quantitation of *sod-3p::GFP* transgene:

Synchronized worms of the indicated genotype were grown at 20°C, and were transferred to new plates every 24h to avoid starvation resulting from progeny production. Images were acquired with an Axioimager Z1 microscope using animals anaesthetized in 50 mM sodium azide. To quantify GFP fluorescence, animals were imaged at 10x magnification, and the total fluorescence of the animal was determined using FIJI software.

Tunicamycin survival assay:

Worms were synchronized by egg laying onto NGM plates containing 0, 2, or 5 $\mu g\,ml^{-1}$

tunicamycin. These plates were made using a 25 mg ml^{-1} stock of tunicamycin dissolved in DMSO, and seeded with *E. coli* OP50. After 72h, worms were evaluated for their development to the L4 larval stage or older.

hsp-4 induction assay:

Worms of the indicated genotype were synchronized by hypochlorite treatment and allowed to develop to the L4 stage on NGM plates. Approximately 2,000 worms per treatment were then washed onto new plates containing 10 μg/mL of tunicamycin for 4h. Worms were then harvested and RNA preparation and qRT-PCR were performed as described (Richardson et al., 2010). *hsp-4* was normalized to the housekeeping gene *act-1*, and each condition was performed in triplicate.

Tagging of _eif-3.K_ by CRISPR/Cas9:

The endogenous locus of eif-3.K was tagged as previously described (Arribere et al., 2014; Farboud and Meyer, 2015). For the homologous repair template, a 2xTY1::GFP::3xFLAG tag was amplified from clone CBGtg9050D0789D from the TransgeneOme project (Sarov et al., 2012), and was subsequently inserted in-frame into a plasmid containing 1.6 kB homology upstream of the eif-3.K stop codon and 1.1 kB homology downstream of the stop codon using Gibson assembly. The gRNA was constructed using the pRB1017 backbone, targeting the sequence gatattaaagagtcaacgg, which is less than 10 bp from the site of insertion.

Generation of Transgenic Animals:

The *eif-3.K* cDNA was amplified from wildtype cDNA by PCR. The *unc-54* 3' UTR was amplified by PCR from Fire Vector pPD95.75. The promoters for *myo-3* (1.3 kb), *dpy-7*

(1.3 kb), *ges-1* (2.9 kb), and *rab-3* (1.4 kb), were amplified from wild-type genomic DNA by PCR. DNA constructs (promoter::cDNA::*unc-54* 3' UTR) were synthesized using Gibson Assembly and sequences were verified using Sanger sequencing. Genomic *eif-3.K* was amplified from fosmid clone WRM0624aG04 (Source BioScience), including 4.6 kb upstream and 1.3 kb downstream of the *eif-3.K* CDS. DNA constructs were injected into animals at a concentration of 25 ng/μl for rescue plasmids or 1 ng/μL for genomic PCR rescue construct, along with a plasmid carrying either *ges-1p::gfp* (25 ng/μL) or *ofm-1p::gfp* (50 ng/ml). At least three independent transgenic lines were analyzed for each rescue construct.

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SUPPLEMENTARY TABLES

Supplementary Table 1

Sample	*C. elegans* CDS reads	*S. cerevisiae* CDS reads	Ratio
WT replicate 1	10283353	574131	17.91
WT replicate 2	12581951	575114	21.88
eif-3.K(qd213) replicate 1	9829209	459678	21.38
eif-3.K(qd213) replicate 2	8902662	434957	20.47
eif-3.K(gk126) replicate 1	6870555	374967	18.32
eif-3.K(gk126) replicate 2	8286071	449641	18.43

Read counts mapped to *C. elegans* or *S. cerevisiae* genome from ribosome profiling experiment.

Supplementary Table 2

Statistics for lifespan experiments.

***eif-3.K/eif-3.L* Longevity Experiments, *daf-16* epistasis**

Experiment	Genotype	Mean lifespan ± SEM	P value vs control (Log rank test)	Percent Increase vs. Control	Number of worms	Comments
1	N2	13.3 ± 0.2	Control	Control	83/90	Performed at 25°C
	eif-3.K(qd213)	19.7 ± 0.3	< 0.0001	48.1	80/90	
	eif-3.K(gk126)	19.6 ± 0.3	< 0.0001	47.4	81/90	
2	N2	14.8 ± 0.2	Control	Control	85/90	Performed at 25°C
	eif-3.K(qd213)	20.4 ± 0.4	< 0.0001	37.8	88/90	Presented in Figure 2C
	eif-3.K(gk126)	19.3 ± 0.3	< 0.0001	30.4	89/90	
	eif-3.L(gk485491)	19.3 ± 0.4	< 0.0001	30.4	87/90	
	daf-16(mu86)	9.8 ± 0.1	Control	Control	89/90	
	daf-16(mu86); eif-3.K(gk126)	10.2 ± 0.1	0.0132	4.1	85/90	
3	N2	15.4 ± 0.2	Control	Control	83/90	Performed at 25°C
	eif-3.K(qd213)	21.0 ± 0.3	< 0.0001	36.4	88/90	
	eif-3.K(gk126)	19.6 ± 0.4	< 0.0001	27.3	85/90	
	eif-3.L(gk485491)	20.6 ± 0.5	< 0.0001	33.8	78/90	
	daf-16(mu86)	9.9 ± 0.1	Control	Control	90/90	
	daf-16(mu86); eif-3.K(gk126)	9.8 ± 0.1	0.7288	-1.0	83/90	
4	N2	15.5 ± 0.2	Control	Control	85/90	Performed at 25°C
	eif-3.K(qd213)	23.0 ± 0.3	< 0.0001	48.4	79/90	Presented in Figure 2A
	eif-3.K(gk126)	21.9 ± 0.4	< 0.0001	41.3	72/90	
	eif-3.L(qd310)	21.9 ± 0.4	< 0.0001	41.3	75/90	
	eif-3.L(gk485491)	22.7 ± 0.5	< 0.0001	46.5	71/90	
5	N2	18.3 ± 0.6	Control	Control	48/90	Performed at 20°C
	eif-3.K(qd213)	26.1 ± 0.5	< 0.0001	42.6	78/90	
	eif-3.K(gk126)	25.3 ± 0.5	< 0.0001	38.3	85/90	
	daf-16(mu86)	11.7 ± 0.2	Control	Control	79/90	
	daf-16(mu86); eif-3.K(gk126)	12.5 ± 0.2	0.0156	6.8	86/90	
6	N2	21.3 ± 0.5	Control	Control	47/90	Performed at 20°C
	eif-3.K(qd213)	34.4 ± 0.9	< 0.0001	61.5	75/90	
	eif-3.K(gk126)	27.4 ± 0.6	< 0.0001	28.6	82/90	
	daf-16(mu86)	12.7 ± 0.2	Control	Control	76/90	
	daf-16(mu86); eif-3.K(gk126)	13.1 ± 0.2	0.1572	3.1	80/90	

RNAi Experiments

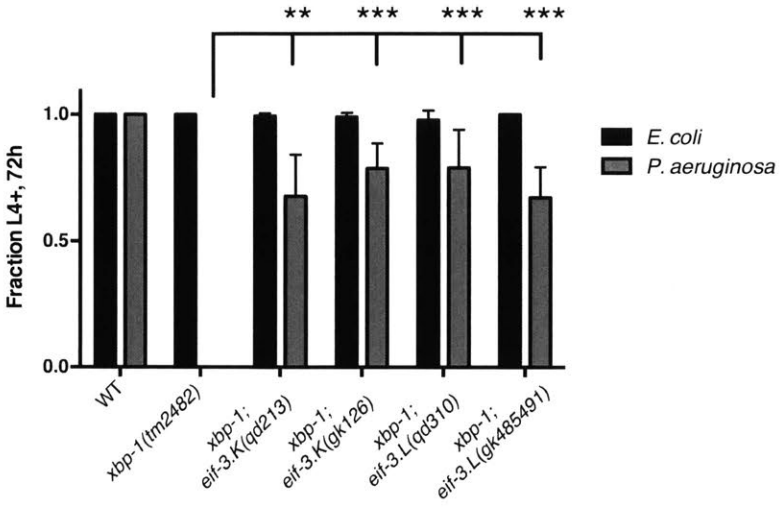
7	*eif-3.K(qd213) on GFP RNAi*	24.6 ± 0.5	Control	Control	89/90	Performed at 20°C
	eif-3.K(qd213) on eif-3.A RNAi	32.6 ± 0.5	< 0.0001	32.5	87/90	Presented in Figure 2B
	eif-3.K(gk126) on GFP RNAi	23.3 ± 0.4	Control	Control	88/90	
	eif-3.K(gk126) on eif-3.A RNAi	33.6 ± 0.6	< 0.0001	44.2	82/90	
8	*eif-3.K(qd213) on GFP RNAi*	24.3 ± 0.5	Control	Control	87/90	Performed at 20°C
	eif-3.K(qd213) on eif-3.A RNAi	35.0 ± 0.7	< 0.0001	44.0	80/90	
	eif-3.K(gk126) on GFP RNAi	22.6 ± 0.5	Control	Control	89/90	
	eif-3.K(gk126) on eif-3.A RNAi	33.3 ± 0.8	< 0.0001	47.3	89/90	
9	*eif-3.K(qd213) on GFP RNAi*	24.0 ± 0.4	Control	Control	89/90	Performed at 20°C
	eif-3.K(qd213) on eif-3.A RNAi	33.5 ± 0.7	< 0.0001	39.6	88/90	
	eif-3.K(gk126) on GFP RNAi	24.2 ± 0.4	Control	Control	90/90	
	eif-3.K(gk126) on eif-3.A RNAi	34.8 ± 0.7	< 0.0001	43.8	89/90	

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Multiple alleles of *eif-3.K* and *eif-3.L* suppresses larval lethality of *xbp-1* mutants on *P. aeruginosa*.

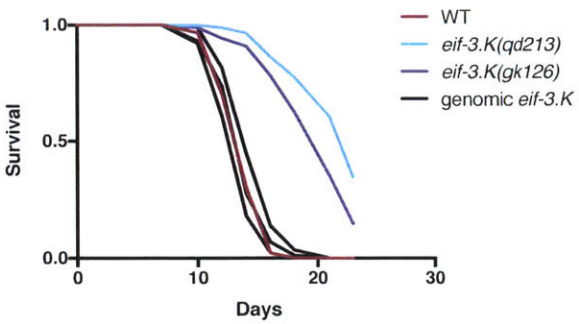
Development assay monitoring the growth and viability of the indicated genotypes on *E. coli* or *P. aeruginosa* at 25°C. 50-100 eggs were laid on each plate and following 72h the fraction reaching the L4 larval stage or older were counted. Error bars reflect the S.D. of 3 plates. A Student's *t*-test was used to assess significance: **P<0.01, ***P < 0.001.

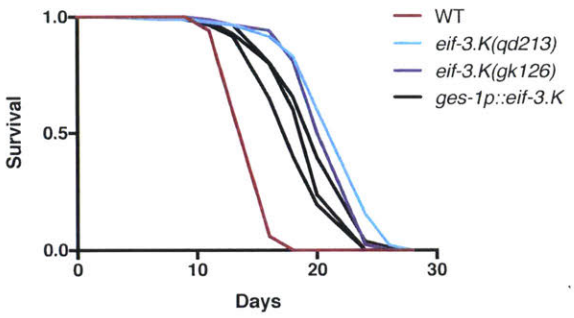
Supplementary Figure 1

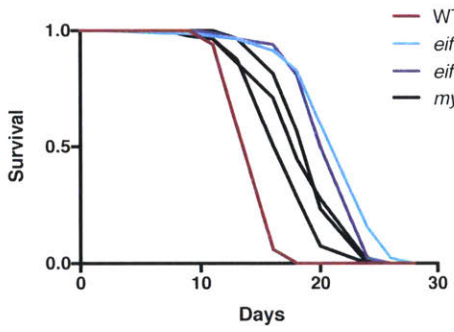


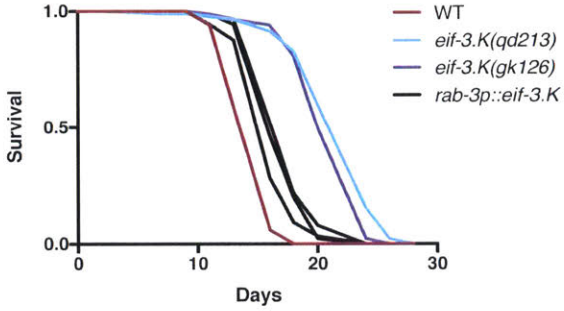
Supplementary Figure 2. Tissue-specific rescue of *eif-3.K* for longevity and development on *P. aeruginosa*. (A) Survival curves of the indicated genotypes at 25°C. Times indicated are days post-L4 stage. (B) Development assay monitoring the growth and viability of the indicated genotypes on *P. aeruginosa* at 25°C. 50-100 eggs were laid on each plate and following 72h the fraction reaching the L4 larval stage or older were counted. Error bars reflect the S.D. of 3 plates.

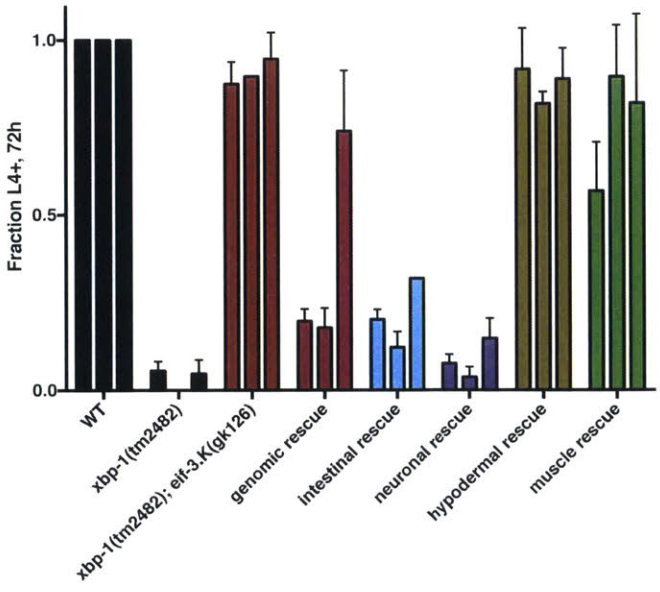
Supplementary Figure 2











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Chapter Three

Conclusions and Future Directions

Conclusions

I have demonstrated that the *eif-3.K* and *eif-3.L* subunits of the eIF3 complex are nonessential in *C. elegans*, despite their broad conservation within the eukaryotic phylogeny, and that loss of these genes confers improvements to ER homeostasis and longevity. The nonessential nature of these genes is consistent with genetic ablation and knockdown of these subunits in other organismal contexts, including fungi and mammalian cells, which also appears well tolerated (Majzoub et al., 2014; Smith et al., 2013). Mutants deficient in these two genes surprisingly show no evidence of attenuated bulk protein synthesis in *C. elegans*, which is corroborated by evaluation of developmental rates as well as quantitative measures of mRNA translation. Given that bulk protein synthesis remains intact in these mutants, I performed ribosome profiling several times in both unstressed and tunicamycin-stressed animals and was unable to identify changes in gene expression that would be consistent with regulation of translation at the level of individual mRNAs (as evaluated by differences in translational efficiency, TE). And yet, mutants deficient in these two subunits exhibit radical physiological changes consistent with a role for *eif-3.K/L* in the regulation of organismal physiology, including improved longevity and ER homeostasis.

Regarding the improved longevity of these mutants, I found that that mutation of these genes produces transcriptional changes that mimic *daf-2* loss-of-function, including upregulation of the superoxide dismutase gene SOD-3

throughout the organism, most noticeably in the intestine, where increased DAF-16 activity has been linked to lifespan extension resulting from reduced IIS signaling (Libina et al., 2003). Consistent with a model in which IIS is reduced in *eif-3.K* and *eif-3.L* mutants, lifespan extension is completely suppressed in the absence of the downstream transcription factor DAF-16. Furthermore, lifespan extension of these mutants was enhanced by RNAi-mediated knockdown of another essential eIF3 subunit, *eif-3.A*, suggesting that the mechanism by which loss of these subunits mediates lifespan extension is not simply a result of diminished eIF3 activity. Therefore, although several studies to date have identified mechanisms that promote longevity by diminishing the activity of translation initiation factors, this is the first to do so without causing a concomitant decrease in protein synthesis.

I found that improvements to ER homeostasis conferred by mutation of *eif-3.K* and *eif-3.L* are independent of each of the three branches of the UPR: XBP-1, ATF-6, and PEK-1, indicating that compensatory upregulation of other branches of the UPR is not the mechanism through which these mutants are resistant to tunicamycin. Surprisingly, DAF-16 is not required for resistance to tunicamycin. This indicates that the influence of these eIF3 subunits on organismal phenotypes has both DAF-16-dependent and DAF-16-independent components.

As reviewed in Chapter 1, inhibition of translation as well as other essential cellular processes can contribute to the lifespan extension of *C.*

elegans, and many of these appear to do so through the activation of the stress-response protein SKN-1 (Wang et al., 2010). This is unlikely to be the case in the *eif-3.K/L* mutants because fluorescence from a transcriptional reporter downstream of SKN-1 activation, *gcs-1p::GFP*, was not elevated in the *eif-3.K* mutant background (data not shown). Furthermore, inhibition of the p38 MAPK pathway—a major regulatory pathway of SKN-1 in *C. elegans* (Inoue et al., 2005)—did not suppress the lifespan extension of the *eif-3.K* mutant (data not shown). Finally, lifespan extension conferred by hyperactivation of SKN-1 occurs independent of DAF-16 (Tullet et al., 2008), which is in contrast to the requirement for DAF-16 in the lifespan extension of mutants lacking *eif-3.K/L*.

Perspective and Future Directions

Given that these eIF3 subunits are nonessential, it was tempting to consider whether the inclusion or exclusion of these subunits may be regulated in order to mediate a specialized translational program. Evidence of eIF3 subcomplexes exhibiting specialized translation based on transcript discrimination has previously been described in fission yeast (Zhou et al., 2005) and zebrafish (Choudhuri et al., 2013). Although we could not identify translational differences in these mutants versus wildtype as assayed by ribosome profiling, these results have several caveats. First, it is possible that EIF-3.K/L exert translational differences during a critical period or stress not evaluated in our experiments. Second, these subunits could be functioning in a small subset of cells or tissues such that a possible translational difference was

obscured by the dominance of intestine- and gonad-derived ribosomes present in whole worm lysates, as these two tissues account for a significant majority of the worm's total volume. An attractive alternative would be to perform profiling on *eIF3k*$^{-/-}$ mammalian cells as this would eliminate complications that may have arisen due to tissue heterogeneity, particularly if these cells were demonstrated to exhibit improved ER homeostasis

However, the ubiquity of *eif-3.K::GFP* expression in *C. elegans* in conjunction with its apparent stoichiometric relationship with the other eIF3 subunits following affinity purification (Zhou et al., 2008) argues against this model. Additionally, although a study that conducted CLIP-seq on mammalian cells identified direct binding of mRNAs to four other eIF3 subunits, eIF3k and eIF3l were not among them (Lee et al., 2015), though it is possible that loss of these subunits enhances or suppresses mRNA binding to its neighboring subunits that do bind mRNA. It is also apparent that these subunits do not serve an important structural role in the assembly of the eIF3 holocomplex, as assembly of a functional eIF3 complex can occur in the absence of these subunits (Smith et al., 2016), which is epitomized by the lack of translation-attenuated phenotypes in *eif-3.K* and *eif-3.L* mutants.

Specialization of translation could alternatively arise independent of mRNA specificity through the interaction of the translational apparatus with other cellular machinery. eIF3 has already been described as a platform that mediates mTOR signaling (Holz et al., 2005), and notably, immunofluorescence

experiments of native eIF3k in human epithelial cells identified strong colocalization with keratin intermediate filaments (Lin et al., 2008). Later high-sensitivity affinity purification of this complex in *S. pombe* has identified hundreds of additional potential interactors of this complex, including proteins of both ribosomal subunits, elongation factors, cytoskeletal proteins, and components of the proteasome machinery (Sha et al., 2009). This potential interaction with the proteasome is particularly compelling, in light of the fact that between 10-30% of nascent peptides are subject to co-translational ubiquitination and degradation (Schubert et al., 2000; Wang et al., 2013), and that modulating this degradation pathway could be a means of promoting proteostasis. Changes in this ratio would not be detected by bioinformatic approaches such as ribosome profiling, which is only able to monitor the status of translating ribosomes and is agnostic to the qualitative features and stability of the resulting synthesized proteins.

An important controversy that must be resolved is whether the eIF3 complex is present on translating ribosomes. Though it was once believed that eIF3 functioned solely as an initiation factor to scaffold productive interactions among various other eIFs and to prevent precocious binding of the 60S ribosomal subunit to the 43S PIC, this anti-association property was later found to require other cofactors such as eIF1, eIF1A, and the eIF2-ternary complex *in vitro* (Kolupaeva et al., 2005), and so eIF3 and 60S binding to the 40S subunit might not be mutually exclusive as was once suspected. In addition, crystallographic evidence from the last year indicates that the eIF3 complex is

dramatically reconfigured in late-stage preinitiation complexes with respect to the 40S subunit, with only the b, g, and i, subunits occupying the inter-subunit face of the 40S ribosomal subunit (Simonetti et al., 2016). The octamer "core" of eIF3, which includes eIF3k and eIF3l, resides on the solvent face of the 40S subunit, and it is therefore not essential that it be displaced following 60S subunit joining. To this point, several genetic studies in yeast have implicated a role for eIF3 in both translation termination (Beznosková et al., 2013) as well as reinitiation of translation following uORF translation (Munzarová et al., 2011; Szamecz et al., 2008), implying that eIF3 must be retained for some length of time on the elongating ribosome, and biochemical study of purified 80S monosomes indicates a significant amount of bound eIF3 (Beznosková et al., 2013).

If eIF3 is similarly found to be present on translating 80S ribosomes within metazoa, it would open up a host of new avenues to explore with regard to potential eIF3 functions outside of translation initiation. One experimental result that has been difficult to rationalize is our observation that acute tunicamycin treatment in *eif-3.K/L* mutants induces the UPR and folding chaperone *hsp-4/BiP* to a lesser degree than in WT animals. This indicates that in addition to being physiologically resilient to this stress (as mutation of *eif-3.K/L* also enhances resistance to development upon tunicamycin exposure), the mutant animals exhibit a lower degree of UPR activation in response to a similar pharmacological insult, which we interpreted as being an indication of an improved folding capacity within the ER.

What could account this enhanced folding capacity? A similar observation has also been observed in *daf-2* mutants (Henis-Korenblit et al., 2010), though these mutants have also been identified as having both diminished protein synthesis and degradation (Dhondt et al., 2016), so a decreased flux of protein into the ER could account for a diminished induction of the UPR. This is distinct from *eif-3.K/L* mutants, which we observe do not exhibit attenuated rates of protein synthesis. One possibility is that eIF3k/l may function within the early translating ribosome to enhance the affinity with which ribosomes bind the translocation machinery of the ER. It was observed over 40 years ago that ribosomes possess an intrinsic affinity for the translocon of the ER, independent of nascent peptide synthesis and the Signal Recognition Particle (SRP) (Borgese et al., 1974). The Nascent polypeptide-Associated Complex (NAC) is therefore required to prevent nonspecific translocation of cytosolic protein into the ER lumen by a principle of mutual antagonism with the SRP (Gamerdinger et al., 2015). If eIF3k/l are enhancing this intrinsic affinity of ribosomes for the translocon, it is possible that their absence would result in the aberrant translation and subsequent degradation of ER-targeted proteins into the cytosol. This hypothesis is appealing because it would be consistent with normal rates of protein synthesis, and a consequence would be reduced flux of protein through the ER by instead shunting some fraction into the cytoplasm where it is degraded. This mechanism would be functionally, though not mechanistically, similar to the UPR-inducible gene $P58^{IPK}$, which instigates cotranslational

degradation of ER-targeted proteins in a manner distinct from ERAD, in that the substrate proteins are ubiquitylated and destroyed prior to entering the ER lumen (Oyadomari et al., 2006). Loss of the $P58^{IPK}$ ortholog in *C. elegans* results in increased *hsp-4::GFP* expression both basically and upon tunicamycin exposure, indicating that $P58^{IPK}$ serves an important role in the maintenance of ER homeostasis. Testing this hypothesis would most simply involve a split-GFP system to evaluate whether ER-targeted protein is being synthesized in the cytosol in *eif-3.K* mutants, and this experimental system has already been used to great effect by Gamerdinger and colleagues (Gamerdinger et al., 2015).

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Appendix I

Evaluating a possible non-translational moonlighting function for eIF3 initiation factor subunits *eif-3.K* and *eif-3.L* in *C. elegans*

Introduction

If the ER-stress resistance and longevity phenotypes observed in mutants lacking *eif-3.K* and *eif-3.L* are not a consequence of differences in bulk or mRNA-specific protein translation, it is possible that these mutations are exerting their effect on physiology through some other means. As mentioned in Chapter 2, several caveats accompany the interpretation of our ribosome profiling experiments, which may have impeded our identification of translational regulation at the mRNA level. One additional hypothesis which we have not yet explored in detail is the possibility that these subunits may have a moonlighting function, as a pair, outside the context of eIF3 and translation initiation. One way to evaluate this possibility is to investigate whether *eif-3.K/L* subcomplexes, which are known to associate in the absence of the complete eIF3 complex (Smith et al., 2016; Zhou et al., 2008), still retain wild-type function when their capacity to bind the rest of the eIF3 complex is compromised. A second possibility that will be explored in this Appendix is that *eif-3.K/L* may exert influence on *C. elegans* physiology through their interaction with a third protein, Sem1, which has been identified as a binding partner of eIF3k and eIF3l homologs in the COP9 Spliceosome (CSN) and the 26S proteasome lid.

Background

Sem1 binds structural homologs of *eif-3.K* and *eif-3.L*

Sem1 is an intrinsically disordered protein that functions as a stoichiometric subunit of the 26S proteasome lid in all eukaryotes (Sone et al., 2004). In addition to this native function, Sem1 has been demonstrated as a physical binding partner of proteins such as the tumor suppressor Brca2 (Yang et al., 2002), integrator complex proteins (Baillat et al., 2005), the nuclear pore-associated TREX-2 transcription-export complex (Ellisdon et al., 2012), as well as a component of the COP9 Spliceosome (CSN) (Wilmes et al., 2008), implicating a role for Sem1 across diverse biological processes that include protein degradation, histone remodeling, DNA repair, mRNA nuclear export, regulation of ubiquitination, and RNA splicing (Figure 1). Intriguingly, the two proteasome lid proteins and one of the CSN proteins that have been shown to bind Sem1 are structural homologs of eIF3 subunits *eif-3.K* and *eif-3.L*. Due to the apparent architectural equivalencies between these three complexes (Figure 2) it is possible that *eif-3.K* and *eif-3.L*, too, might bind Sem1 in order to regulate biological processes out of the context of the initiation complex eIF3. Additionally, the phenotypes exhibited by worms lacking *eif-3.K* and *eif-3.L* may be explained by the already-known biological functions of Sem1, such as its role in ubiquitination, protein degradation, and gene expression.

Figure 1

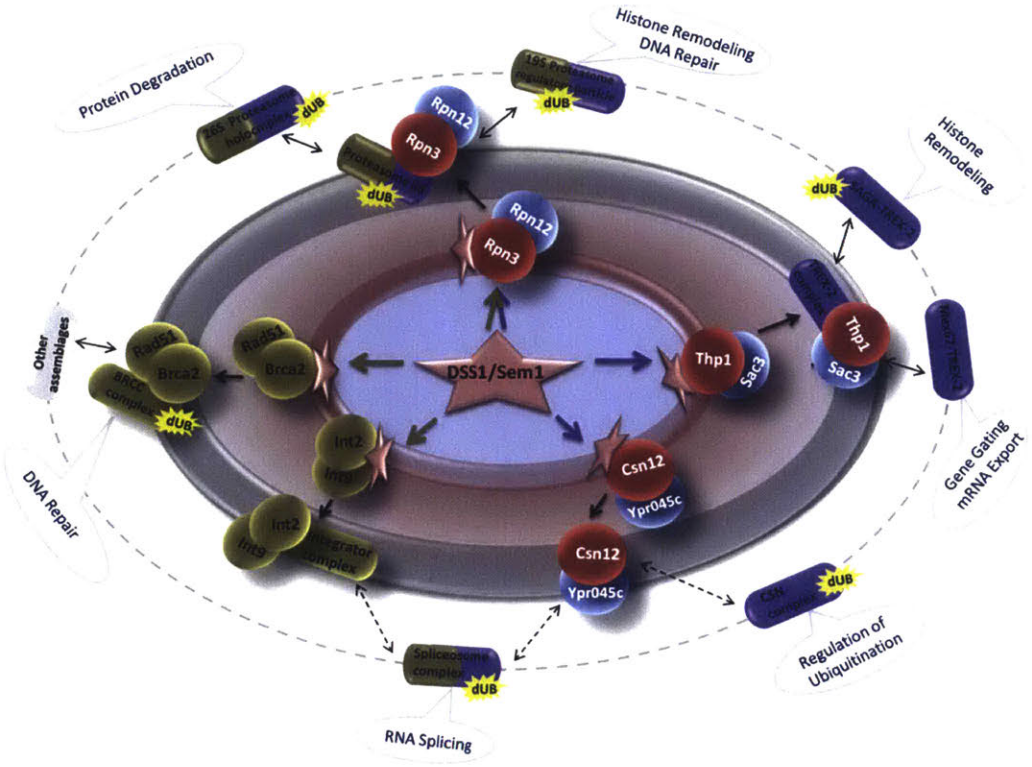


Figure 1 The intrinsically disordered protein Sem1 binds to eIF3k and eIF3l homologs in the RPN and CSN, and regulates many elements of cellular physiology. Adapted from Pick, et al. 2009.

Figure 2

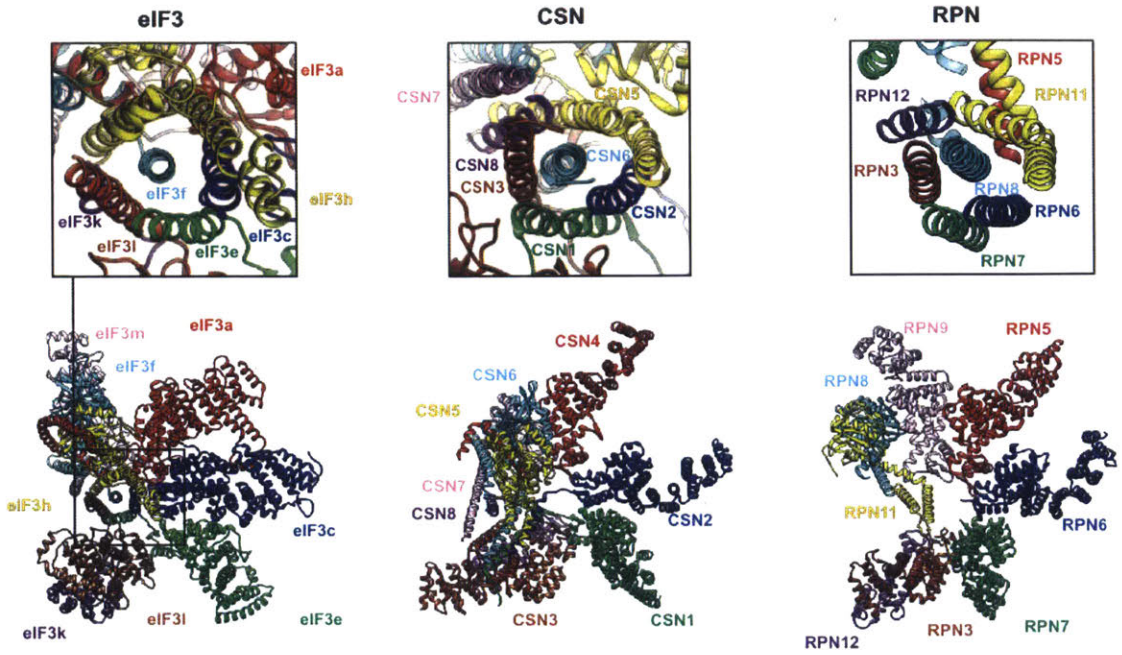


Figure 2. The 8-subunit core of eIF3, COP9 Spliceosome (CSN), and 26S Proteasome Lid (RPN) complexes are structurally similar, including an alpha-helical-bundle. Adapted from des Georges, et al. 2015.

The domain structure of Sem1-interacting proteins suggests a model for how Sem1 associates with each of these complexes. The *Saccharomyces cerevisiae* genome has three proteins that harbor a PAM (PCI-Associated Module) domain, which is a highly conserved region upstream of a subset of PCI domains (Figure 3). These PAM-containing proteins are Rpn3, Thp1, and Csn12, which all physically bind Sem1. While the *S. cerevisiae* genome does not contain an ortholog of *eif-3.L*, this gene is nevertheless contained within this gene family (Figure 3). Similarly, Rpn12, Sac3, and Ypr045c are the only three proteins in *S. cerevisiae* that contain a conserved variant of the PCI domain, here referred to as an Atypical PCI domain. *eif-3.K* is a member of this latter gene family, and an ortholog of this gene is similarly not present in the *S. cerevisiae* genome. Members of each gene family then form a heterodimer in the presence of Sem1 with the following pairing: Csn12/Ypr045c, Thp1/Sac3, and Rpn3/Rpn12 which then participate in the biological processes of mRNA splicing, mRNA export/transcription, and proteolysis/DNA repair/transcription, respectively (Figure 2).

Figure 3

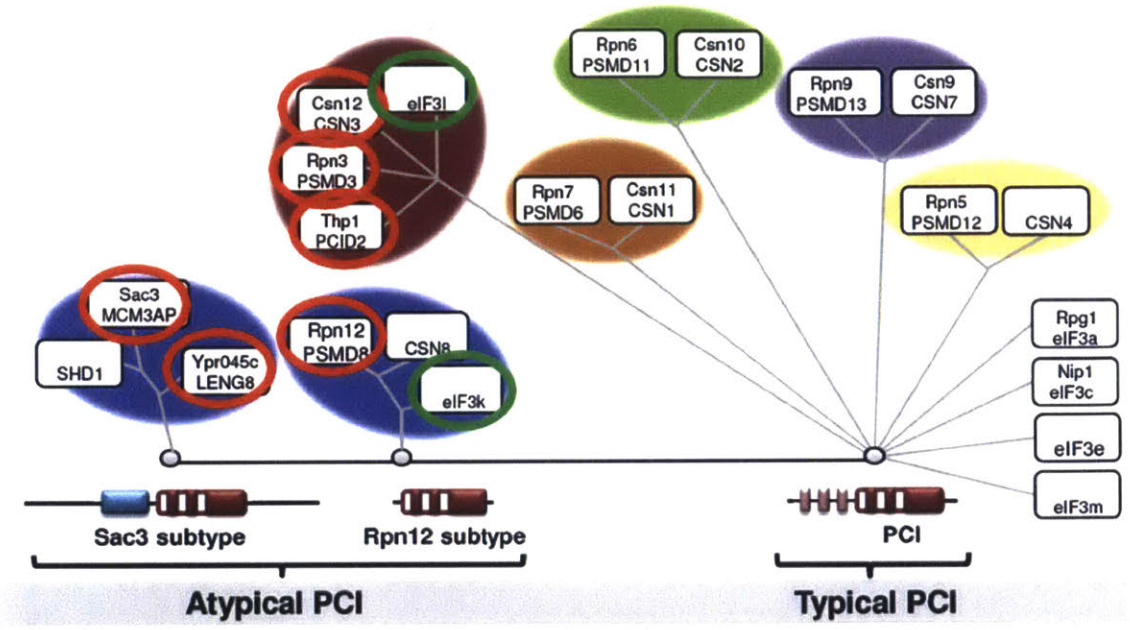


Figure 3 Classification and arrangement of PCI domains. Each box contains yeast (top) and human (bottom) nomenclature. Genes known to bind Sem1 are circled in red, and eIF3k/l are circled in green. Adapted from Pick, et al. (2009).

Additional observations in the literature support the hypothesis that eIF3k and eIF3l may bind Sem1. Deletions of eIF3k or eIF3l is tolerated in the filamentous fungi *N. crassa*, and the strains are superficially wild-type. Surprisingly, mixing lysates from both of these strains does not result in the formation of the full eIF3 complex containing eIF3k and eIF3l, though a heterokaryon that is the diploid mate of these two strains does form the full eIF3 complex (Smith et al., 2013). This implies that eIF3k and eIF3l require each other as well as other *in vivo* factors to be incorporated onto the complex, and this other *in vivo* factor may be Sem1.

Given the lack of phenotypic and biochemical evidence connecting *eif-3.K* and *eif-3.L* to the process of translation initiation in *C. elegans*, it is possible that both of these proteins are exerting effects on longevity and ER homeostasis through their interaction with Sem1.

Results

C-terminal truncation of *eif-3.K/L* proteins is unable to rescue tunicamycin resistance of *eif-3.K/L* mutants

The first hypothesis I wanted to test was whether EIF-3.K and EIF-3.L could retain function when their interaction with the rest of the eIF3 complex is disrupted. By analyzing the cryo-EM structure of the mammalian eIF3 complex, I identified a single C-terminal alpha helix of eIF3l (Figure 4, pink) as mediating the majority of molecular contact between the eif3k:eIF3l heterodimer (yellow and red, respectively) and the rest of the eIF3 complex (green). The subunit eIF3k (yellow) subsequently makes almost all of its molecular contacts to eIF3 through the subunit eIF3l. Furthermore, this alpha helix does not seem to promote k/l heterodimer formation itself, as it is situated on the opposite side of eIF3l as the region that forms the k/l interface. I leveraged these structural insights to query the requirement for eIF3 complex binding for eIF3k/l function by transgenically expressing an *eif-3.L(tr)* construct lacking this C-terminal alpha-helix in an *eif-3.L* mutant, which I hypothesized would be able to bind *eif-3.K* but not the rest of the eIF3 complex.

While these experiments were in progress, a paper was published that took a complementary approach by instead truncating the C-terminal helix of eIF3k (Smith et al., 2016). They observe that this truncated subunit is able to bind eIF3l but not the rest of the eIF3 complex, as assayed by immunoprecipitation. I set about generating a similar construct using the *C.*

elegans ortholog, as the published construct was already known to form a stable protein product that is able to form an eIF3k/l heterodimer. I found that expression of the *eif-3.K(tr)* construct was incapable of suppressing the tunicamycin resistance of *eif-3.K* mutants, which suggests that these genes function within the context of the eIF3 complex (Figure 5). The result was similar with the *eif-3.L(tr)* construct (data not shown).

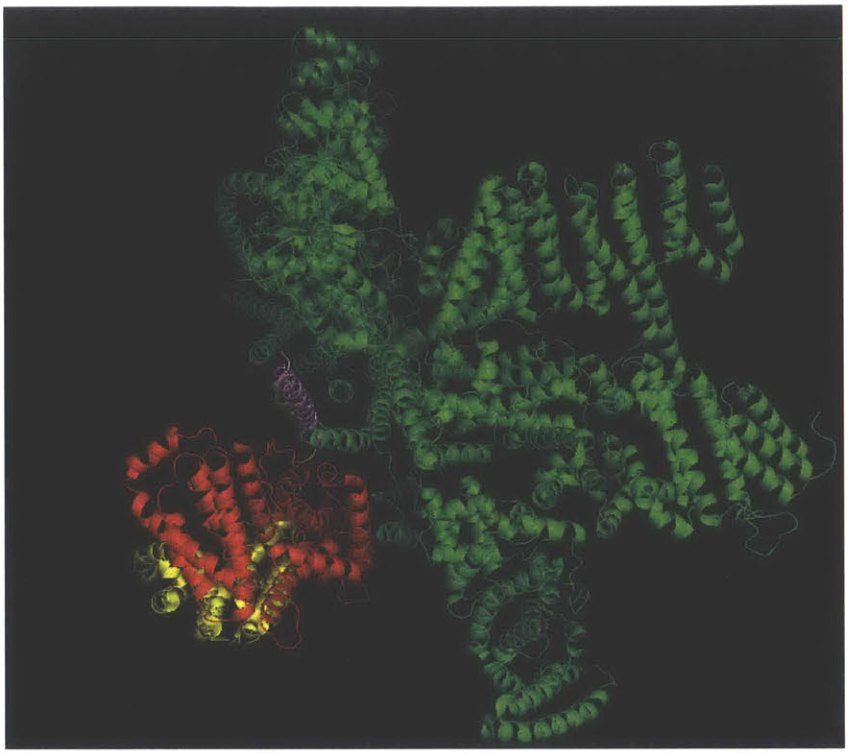


Figure 4 Cryo-EM structure of the mammalian eIF3 complex. eIF3k is pictured in yellow, eIF3l in red/pink, and the remaining subunits of the 8-subunit eIF3 core are pictured in green (PDB 5A5T).

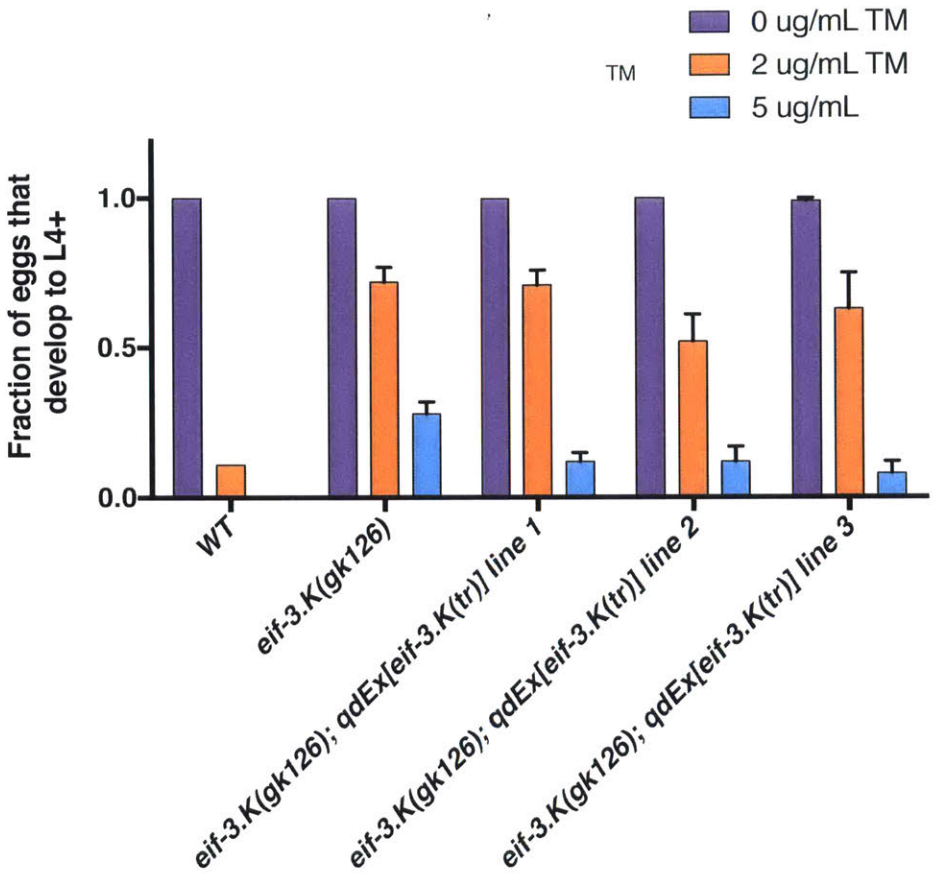


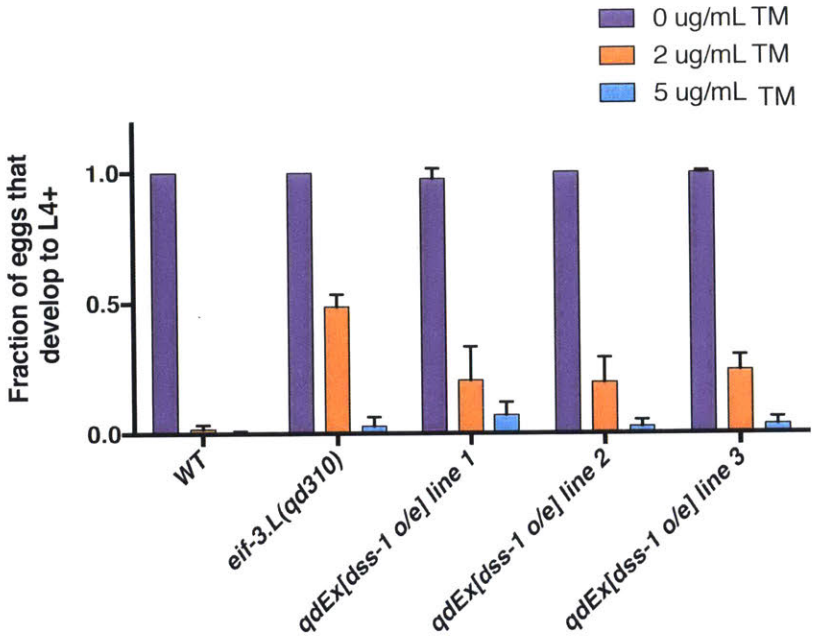
Figure 5. Expression of truncated *eif-3.K(tr)* is unable to suppress tunicamycin resistance of an *eif-3.K* mutant.

Sem1 overexpression confers resistance to tunicamycin but not lifespan extension

One possible hypothesis as to why loss of *eif-3.K* and *eif-3.L* contributes to ER stress resistance and longevity extension is that if Sem1 is typically bound and sequestered by these proteins, it is therefore unavailable for participation with its other binding partners. This hypothesis is appealing because EIF-3.K/L are abundant proteins whose loss could cause a substantial shift in the equilibrium of various other Sem1 binding interactions, additionally because many of the other complexes available to Sem1 involve the maintenance of proteostasis. Another protein that is bound and stabilized by Sem1 is Brca2, a protein required for double strand break repair, and it has been observed previously that siRNA mediated knockdown of eIF3k in mammalian cells is results in increased resistance to UV irradiation (Lin et al., 2008). By this model, phenotypes present in the *eif-3.K/L* mutant worms should be phenocopied by overexpression of Sem1.

To this end I overexpressed the *C. elegans* ortholog *dss-1* and evaluated its effect on the tunicamycin resistance phenotype. I find that overexpression of *dss-1* does, in fact, confer resistance to tunicamycin, though the effect is intermediate as compared to that of EIF-3.L loss-of-function. I additionally find that overexpression of *dss-1* is not able to improve longevity.

A



B

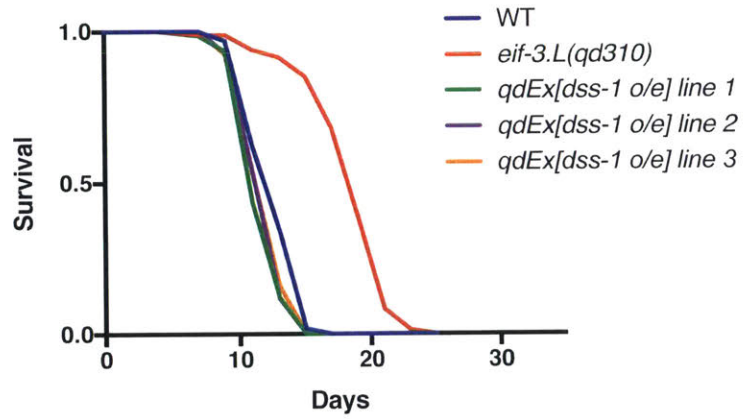


Figure 6. Overexpression of *dss-1* promotes resistance to tunicamycin but not does not confer longevity extension. (A) The indicated genotypes were egg laid onto plates containing 0, 2, and 5 μg/mL tunicamycin. After 72 hours worms were scored for their ability to reach the L4 larval stage or older. (B) Lifespan assay of the indicated assays, performed at 25 °C.

Conclusions and Future Directions

The binding of EIF-3.K/L to Sem1 seems likely given that billions of years of co-evolution of CSN and 26S proteasome lid complex proteins towards their respective roles has surprisingly maintained this shared binding interaction. It is possible that a physical or functional interaction of *eif-3.K/L* with Sem1 has gone unobserved due to the lack of orthologs of these genes in *S. cerevisiae*, the model system in which Sem1 function has been best characterized. If a functional interaction between EIF-3.K/L and Sem1 can be demonstrated, and if this interaction can be shown to mediate the phenotypes associated with *C. elegans* mutants lacking *eif-3.K/L*, these nonessential initiation factors could become appealing candidates for pharmacological inhibition, and genetic screens that identify activators of Sem1 activity or expression could also prove informative.

Sem1 is not essential in yeast and a deletion in this gene does not exhibit any growth defects, despite being a stoichiometric subunit of the proteasome regulatory particle (Jäntti et al., 1999). One unexpected phenotype of these mutants is that deletion of Sem1 completely rescues the secretory phenotype (impaired secretion) of late-stage *sec* mutants, which mediate the golgi-to-plasma membrane secretion pathway, suggesting a role for this protein in exocytosis. Given that *eif-3.K/L* mutant worms are resistant to the drug tunicamycin, which inhibits N-linked glycosylation (thereby preventing proper

trafficking through the ER and golgi), it is tempting to suspect some role of Sem1 in the remediation of protein secretion. Sem1 in yeast was found not only in the cytosol, but was found in the microsomal pellet, indicating a physical interaction with the membrane.

Though nonessential in *S. cerevisiae*, the Sem1 ortholog *dss*-1 was reported to be essential in *C. elegans*, as a deletion in this gene produces larval lethality (Pispa et al., 2008). This is in contrast with RNAi-mediated knockdown of this gene, which was associated with no phenotypes under normal growth conditions. A closer inspection of the *dss-1* deletion allele used in this study, *tm370*, reveals that in addition to deleting the second exon of *dss-1*, this variation additionally removes the entire upstream promoter region of a downstream gene that encodes the mitochondrial translation elongation factor G2 (EFG2), an essential protein for the elongation phase of protein synthesis. It is very likely that the disruption of this second gene is responsible for the phenotypes associated with this deletion, and so a CRISPR-mediated knockout of *dss-1* could prove informative in genetic epistasis experiments with *eif-3.K/L* mutants in determining if the phenotypes associated with *eif-3.K/L* mutation require the activity of *dss-1*.

Materials and Methods

Lifespan analysis:

Lifespan assays were carried out as previously described (Youngman et al., 2011). Briefly, 30 L4 worms were transferred to NGM plates containing 50 $\mu g\,ml^{-1}$ 5-fluoro-2'-deoxyuridine (FUDR) in triplicate and the assay was carried out at 25°C. Worms were scored every 1-2 days for survival. For lifespan on RNAi bacteria, worms were transferred to RNAi plates containing 50 $\mu g\,ml^{-1}$ FUDR, and plates were shifted to 20°C after 3d in order to minimize explosion.

Tunicamycin survival assay:

Worms were synchronized by egg laying onto NGM plates containing 0, 2, or 5 $\mu g\,ml^{-1}$ tunicamycin. These plates were made using a 25 mg ml^{-1} stock of tunicamycin dissolved in DMSO, and seeded with *E. coli* OP50. After 72h, worms were evaluated for their development to the L4 larval stage or older.

Generation of Transgenic Animals:

For *eif-3.K(tr),* two gene fragments were amplified in two parts to exclude the CDS for the 30 residues immediately before the stop codon, and that include 1Kb in upstream sequence and 1Kb of downstream sequence. A similar approach was used to synthesize the *eif-3.L(tr) construct.* PCR fusion was used in order to fuse these fragments, and the resulting DNA constructs were injected into animals at a concentration of 25 ng/μl along with a plasmid carrying *ges-1p::gfp* (25 ng/μL), and 50 ng/μL 1Kb ladder. At least three independent transgenic lines

were analyzed for each rescue construct. For *dss-1* genomic rescue, a PCR product was amplified which included 1.2 Kb upstream of the CDS and 900 bp downstream of the CDS.

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