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Investigating the role of RNA Polymerase III and the Unfolded Protein Response

By

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Submitted to the Division of Natural Sciences

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Rene Rivera

Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent or any other University or Institution of learning.

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Abstract

Over time, the transcriptome of eukaryotes ranging from yeast to humans has been shown to become dysregulated, and ensuing changes in gene expression have been linked to several hallmarks of ageing, such as mitochondrial dysfunction, altered intracellular communication, and deregulated nutrient sensing. Three RNA polymerases generate the transcripts that make up the transcriptome, with each polymerase synthesising specific RNA products. RNA Polymerase III (Pol III) synthesises various non-coding RNAs: tRNAs and 5S rRNAs, which are fundamental components of the translation machinery, and other small RNAs such as snoRNA, SINEs, 7SL RNA, Y RNA, and U6 spliceosomal RNA that are involved in RNA processing and regulatory functions. Pol III transcription is regulated by cell cycle stage, nutrient availability, and cell stress. Dysregulation of Pol III is implicated in several pathological states, including Alzheimer's, Fragile X syndrome and many forms of cancer. Pol III inhibition has recently been shown to extend lifespan in yeast, worms and flies. However, the mechanism underpinning longevity is not known.

The unfolded protein response (UPR) is a signalling system that detects misfolded proteins within the endoplasmic reticulum (ER) and coordinates a cellular response to restore protein homeostasis. Three key UPR signal activators—IRE1, PERK and ATF6—give rise to separate branches of the response. All the branches are conserved in the soil-dwelling nematode *Caenorhabditis elegans* (*C. elegans*) and have been shown to respond to elevated levels of misfolded proteins. This report explores the relationship between Pol III and the UPR in *C. elegans*. Using mutant strains, RNAi and microscopy, it was found that Pol III inhibition induced levels of the protein chaperone HSP-4 (mammalian BiP). This activation of the UPR was found to be specific to the ER and dependent on downstream transcription factors XBP-1 and ATF-4. Also explored in this report is how Pol III targets—tRNA synthetases—control the UPR-ER.

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The ability to activate the UPR-ER declines with age, and the inability to ensure proper folding of proteins has been implicated as a contributing factor to the ageing process. Linking RNA Pol III and the UPR-ER uncovers a novel regulatory mechanism that may have implications for age-related diseases, particularly those involving protein misfolding.

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Chapter 1: Introduction

Ageing is a course of biological certainty beginning at conception and ending with death. It can be characterised by a deterioration of physiological integrity, leading to loss of function and increased vulnerability to several pathological states. Progression in the ageing process onsets the decline of key functional components that are accentuated in the elderly and have negative implications for public health. Sensory factors, muscular strength, fat stores, the immune system, and urologic factors all experience changes that can impact well-being and quality of life during ageing (Jaul and Barron, 2017). The prevalence of diseases such as cardiovascular disease, hypertension, cancer, osteoarthritis, diabetes, and osteoporosis, as well as instances of multiple chronic conditions, also increase as we age (Jaul and Barron, 2017).

Due to declining fertility rates and increased longevity, the world population is rapidly growing older. Current population demographics show that by 2030 12% of the population will be 65 years or older, and by 2050 it will rise to 16.7% (United States Census Bureau, He, Goodkind and Kowal, 2016). In many countries, including the UK, the burden placed on healthcare is already unsustainable and will only worsen with the increased frequency of ailments in an elderly population. Understanding the molecular mechanisms underpinning longevity will lead to the formulation of novel treatments to battle ageing, which will alleviate healthcare demands and allow people to live healthier for longer.

1.1 The nematode *Caenorhabditis elegans* (*C. elegans*) is a good model organism for studying longevity

To understand the molecular mechanisms contributing to longevity, an appropriate model is needed for its study. The tiny, free-living nematode *Caenorhabditis elegans* (*C. elegans*) has many benefits as a model organism (Figure 1); they have a rapid life cycle (3 days from egg to egg-laying adult at 25 °), can be kept at a low cost, and have high homology with humans (83% of the *C. elegans* proteome has

human homologs) (Lai et al., 2000; Corsi, Wightman and Chalfie, 2015). Also, their transparent, self-fertilising, hermaphroditic nature enables the in-vivo study of fluorescently tagged proteins in large populations of genetically identical worms, providing an advantage over other organisms when studying the pathways involved in longevity (Corsi, Wightman and Chalfie, 2015).

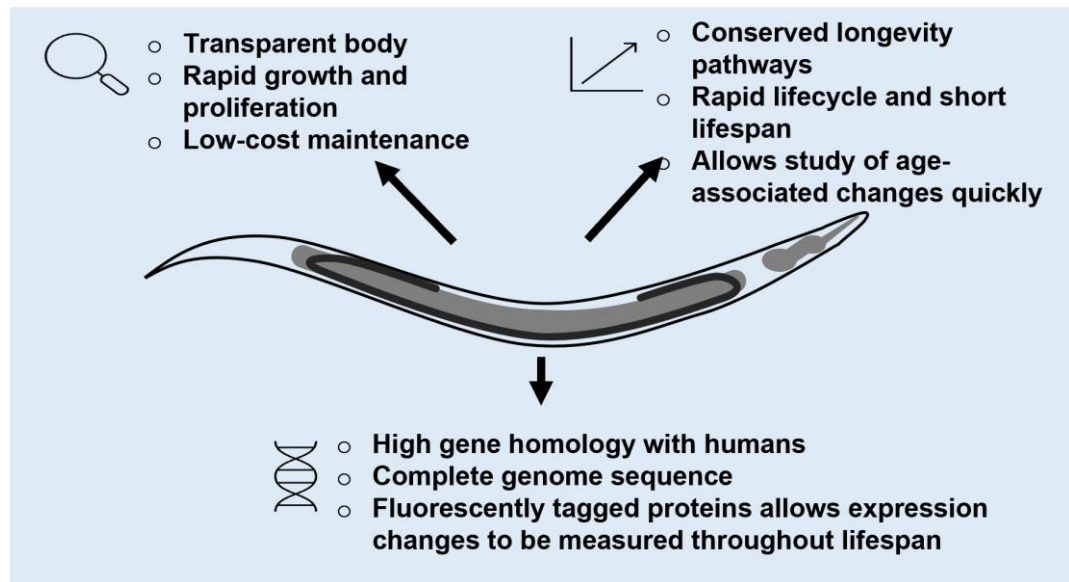


Figure 1. *Caenorhabditis elegans* as a model organism for studying longevity.

C. elegans offers various technical and biological advantages as a model organism. Studies in the worms can give insights into the mechanisms that mediate ageing in humans due to having well-conserved longevity pathways.

Many of the changes that occur with age at the molecular, cellular, and tissue level in *C. elegans* have been documented in-depth and are well reviewed by (Son *et al.*, 2019). This has led to the formation of different physiological, cellular, and molecular biomarkers that are predictive of lifespan in the worm (Son *et al.*, 2019). For example, with regards to physiological function, locomotion can decrease with age and maintenance of youthful locomotion into middle age can predict a longer lifespan (Hsu *et al.*, 2009). Similarly, the number of progeny post-mating and the pharyngeal pumping rate can predict the remaining lifespan (Huang, Xiong and Kornfeld, 2004; Pickett *et al.*, 2013). At the cellular level, nucleolar size is predictive of longevity; small nucleolar size is seen in significant longevity

pathways in the worm (Tiku *et al.*, 2017). Lastly, molecular genetic changes that impact expression levels of specific genes, such as heat-shock protein 16.2 (hsp-16.2) and superoxide dismutase 3 (sod-3), as well as varying miRNA levels, also correlate with longevity (Son *et al.*, 2019).

Various pathways that regulate ageing have been discovered in *C. elegans*. The insulin/IGF-1 pathway was the first shown to influence longevity; mutations decreasing the activity of the known regulatory gene, *daf-2*, more than double the lifespan of the worm (Kenyon *et al.*, 1993; Kenyon, 2005).

Inhibiting TOR signalling in the nematode has also been shown to increase longevity (Vellai *et al.*, 2003). TOR (target of rapamycin) kinases are a group of enzymes that regulate cell growth and proliferation in response to nutrients and growth factors (Jia, Chen and Riddle, 2004). Inhibiting the TOR pathway in *C. elegans* is enough to double its natural lifespan (Vellai *et al.*, 2003). Similar extensions can also be seen by overexpressing the nutrient and energy sensor AMP kinase and the NAD⁺-dependent protein deacetylase, sirtuin gene *sir-2.1* (Kenyon, 2010). Both seem to function similarly - by activating the longevity regulator DAF-16/FOXO (Kenyon, 2010).

1.2 RNA Polymerase III

Three distinct RNA polymerases (Pols) are present in eukaryotic cells and transcribe different classes of genes from DNA to RNA. RNA Polymerase II (Pol II) transcribes a large portion of the genome and is responsible for generating mRNA from protein-coding genes. RNA polymerase I (Pol I) solely transcribes rRNA genes, synthesising a sizeable 45S pre-rRNA, which is then processed into smaller 28S, 18S, and 5.8S rRNAs. RNA Polymerase III (Pol III) transcribes highly abundant noncoding RNA genes involved in fundamental cellular processes. The most copious products of Pol III-dependent transcription are the different species of tRNAs and the 5S rRNA, which along with Pol I generated 28S, 18S, and 5.8S rRNAs are essential components of the protein synthesis machinery (Dieci *et al.*, 2013). Other Pol III products are involved in protein translocation as part of the signal recognition

particle (7SL RNA); regulation of Pol II elongation (7SK RNA); the processing of other RNA molecules (U6 spliceosomal snRNA; RNase P RNA and RNase MRP RNA); macromolecular assembly/transport (Vault RNA); neuronal disease (BC200); Alu and MIR repeats (SINE elements) and chromosomal DNA replication in human cells (Y RNA) (Rosenblad *et al.*, 2003; Christov *et al.*, 2006; Zhu *et al.*, 2006; Marvin and Engelke, 2009; Peterlin, Brogie and Price, 2012; Dieci *et al.*, 2013; Alla and Cairns, 2014; Samson, Cronin and Dean, 2018; Yeganeh and Hernandez, 2020). However, the role of many of these Pol III products are still poorly understood, and new functions are continuously being discovered.

Pol III is regulated by various biological processes such as development (Marshall, Rideout and Grewal, 2012; Rideout, Marshall and Grewal, 2012), cell cycle (White *et al.*, 1995), differentiation (Alla and Cairns, 2014; van Bortle, Phanstiel and Snyder, 2017), cellular stress (Upadhyaya, Lee and Willis, 2002; Gouge *et al.*, 2015), and regeneration (Yeganeh *et al.*, 2019). It is not then surprising that dysregulated Pol III activity is implicated in diseases such as cancer (White, 2004) and mutations in Pol III subunits have been found to cause a wide range of rare inherited disorders (Bernard *et al.*, 2011; Saitsu *et al.*, 2011; Tétreault *et al.*, 2011; Thiffault *et al.*, 2015; Dorboz *et al.*, 2018; Beauregard-Lacroix *et al.*, 2019; Franca *et al.*, 2019; Terhal *et al.*, 2019).

1.3 Pol III transcriptional machinery

Pol III recognises three types of genes based on their promoter structure (Figure 2). Type I promoters are gene internal and only include 5s rRNA genes. Similarly, Type II genes have internal promoters and consist of tRNAs, 7SL RNA, BC200, vault RNA, and SINE elements. Type III promoters reside in the 5' flanking region upstream of the transcription start site and include genes such as U6 snRNA, RNase P, RNase MRP, Y RNA and 7SK RNA (Shen, 2019). Pol III relies on a combination of different transcription factors to bind to a specific type of promoter. They all require the binding of TFIIB, a three-polypeptide transcription factor that includes the TATA-binding protein, TBP, BDP1, and BRF1

or BRF2 (Schramm and Hernandez, 2002). In Type II promoters, the multisubunit transcription factor TFIIC binds A and B box promoter elements to recruit BRF1-containing TFIIB (Schramm and Hernandez, 2002; Alla and Cairns, 2014). Type I promoters further require the transcription factor TFIIA, which binds A and C box promoter sequences to recruit TFIIC and a TFIIB complex containing BRF1, similarly to Type II promoters (Schramm and Hernandez, 2002). Type III promoters differ from Type I and II in that they do not require the TFIIC transcription factor and instead employ the snRNA activating protein complex (SNAPc) to recognise the proximal sequence element (PSE) and a BRF2-containing TFIIB to aid Pol III transcription (Alla and Cairns, 2014).

Other factors have also been found that regulate Pol III activity. The protein, MAF-1, as well as several tumour suppressor genes (RB1 (Gjioda and Henry, 2013); TP53 (Chesnokov *et al.*, 1996); PTEN (Woiwode *et al.*, 2008); CDK2A (Morton, Kantidakis and White, 2007)) function as repressors of Pol III transcription, while components of the MAP kinase pathway (JNK1 (Zhong *et al.*, 2011); ERK (Felton-Edkins *et al.*, 2003)) and proto-oncogene MYC (Gomez-Roman *et al.*, 2006) activate Pol III activity.

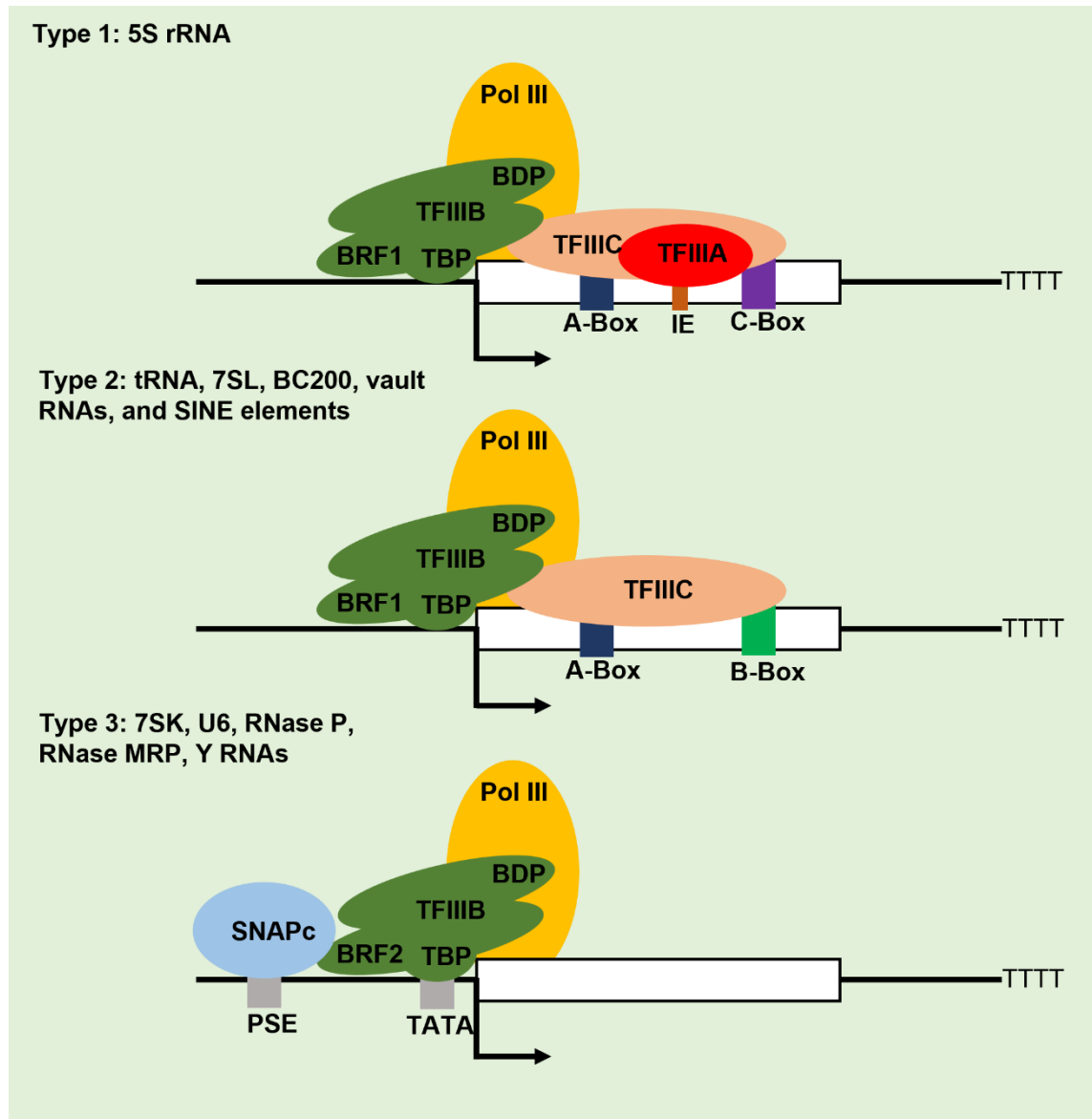


Figure 2. RNA Polymerase III genes and their corresponding promoter structure and transcription factors.

1.4 Partial inhibition of Pol III activity promotes longevity

Recently, Pol III was found to regulate organismal ageing; reducing Pol III activity in yeast, worms, and flies led to significantly increased lifespans (Filer *et al.*, 2017). Targeting individual subunits of the 17-subunit Pol III complex makes it possible to reduce its function without leading to organismal lethality (Kulaberoglu *et al.*, 2021). Pol III depletion via inducible loss of the largest core subunit *RPC160* in *S. cerevisiae* led to increased chronological lifespan (Filer *et al.*, 2017). Similarly, RNAi targeting *RPC160*

orthologues *rpc-1* in *C. elegans* and *dC160* in *D. melanogaster* led to Pol III-mediated extension in organismal lifespan (Figure 3)(Filer *et al.*, 2017). Also, tissue-specific inhibition of Pol III in the worm and fly gut was enough to increase lifespan (Filer *et al.*, 2017). This effect was not seen in the fly neurons and fat body, suggesting Pol III acts in a tissue-specific manner to mediate longevity.

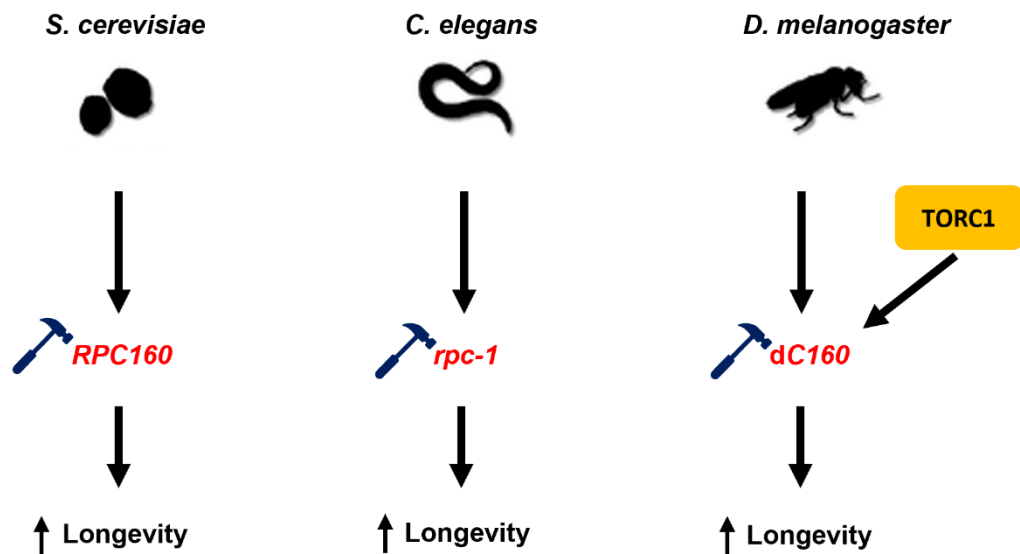


Figure 3. RNA Polymerase III inhibition in yeast, worms and flies promotes longevity.

In *S. cerevisiae*, the gene *RPC160*, which encodes for the largest Pol III subunit C160, was fused to the auxin-inducible degron and then targeted for degradation, achieving Pol III inhibition; leading to increased chronological lifespan. *RPC160* homologs *rpc-1* and *dC160*, in *C. elegans* and *D. melanogaster*, respectively, were targeted using RNAi, leading to increased longevity. In *D. melanogaster*, Pol III-mediated longevity is acting downstream of TORC1. Results in this Figure use data from (Filer *et al.*, 2017).

In flies, Pol III-mediated effects on lifespan act downstream of target of rapamycin complex 1 (TORC1) (Filer *et al.*, 2017). The TOR kinase in TORC1 and TORC2 responds to nutrient and anabolic signals to promote growth and proliferation, cell survival, and protein synthesis (Wullschleger, Loewith and Hall, 2006; Ma and Blenis, 2009; Zoncu, Efeyan and Sabatini, 2011). Dysregulated TOR signalling is implicated in various diseases, including diabetes, obesity, neurodegenerative disorders, cancer, and

ageing (Stanfel *et al.*, 2009; Zoncu, Efeyan and Sabatini, 2011; Blenis, 2017). Notably, inhibition of TOR signalling promotes longevity in model organisms ranging from yeast to mice (Harrison *et al.*, 2009; Selman *et al.*, 2009; Stanfel *et al.*, 2009; Bjedov *et al.*, 2010). TOR inhibition is thought to mediate longevity via reduced global mRNA translation and preferential activation of genes that regulate protective mechanisms and promote longevity (Robida-Stubbs *et al.*, 2012). Interestingly, TORC1 is implicated in Pol III regulation; TORC1 mediates the transcription of several rRNA and tRNA genes by localising to their promoters (Li *et al.*, 2006). Inhibition of TORC1 in tandem with Pol III knockdown produces a non-additive lifespan extension that suggests they act on the same pathway to control longevity, with Pol III inhibition acting downstream of TORC1 (Filer *et al.*, 2017). However, this effect has not been observed in any other model organism.

The study by Filer *et al.*, 2017 uncovers a novel pathway linking Pol III to longevity and identifies Pol III as a potential therapeutic target for ageing and age-related diseases. To validate this, more work is needed to understand the mechanisms underpinning longevity.

1.5 The aminoacyl tRNA synthetases' role in protein synthesis

Translation of the genetic code contained in mRNA into proteins is a highly conserved mechanism essential for all organisms. Among Pol III targets are the aminoacyl tRNA synthetases (ARSs), which are comprised of a family of enzymes that catalyse the esterification of a tRNA to its cognate amino acid during translation (Ibba, Curnow and Söll, 1997; Pang, Poruri and Martinis, 2014). This reaction is a two-step process that results in an aminoacyl-tRNA (aa-tRNA) that is delivered into the ribosome to be used in protein synthesis (Gomez and Ibba, 2020) (Figure 4). In the first step, an amino acid is activated by ATP, forming an aminoacyl adenylate and releasing an inorganic pyrophosphate (PPi). The second step then involves the transfer of the activated amino acid to the 2' – 3'-OH group of the terminal adenosine of its cognate tRNA, releasing AMP (Schimmel and Söll, 2003). ARSs can then be

described as the linchpin of translation, providing the building blocks for protein synthesis. However, they also mediate other non-canonical functions, such as regulation of gene expression; angiogenesis; and cellular signalling, that stem from cellular interactions distinct from those involved in translation (Hausmann and Ibba, 2008; Guo, Schimmel and Yang, 2010; Guo, Yang and Schimmel, 2010). Mutations in the ARSs genes involved in translation and non-translational activity have also been linked to neurogenetic and tissue-specific disease phenotypes, reviewed by (Rajendran *et al.*, 2018).

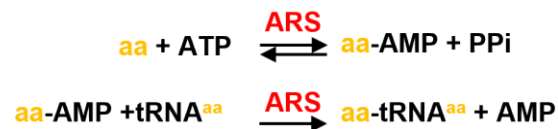


Figure 4. Two-step aminoacylation reaction by ARS.

The first step is reversible and involves the activation of an amino acid (aa) by ATP and the release of inorganic pyrophosphate (PPi). In the second step, the amino acid is transferred to the tRNA and AMP is released.

1.6 ARSs classification

ARSs are classified based on the architectures of their catalytic domains (Cusack *et al.*, 1990) and the chemical structures of their primary sequence homologies (Eriani *et al.*, 1990; Ribas de Pouplana and Schimmel, 2001). In eukaryotic cells, a little more than twenty ARSs are generally found to activate twenty amino acids, and they are split into two groups, class I and class II (Poruri, 2020). Class I ARSs contain a Rossmann fold that binds the minor groove of the tRNA receptor stem and comprises the catalytic site (Webster *et al.*, 1984; Hountondji, Dessen and Blanquet, 1986). They characteristically aminoacylate the 2'-hydroxyl position of the terminal ribose of the cognate tRNAs (Yao and Fox, 2020). Comparatively, class II enzymes contain a more unique catalytic core; they have an antiparallel β -sheet that binds the major groove of the acceptor stem and aminoacylate the terminal ribose of tRNA substrates at the 3'-hydroxyl moiety (Yao and Fox, 2020). Another structural difference that separates the two groups is that class I ARSs are usually monomeric, while class II ARSs are dimeric or

oligomeric (Kwon, Fox and Kim, 2019). They can also be grouped into additional subclasses based on their recognition of related amino acid substrates and various structural features (Kwon, Fox and Kim, 2019).

1.7 ARSs in ageing and lifespan

In higher eukaryotic systems, ARSs usually exist in free form or as a multi-tRNA synthetase complex (MSC) formed from eight ARSs, and three non-enzymatic ARS-interacting multi-functional proteins (AIMP1/p43, AIMP2/P38, and AIMP3/p18) (Zhou *et al.*, 2020). Not much is known regarding how ARSs contribute to ageing or regulate lifespan. However, multiple studies using different models have shown a close association between ARSs, AIMPs and longevity (Lee *et al.*, 2003, 2013; Bayat *et al.*, 2012; Niehues *et al.*, 2015; Nakamura *et al.*, 2016; Oh *et al.*, 2016; Arif *et al.*, 2017; Son *et al.*, 2017; Kim *et al.*, 2019; D'Hulst *et al.*, 2020; Suh *et al.*, 2020). For example, in *Drosophila*, mutations in the ARSs gene, glycyl-tRNA synthetase (GARS), shortened lifespan in a dosage-dependent manner that was linked with impaired protein synthesis (Niehues *et al.*, 2015). Similarly, in flies, inhibition of Methionyl-tRNA synthetase (MARS) decreased longevity by reducing the expression of anti-microbial peptide genes (Suh *et al.*, 2020). However, ARSs genes have also been shown to promote longevity (Lee *et al.*, 2003; Nakamura *et al.*, 2016; Son *et al.*, 2017). A null mutation in leucyl-tRNA synthetase (*lars-2*) was associated with increased longevity in *C. elegans*, which was linked to impaired mitochondrial function due to reduced ATP content and oxygen consumption (Lee *et al.*, 2003). Similarly, in worms, downregulation of *lars-1* represses TOR activity and activates preferential targets to regulate longevity (Nakamura *et al.*, 2016). Also, a decrease in Tyrosyl-tRNA synthetase (*yars-2*), facilitated by nonsense-mediated mRNA decay (NMD), is crucial for the longevity of *daf-2* mutant worms. How specifically ARSs interactions affect ageing requires further work, especially in mammalian systems where their relationship has yet to be well studied.

1.8 The UPR-ER

One of the significant stress responses linked with ageing involves activating the unfolded protein response (UPR) to resolve perturbations in the endoplasmic reticulum (ER). The ER is a membrane-bound organelle that is a major site for protein synthesis, transport, and folding (Schwarz and Blower, 2016). This protein synthesis and folding process are highly susceptible to homeostatic changes in the ER, leading to an increase in ER stress due to the accumulation of misfolded proteins (Read and Schröder, 2021). In response, the cell has developed the UPR, a system of regulated signalling pathways that detects the build-up of misfolded proteins in the ER and coordinates an adaptive cellular response to return homeostasis to the ER (Taylor, 2016; Read and Schröder, 2021). The UPR reduces global protein synthesis, increases protein-folding capacity, and enhances ER-associated degradation (ERAD) (Cao and Kaufman, 2012).

The UPR functions through three pathways, each containing individual upstream transmembrane receptors in the ER: inositol requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF-6) (Cao and Kaufman, 2012; Taylor, 2016; Read and Schröder, 2021)(Figure 5). The uncoupling of ER-resident chaperones that facilitate proper protein folding, such as glucose-regulated protein 78 (Grp-78 or binding immunoglobulin protein, BiP) from the three transmembrane receptors, activates the UPR signalling cascade (Bell *et al.*, 2016). IRE1 is the most evolutionary conserved receptor, and the presence of misfolded proteins in the ER directly promotes its oligomerisation and activation (Acosta-Alvear *et al.*, 2018). Active IRE1 functions as an endoribonuclease and cleaves an unconventional intron from the mRNA that encodes for the transcription factor X-box binding protein 1 (XBP-1) (Acosta-Alvear *et al.*, 2018). XBP-1 is a dynamic transcription factor that regulates target genes involved in processes such as protein folding and degradation (Acosta-Alvear *et al.*, 2007). IRE1 also cleaves ER-localised mRNAs in a process known as

regulated IRE1-dependent decay (RIDD), thus reducing ER-protein folding load and activating other downstream effector molecules (Hollien and Weissman, 2006; Hollien *et al.*, 2009).

The PERK pathway's primary function is to reduce translation of RNA to restrict the input of new proteins in the ER (Bell *et al.*, 2016). In the presence of misfolded proteins, BiP uncouples from PERK, leading to its dimerisation and autophosphorylation, activating the kinase domain that interacts with downstream substrates (Bell *et al.*, 2016). For example, PERK-mediated phosphorylation of elongation initiation factor 2 α (eIF2 α) reduces protein synthesis by preventing a step needed to initiate the translation of mRNA (Harding, Zhang and Ron, 1999). However, substrates such as activating transcription factor 4 (ATF-4) avoid suppression of translation and are preferentially targeted (Bell *et al.*, 2016; Taylor, 2016). ATF-4 is essential for activating the expression of proteins needed to resolve ER stress, such as ER chaperones and pro-apoptotic transcription factor CHOP (Harding *et al.*, 2003; Marciniak *et al.*, 2004).

Finally, upon dissociation with BiP, ATF-6 translocates to the Golgi, where it is then cleaved by site-specific proteases S1P and S2P, releasing an activated cytosolic transcription factor that migrates to the nucleus (Haze *et al.*, 1999; Shen *et al.*, 2002; Parmar and Schröder, 2012). Once in the nucleus, ATF-6 activates the transcription of a range of UPR target genes, such as other transcription factors XBP-1 and ATF-4, as well as ER protein chaperone BiP (Taouji, Wolf and Chevet, 2013).

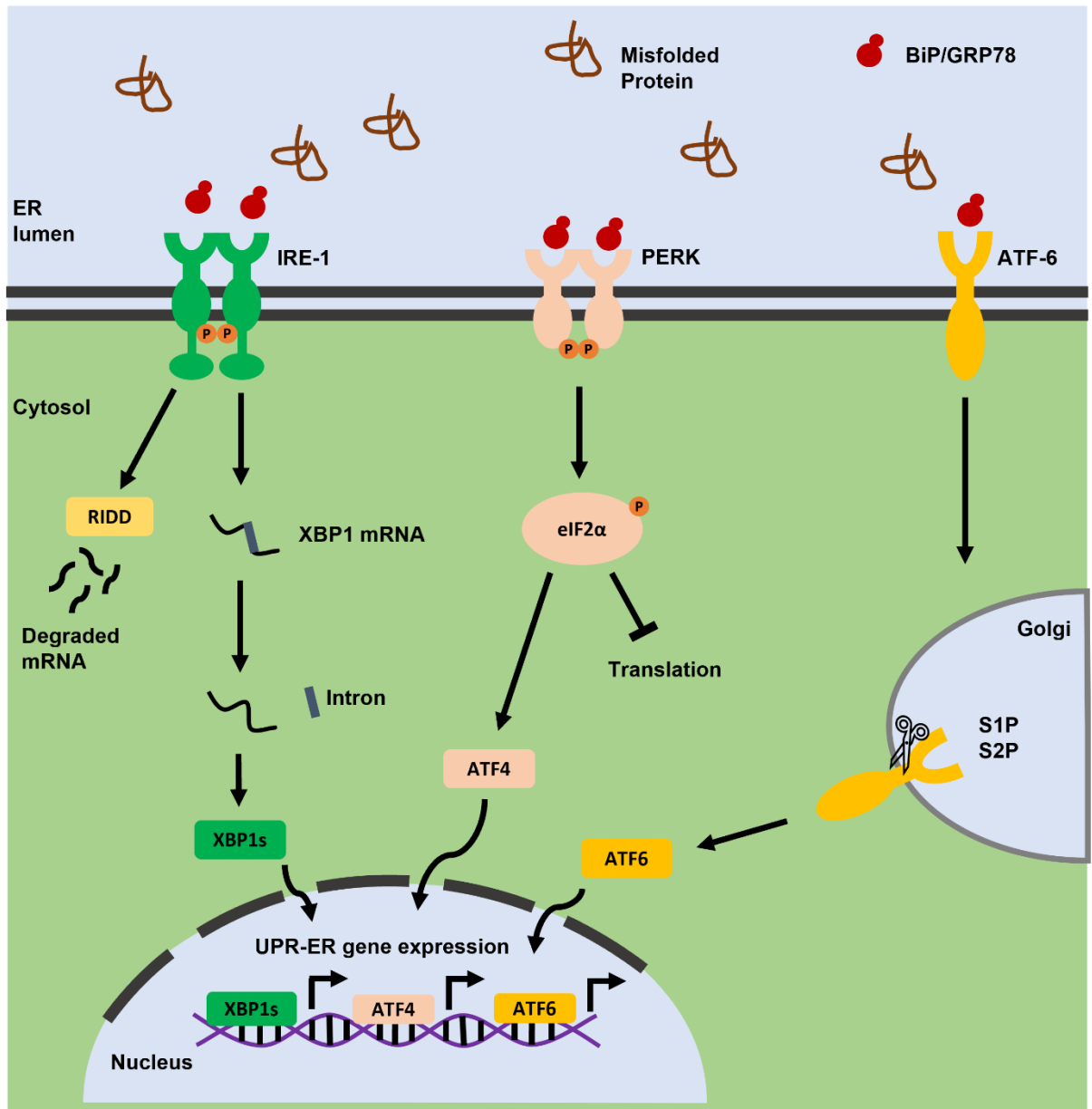


Figure 5. Overview of the UPR-ER signalling pathway.

A buildup of misfolded proteins in the ER initiates a UPR-driven cellular transcriptional and translational response to maintain ER proteostasis. Three transmembrane proteins, IRE1, PERK, and ATF6, are activated by ER stress and facilitate three separate branches of the response. IRE1 pathway: Upon oligomerisation and autophosphorylation in the presence of misfolded proteins, activated IRE1 splices XBP1. The activated transcription factor, XBP1s, then regulates UPR-related genes. PERK pathway: activated PERK phosphorylates eIF1 α , inhibiting protein translation. However, ATF4 avoids inhibition and translocates to the nucleus, where it induces UPR-related genes. ATF6 pathway: ER stress causes ATF6 to relocate to the Golgi, where it is cleaved by S1P and S2P proteases. The activated ATF6 transcription factor then translocates into the nucleus and induces genes to resolve ER stress.

1.9 Ageing and the UPR-ER

During ageing, the protective stress response facilitated by the UPR-ER is significantly diminished, and there is an increased focus on pro-apoptotic signalling (Paz Gavilán et al., 2006; Hussain and Ramaiah, 2007; Naidoo et al., 2008). Critical ER resident chaperones such as BiP, heat shock protein 90 (HSP90)-like GRP94, calreticulin, calnexin, thiol-disulfide oxidoreductases, and protein disulfide isomerases (PDIs), which are required for proper protein folding, become progressively oxidised with age, thus impairing their function (Nuss *et al.*, 2008; Taylor, 2016). In addition, the total expression levels of these chaperones also reduce with age. For example, in the cerebral cortex of aged mice, levels of BiP were shown to have decreased by 30% compared to young mice (Naidoo *et al.*, 2008). Similarly, in the brain and peripheral tissue of Wistar rats, levels of BiP declined with age (Hussain and Ramaiah, 2007).

In addition, the ability of the UPR-ER to regulate downstream targets diminishes with age (Paz Gavilán et al., 2006; Hussain and Ramaiah, 2007; Taylor and Dillin, 2013). This has been shown in the hippocampus of aged rats; PERK mRNA was significantly reduced, and GADD34 was increased, which facilitates the synthesis of pro-apoptotic proteins like CHOP by removing the translational block imposed by eIF2 α (Paz Gavilán et al., 2006). Also, the ability to activate the IRE-1 arm of the UPR-ER was shown to decline during the fertile period of the nematode *C. elegans* (Taylor and Dillin, 2013a). The combination of these studies suggests that ageing hinders UPR-ER activation, compromising its ability to coordinate an adaptive cellular response to ER stress, thus allowing the accumulation of misfolded proteins and, eventually, cellular dysfunction and disease.

1.10 Activating the UPR-ER can extend lifespan

In yeast and worms, activation of the UPR can facilitate an increase in lifespan. Deletion of genes targeted by the UPR signalling cascade in yeast extended replicative lifespan via constitutive

activation of the UPR (Labunskyy *et al.*, 2014; Cui *et al.*, 2015). In *C. elegans*, activation of the UPR using pharmacological reagents that induce ER stress also extends lifespan (Matai *et al.*, 2019). Similarly, expressing the spliced activated form of XBP-1, XBP-1s in worms increases longevity, mediated by UPR activation (Taylor and Dillin, 2013a; Imanikia, Özbey, *et al.*, 2019). The likely mechanism behind this improved longevity is the ability to protect against proteotoxic stress and maintain ER homeostasis. Neuronal *xbp-1s* in *C. elegans* induced the expression of lysosomal genes in the intestine with improved acidity and activity levels, thus enhancing their ability to remove toxic, misfolded protein species (Imanikia, Özbey, *et al.*, 2019; Taylor *et al.*, 2020). However, activation of the intestinal UPR also promotes ER restructuring associated with lipophagy, which is also required for longevity (Daniele *et al.*, 2020). This suggests that metabolism and proteostasis interact distinctly with UPR-ER activation to promote longevity.

Project Aims

There are many unanswered questions relating to how Pol III modulates lifespan. What pathways or processes is Pol III inhibition collaborating with to promote longevity? What small RNA targets are being affected by Pol III inhibition? And how do these things interact to modulate longevity? To address specific aspects of these questions, this project focused on two key aims:

Aim 1: Define the impact of Pol III knockdown on the unfolded protein response (UPR)

The Tullet lab has previously determined that Pol III inhibition can increase survival compared to WT when worms are exposed to conditions that generate toxic ER stress. Homeostasis in the ER is maintained by the UPR signalling cascade. Thus, to test whether the UPR-ER response was altered by Pol III inhibition, RNAi was performed on a *C. elegans* strain expressing a UPR-ER reporter *hsp-4p::GFP*, and fluorescence compared to control RNAi conditions using quantitative fluorescence microscopy. The specific UPR signalling pathway involved in this mechanism was then explored.

Aim 2: Define the link between Pol III's transcriptional function and the unfolded protein response (UPR)

RNA-seq data examining Pol III targets from the Tullet, Alic and Selman Labs has shown that Pol III controls the expression of tRNAs and other small RNAs. To test whether alterations in tRNA levels could account for the UPR response (Aim 1), a mini-screen of 20 individual tRNA Synthetase genes were targeted using RNAi and UPR activity was quantified using the UPR *hsp-4p::GFP* reporter.

Chapter 2: Materials and Methods

2.1 Worm husbandry

Worm husbandry was performed following techniques from the standard protocol (Stiernagle, 2006).

Worms were maintained at 20°C and fed OP50-1 (streptomycin-resistant) *E. coli* (OP50-1). OP50-1 was grown overnight for ~16 h in 250 mL conical flasks in 50 mL LB + 100 µL streptomycin sulfate (17.2 mM in dH₂O) and 50 µL nystatin (10.8 mM in DMSO). A platinum wire pick was used for transferring worms between agar plates.

Nematode Growth Medium (NGM) plates were made following a well-described standard protocol (Stiernagle, 2006). For a typical 2 L NGM solution: 6 g NaCl, 34 g agar, 5 g Bacto™ Peptone and 2 L of dH₂O were mixed, autoclaved, and then cooled to 55°C. 50 mL KH₂PO₄ (1 M, pH 6) was added, and 2 mL each of the following: MgSO₄ (1 M), CaCl₂ · 2H₂O (1 M), cholesterol (12.93 mM in EtOH), nystatin and 4 mL streptomycin. 10 cm agar plates were filled with 10 mL NGM, respectively and dried overnight before seeding with bacteria.

M9 buffer was made (1 L) using 7g Na₂HPO₄ · 2H₂O, 3 g KH₂PO₄, 5 g NaCl, 0.25 g MgSO₄ · 7H₂O and 1 L dH₂O, mixed, then autoclaved. M9 is a non-toxic buffer used in the bleaching protocol described below.

All reagents used are shown in Table 1.

Name	Supplier	Serial number/CAS/code
Agar	Melford	9002-18-0
Ampicillin	Melford	39372-40192
Bacto™ Peptone	Gibco™	211677
Calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	Melford	10035-04-8
Cholesterol	Sigma	57-88-5
Isopropyl β - d-1-thiogalactopyranoside (ITPG)	Melford	367-93-1
Magnesium sulfate anhydrous (MgSO_4)	Melford	7487-88-9
Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	Sigma Aldrich (BioXtra)	10034-99-8
Miller's Luria Broth (LB)	FisherScientific	231-598-3
Nystatin	Melford	1400-61-9
Potassium phosphate monobasic (KH_2PO_4)	Supelco	7778-77-8
Sodium chloride (NaCl)	Fisher Chemical	7647-14-5
Sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)	Sigma Aldrich (Fluka/BioXtra)	7558-79-4
Streptomycin sulfate	Melford	3810-74-0
Tetramisole hydrochloride	Sigma	16595-80-5
Tunicamycin	Sigma	11089-65-9

Table 1. Reagent List.

2.2 Strains and mutants

Strains used were: SJ4005 *zcls4* [*hsp-4::GFP*] V, SJ17 *xbp-1*(*zc12*) III; *zcls4* [*hsp-4::GFP*] V, RB790 *atf-4*(*ok576*) X, RB772 *atf-6*(*ok551*) X, SJ4058 *zcls9* [*hsp-60::GFP+lin15(+)*] V, *atf-4*(*ok576*) X; *zcls4* [*hsp-4::GFP*] V. All strains except for *atf-4*(*ok576*) X; *zcls4* [*hsp-4::GFP*] V were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

atf-4(*ok576*) X; *zcls4* [*hsp-4::GFP*] V genetic cross was confirmed using PCR (Figure 7). *Ok576* deletion and primer combination are shown in Figure 6. The sequence was visualised, and primer constructs were created using ApE. Primers were ordered from (Integrated DNA Technologies | IDT). GFP was screened using microscopy due to not having GFP primers at the time, so all data from chapter 3.2 and half of 3.3 could be incorrect.

To create the *atf-4*(*ok576*) X; *zcls4* [*hsp-4::GFP*] V strain, the *atf-4*(*ok576*) strain was crossed with the *zcls4* [*hsp-4::GFP*] UPR-ER reporter strain, as described in Figure 7. The *ok576* mutation is a 484 base pair deletion in the T04C10. 11 (*atf-4*) gene, shown in Figure 6A. A three-primer strategy was employed; the primer combination and corresponding sequence are shown in Figure 6B. The following PCR protocol was performed: 94° for 2 minutes (cycle one only), 94° for 30 seconds, 60° annealing temperature for 30 seconds, and 72° for 45 seconds for 32 cycles. The annealing temperature was determined for the primers in Figure 6B using a gradient PCR. Gradient temperatures were from 10°; T_m minus 5° (lowest temperature) to T_m plus 5° (highest temperature), a 55° to 65° gradient was used. PCR products were resolved on 1% agarose gels and visualised with GeneSys (Version 1.5.2.0).

A

Wildtype ok576

Legend: **a** Flanking sequence **A** Mutation

ccatttgaagttgacatcggagttcaaatgatgctgttgataagattgatgggtaggggtgaaatgatgaggatgctcggagattgatggtg
atgatgatgatgggtatgatattgtgggtggaatgggtttgattgtgcatccattataaagcaagaactgtggattttgttcattacataagccatttgtt
ggtaatcattttgtccgtatgcatgacccgtaggctcaacaacacagcacgtgatttcctggaaggatcattgttaaactaattattaatcaatataa
agttaaataaacttactgtacaaatggcgtgtggcggaggagcgttcgcgatgacgtaagattggtattgagacgggtcgggtatagttcatgtt
gaagttcataatttcagatgagatgtttctgcgagaaagtggcgtgtatgtgtccagtgatgtgttgattgtgaaatgacagaagaggaaaggggaGAC
GACTAGCGCTTGGTGTAGTTTGGCTGCCCCCAATCTTTCCACCTCCGGCGTTTGGCTCGATAATTTTGTATG
TTCTCTGATCGCACCCGTTTCTTATACTATTTCTCTTAGTTTATCAGGGCTGTCGCGTGCTATCTGCATCTCTC
GCGGTCTCGTACGTCATCAATCGATGTGGATTAGTGGAGGAAATTCAAATATCAAAGACGTTAAGCTGATCT
TTGGATTGAAAAAAGTTTCAGATTAAATTTTAAATAAAATGTGTAAGTAATTCAAAAAGCTCCCAGAGTGTTTT
CTTACAGTTAGGTATAATTAAGGTAGGGCTTCTCTAAACTTTCTTATCTAAATTTCCCGATATTCGCTATTTCCG
GATATCGGTTATTTTAGATTTTTAGCGACAGATAGCTCAGTCGGTAGTGGTCCGCCGCTAACAATCTGGAGAT
CACGAGTTGAAGTTCAAGACCCCTAGGTTTAACCAGCCTCTAAttggagagtggagcaatccactacaggattatcgcca
cagtcacaggttaggatgtggcttaaattatagccagtgagatcaccaccagtcagttacctaataccagttccgcagagcatagcactgaag
aacggatcgtctttaaactccttaactccttagttcgtgttcgaataaaataatTTTTTgaatcggatattttcggacaattggatatttcggatattttctt
aaatttggtattttctgtttcaactaattttattgaaaaatgaacagttattgaaaaccgacacaaaaacatcagaaaaaactgtttaacaaaaaca
acatgttagaaattaaagaaaaataatcaataatttcagtcacacagtagcctctggtcatcaatagattcattagacgaataattttgctcaaaaat
aggagcattttaatttcagggtgacaaagccttttcggtatttagtaaga

B

Primer name	Primer sequence
<i>atf-4</i> External Forward 1	ggatgaatgggtttgattgtg
<i>atf-4</i> Internal Forward 1	GCGCTTGGTGTAGTTTGGCTGC
<i>atf-4</i> External Reverse 1	gtgctatgctctcggaactg

Figure 6. Sequence for wild-type ok576 strain and primer combination.

(A) Sequence showing wild-type ok576 strain. The mutation portraying the Ok576 deletion is displayed in red, and the flanking sequence is shown in yellow.

(B) Primer combination used for genotyping the *atf-4(ok576)* strain.

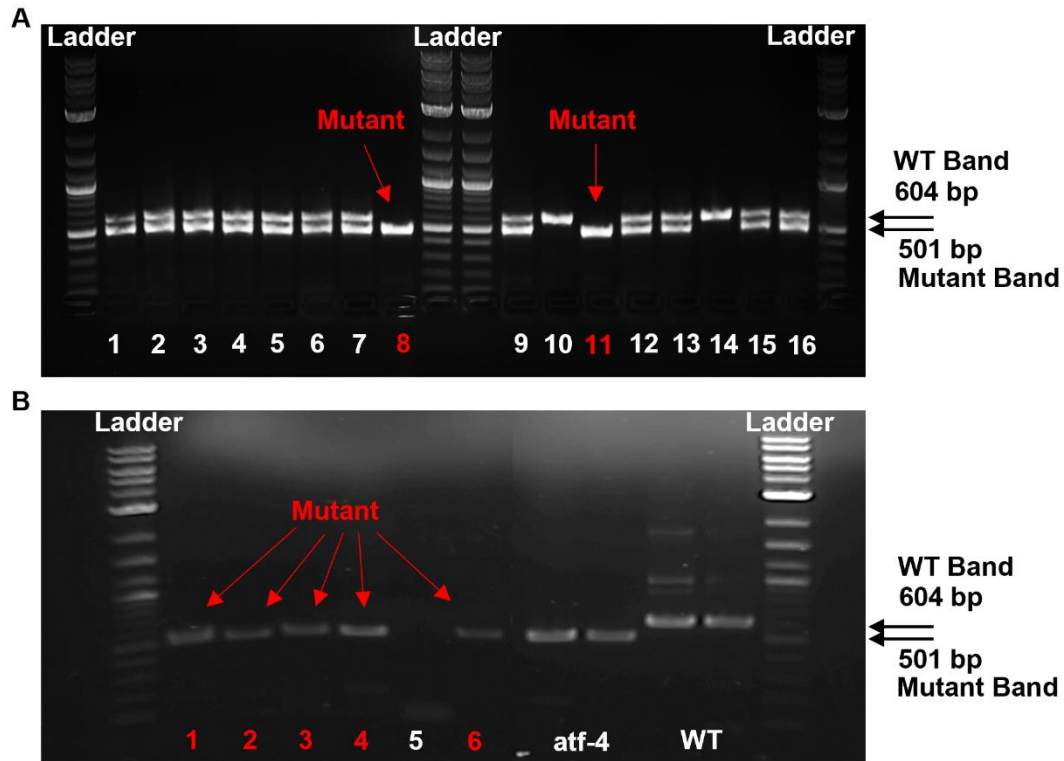


Figure 7. Creation of *atf-4(ok576) X; zcls4 [hsp-4::GFP]* V strain.

(A) PCR genotype of F2 progeny. *Atf-4* (ok576) males were crossed with a single *zcls4* [*hsp-4::GFP*] hermaphrodite. The presence of males in the F1 generation (around 50%) confirmed that the cross had been successful. Three heterozygous F1 hermaphrodites were then transferred onto a separate NGM plate and allowed to lay eggs. Once the F2 generation had reached the L4 stage, worms containing GFP (screened using microscopy) were placed into individual NGM plates and allowed to lay eggs. After confirming that each worm had laid sufficient eggs, the F2 parent was removed and genotyped using PCR; the results are shown above. The numbers match the lane with the corresponding worm sample; 604 base pair represent the wild-type band; 501 base pairs represents the mutant *atf-4* (ok576) band; control lanes were present but are not shown. Homozygous mutant *atf-4* (ok576) worms are shown in red, and the corresponding progeny were chosen for further screening, as shown in Figure 7B. PCR products resolved on 1% agarose gel with a 1 kb plus DNA size ladder.

(B) PCR genotype of F3 progeny. After checking for GFP using microscopy, the progeny from the worm sample in lane 8 in Figure 7A were chosen for further screening. Multiple worms were then genotyped using PCR to ensure they were all homozygous mutant *atf-4* (ok576); the results are shown above. The numbers match the lane with the corresponding worm sample; 604 base pair represent the wild-type band; 501 base pairs represents the mutant *atf-4* (ok576) band. Homozygous *atf-4* (ok576) samples are shown in red, confirming we have the required *atf-4* (ok576); *zcls4* [*hsp-4::GFP*] mutant. There was likely an insufficient amount of worm DNA in lane 5 for the primers to bind to, which is why there was no band. PCR products resolved on 1% agarose gel with a 1 kb plus DNA size ladder.

2.3 RNAi

Whole organisms RNA interference (RNAi) was performed following standard protocol (Conte *et al.*, 2015). Worms were fed HT115 (DE3) RNase III-deficient *E. coli* expressing RNAi in vector L4440 (HT115 (DE3)). RNAi was obtained from the library created by Dr Julie Ahringer's group (Kamath and Ahringer, 2003) and supplied by Source BioScience.

RNAi NGM plates follow the standard NGM protocol described above with changes in the added antibiotics; ampicillin (134.6 mM in dH₂O) and IPTG (1 M in Milli-Q®) were added instead of streptomycin and nystatin at 2 mL each in a 2 L NGM solution.

Luria Broth (LB) ampicillin and tetracycline plates for RNAi were made (1 L) using LB broth (25 g), agar (17 g) and dH₂O (1 L), mixed, autoclaved then cooled to 55°C. 1 mL ampicillin and 1 L tetracycline (20.8 mM in DMSO) were added. 6 cm agar plates were filled to 10 mL with LB ampicillin and tetracycline mixture and dried overnight before use.

HT115 (DE3) *E. coli* (ampicillin and tetracycline-resistant) were grown on ampicillin and tetracycline LB agar plates for 16 h at 37°C. Post growth, 2-3 colonies were taken and placed into a 250 mL conical flask containing 50 mL LB + ampicillin (50 µL), and left at 37°C in a shaking incubator for 16 h. After incubation, to concentrate bacterial density, cultures were poured into a 50 mL conical tube, centrifuged at 4500 RPM for 15 mins to form a pellet. The supernatant was then removed to 10 mL, and the pellet was resuspended and seeded with 250 µL onto 10 cm RNAi agar plates.

2.4 RNAi screen experimental design

Well-fed worms were allowed to grow on 3, 10 cm NGM agar plates seeded with OP50-1 at 20°C until they reached gravid adulthood. Well-fed worms are worms grown in the presence of a constant

readily-available food source, and were used as starvation can epigenetically influence progeny behaviour for up to 3 generations (Rechavi *et al.*, 2014).

Worms were then bleached following standard protocol (Bischof, Huffman and Aroian, 2006), and released eggs were laid on 3, 10 cm NGM agar plates seeded with OP50-1. After ensuring proper aeration to remove leftover M9 from the bleaching, the plates were placed at 20°C and allowed to grow until reaching the L4 larval stage (approx. 47 hrs $\pm$ 4).

L4 worms were then transferred using a platinum wire pick onto 3 RNAi NGM plates (approx. 30 worms per plate) seeded with HT115 (DE3) containing the desired vector construct and 3 RNAi NGM plates seeded with HT115 (DE3) containing an empty vector construct to be used as a control. So overall, 3 sets of plates for the test and 3 for the control, and if several types of vector constructs are being used; 3 plates per test condition. Both sets of plates were placed at 25°C, and worms were imaged (approx. 10-15 worms) using microscopy on days 2, 5, and 8 adulthood (Figure 8).

Each experiment in this report has been repeated for accuracy at least 3 times, barring the results in chapter 4.1.

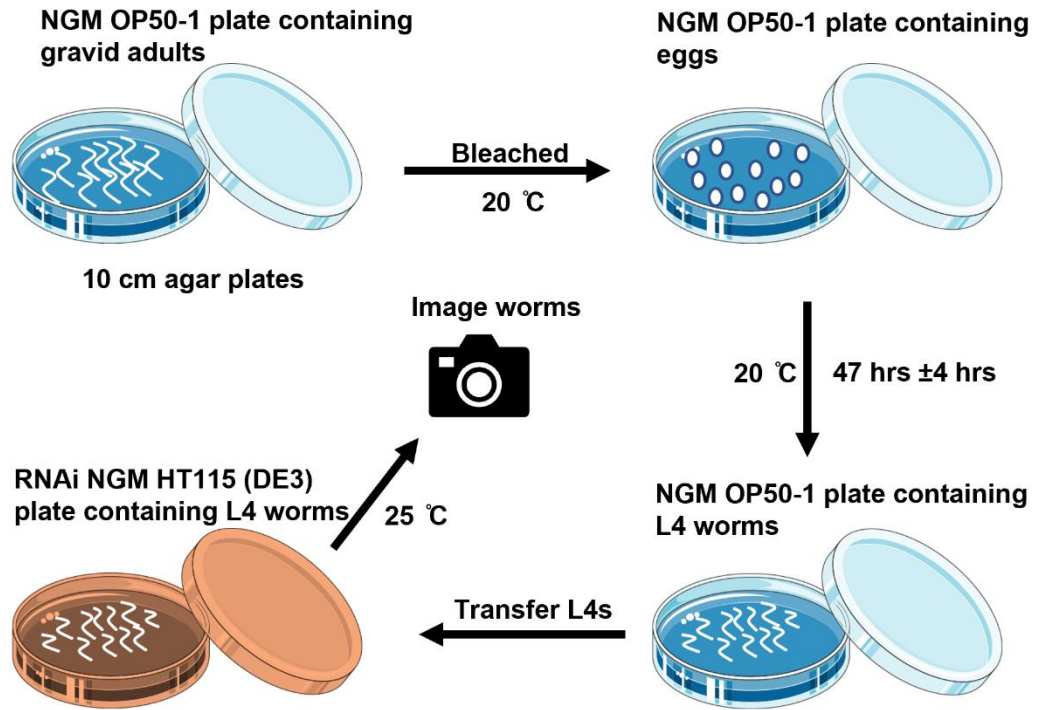


Figure 8. Schematic for RNAi screen.

Well-fed worms were allowed to grow on 10 cm NGM agar plates seeded with *OP50-1* at 20 °C until they reached gravid adulthood. Worms were then bleached, and released eggs were laid on 10 cm NGM agar plates seeded with *OP50-1*. Post aeration, plates were placed at 20 °C and allowed to grow until reaching the L4 larval stage (approx. 47 hrs ± 4). L4 worms were then transferred using a platinum wire pick onto RNAi NGM plates (approx. 30 worms per plate) seeded with HT115 (DE3) containing the desired vector construct, and RNAi NGM plates seeded with HT115 (DE3) containing an empty vector construct to be used as a control. Both sets of plates were then placed at 25 °C, and worms were imaged (approx. 10-15 worms) using microscopy on days 2, 5, and 8 adulthood.

2.5 Fluorescent microscopy

To prepare worms for microscopy, briefly, 2 microscope slides with thin tape were placed vertically onto a surface; then, another slide was placed vertically between both slides without tape. 30 μ L of 2% agarose was pipetted onto the non-taped slide. Another slide was then placed horizontally on top to create an agarose pad. 4 μ L tetramisole hydrochloride (25 mM in M9) was added to the agarose pad. Worms were transferred to the pad using a platinum wire pick, covered with a rectangular coverslip then imaged on the Leica DMR fluorescence microscope using HC PLAN S 10x /22 eyepieces with the appropriate Leica lens (HC PL Fluotar 10 x/0.30 and 20 x/0.50 lens, HCX PL Fluotar 40 x/0.75, and HCX PL APO 63x/1.40-0.60 oil), and imaged with a Leica DFC9000 GT using Leica Application Suite X software. The filter used was GFP 495 525/50 (green).

2.6 Worm genotyping PCR protocol

Worm preparation: Single desired worms were cloned out onto separate NGM agar plates seeded with OP50-1. Once eggs had been laid, the adult worms were removed using a platinum wire pick and placed in individual PCR tubes containing 5 μ l worm lysis buffer + 0.1 mM proteinase K (5 μ l/ml). The progeny were kept.

Worm lysis: PCR tubes were spun down to ensure worms were in the lysis buffer. Standard lysis programme was then used to lyse the worms in the thermocycler (Williams *et al.*, 1992).

Worm lysis programme: Heat tube to 65 degrees for 60 minutes. Inactivate proteinase K by heating to 95 degrees for 15 minutes. Store DNA at -80C.

PCR: For each primer combination, a primer mix was prepared that assumed a 10 μ M concentration of each primer in the solution. Using this, a master PCR mix was prepared (1X = 10 μ l Taq Mix Red, 3 μ l primer mix, 2 μ l nuclease-free water). 15 μ l of the master mix was then pipetted into each lysis

tube, and the lids were placed on the tubes. The desired PCR protocol was then run; this was determined using a gradient PCR.

Gel run: 1% agarose gel was made with 1X TAE buffer (Tris-acetate-EDTA) containing 0.25 µg/ml ethidium bromide. Once properly dried, the samples were loaded into the gel and ran at 100 V for 30 minutes ± 10 minutes. The image was then visualised using UV.

2.7 Image Analysis

Total worm fluorescence was analysed using ImageJ2 (Rueden *et al.*, 2017). Images were processed, and fluorescence intensity was measured using standard protocol (Rueden *et al.*, 2017).

2.8 Statistics

All calculations were performed using Microsoft® Excel® for Microsoft 365 MSO (Version 2202 Build 16.0.14931.20806). Welch's t-test (two-tailed t-test with unequal variance/heteroscedasticity) was used to determine if sample fluorescence was significant. Statistical significance for all tests was $p = \leq 0.05$. Error bars represent the variability from specific data points to the mean ± SDM.

Chapter 3: Defining the impact of Pol III knockdown on the UPR

Introduction

Previous studies in the lab found that when exposed to mild heat shock, worms with Pol III inhibited survived longer than worms that did not experience inhibition. Heat shock is a widely used experimental design that leads to the accumulation of proteotoxic stress and induces heat shock proteins (HSPs) to ensure proper protein folding (Zevian and Yanowitz, 2014). This experiment suggested that the ability to maintain protein homeostasis throughout ageing was a mechanism of Pol III inhibition and contributed to the phenotype of increased longevity. Another experimental design that increases misfolded protein species is the use of the pharmacological reagent, tunicamycin (Bassik and Kampmann, 2011). However, this effect is more specific to the ER. Similar to the heat shock experiment, our lab determined that Pol III inhibition improved survivability in the presence of tunicamycin. The combination of these experiments reinforced the notion that coping with proteotoxic stress throughout ageing contributed to Pol III-mediated longevity and that this effect might be specific to the ER.

As previously described, proteostasis in the ER is maintained by the series of signalling pathways that comprise the UPR, and the UPR-ER stress response has been linked to ageing and longevity. Therefore, to test the theory that Pol III inhibition was improving proteostasis in the ER and contributing to improved longevity, we needed to measure the effect of Pol III inhibition on the UPR. Furthermore, if Pol III inhibition was found to modulate the UPR, we needed to determine through which of the three signalling cascades this was occurring and if this effect is specific to UPR-ER.

Results

3. 1 Pol III knockdown induces *hsp-4* expression

The chaperone protein BiP is critical for ensuring protein quality control in the ER and activating ER-transmembrane signalling molecules (Wang *et al.*, 2009). Present in *C. elegans* is the BiP homologue HSP-4, which is strongly induced by UPR signalling (Zha *et al.*, 2019). To determine the effect Pol III knockdown has on the UPR-ER, we used the well-characterised transcriptional reporter expressing green fluorescent protein (GFP) from the *hsp-4* promoter (*hsp-4p::GFP*) in tandem with the previously described method of inhibiting Pol III activity using RNAi (RNAi)(Figure 8).

Initial results suggested that the *hsp-4p::GFP* reporter was induced in middle and late adulthood in worms experiencing *rpc-1* RNAi compared to control RNAi (Figure 9A). The same effect was observed in every biological repeat and was confirmed using statistical analysis (Figure 9B). As is shown in Figure 9B, worms feeding on bacteria containing the *rpc-1* RNAi vector experience significant induction of *hsp-4p::GFP* on days 5 and 8 of adulthood compared to worms feeding on the control RNAi. There is no apparent difference in *hsp-4p::GFP* induction on day 2 of adulthood, which is likely explained by the nature of RNAi; gene knockdown can often take up to 96 hours to take effect and usually lasts between 5-7 days (Han, 2018).

In nematodes, the ability to activate the UPR-ER decreases with age and salvaging this response has been shown to increase longevity (Taylor and Dillin, 2013b). Our data shows that partial inhibition of Pol III via *rpc-1* knockdown induces levels of *hsp-4* in middle and late adulthood. This suggests that Pol III inhibition activates the UPR-ER in worms into late adulthood and contributes to Pol III-mediated longevity. However, any potential mechanism remains largely unknown; therefore, to establish a possible link, we decided to study the signalling pathways that comprise the UPR-ER.

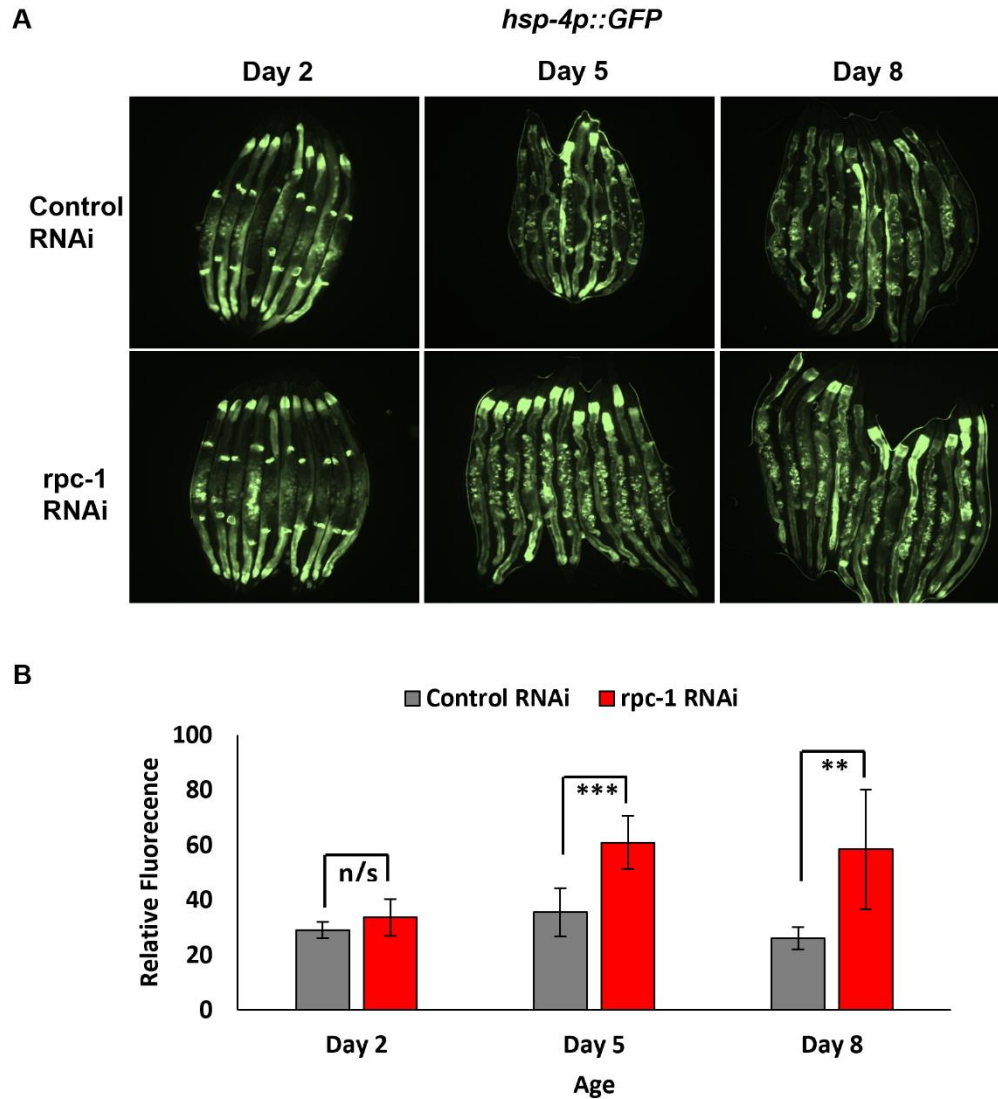


Figure 9. *Pol III* knockdown induces *hsp-4p::GFP* expression.

(A) *hsp-4p::GFP* UPR-ER reporter worms were placed on *rpc-1* RNAi and control RNAi plates at the L4 larval stage, and GFP fluorescence was assessed on days 2, 5, and 8 of adulthood. Representative images of 3 biological repeats, each with a sample size of 10 – 15 worms are shown. Images were taken at 10X magnification.

(B) *hsp-4p::GFP* UPR-ER reporter worms were treated as above, and GFP fluorescence was measured on days 2, 5, and 8 of adulthood using fluorescent microscopy. Relative fluorescence of worms on *rpc-1* RNAi and control RNAi is shown, with error bars indicating standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, n/s = not significant. Representative data of 3 biological repeats, each with a sample size of 10 – 15 worms are shown.

3.2 The UPR-ER is induced by tunicamycin, but this is non-additive with Pol III knockdown

Tunicamycin is widely used to observe the effect of increasing misfolded protein load in the ER. The drug activates the UPR by accumulating large-scale protein misfolding, facilitated by preventing the first step of N-linked glycosylation of proteins by DPAGT1 in the ER (Bassik and Kampmann, 2011). Previously in the lab, it was found that in the presence of tunicamycin, Pol III inhibition via *rpc-1* targeted RNAi increased survivability compared to worms on control RNAi (Supplementary Figure 1). Thus, we wanted to determine if the ability to maintain proteostasis in the ER, enabled by an additive activation of the UPR through Pol III inhibition, was the mechanism behind Pol III-mediated increased survivability in the presence of tunicamycin.

Using worms tagged with the well-characterised UPR-ER transcriptional reporter, *hsp-4p::GFP*, we studied the effect of Pol III inhibition on the UPR-ER in the presence of tunicamycin. Early observations suggested that tunicamycin was significantly inducing the *hsp-4p::GFP* reporter at all stages of adulthood in worms on *rpc-1* RNAi plates and control RNAi (Figure 10A). This same effect was also observed in every consequent repeat, and Pol III inhibition did not appear to be modulating any significant *hsp-4p::GFP* response in the presence of tunicamycin. However, by visually observing the plates, it was clear that worms feeding on *rpc-1* RNAi were coping better with the increased toxicity than worms feeding on control RNAi; worms on control RNAi appeared underdeveloped and had reduced locomotion compared to worms on *rpc-1* RNAi. Statistical analysis of fluorescence levels determined that tunicamycin significantly induced *hsp-4p::GFP* levels at all stages of adulthood in both *rpc-1* RNAi-fed worms and control RNAi-fed worms (Figure 10B). However, partial inhibition of Pol III did not provide any significant modulation in *hsp-4p::GFP* induction compared to fully functioning Pol III at any stage of adulthood in worms treated with tunicamycin (Figure 10B).

Unsurprisingly, treatment by tunicamycin induces great ER stress that causes a significant activation in *hsp-4*, yet Pol III inhibition does not produce an additive increase in *hsp-4*. This contradicts previous results that showed Pol III inhibition increased *hsp-4* induction. Also, this does not explain why Pol III inhibition increases survivability and modulates better physical condition compared to worms with fully functioning Pol III in the presence of tunicamycin. Initially, we thought Pol III inhibition might instigate a hormetic response that mildly induced ER stress to promote longevity and that tunicamycin-induced damaging ER stress led to cellular dysfunction. However, if this were the case, tunicamycin would likely over-run the Pol III hormetic response and cause similar phenotypes in both worms fed *rpc-1* RNAi and control RNAi. Indeed, it appears that Pol III inhibition is providing a protective reaction against cellular dysfunction caused by ER stress. Still, this data suggests it is not by further activating the UPR-ER.

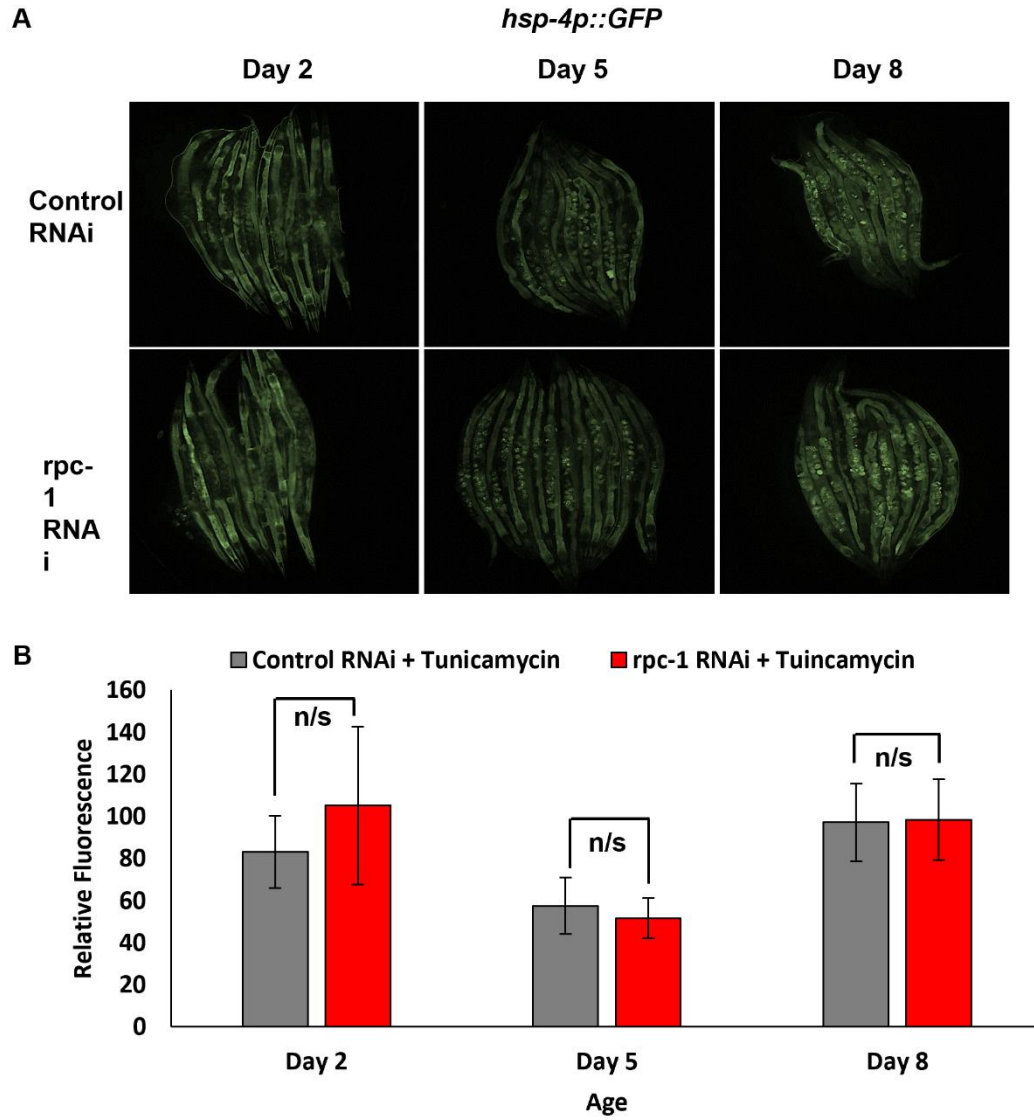


Figure 10. The UPR-ER is induced by tunicamycin, but this is non-additive with Pol III knockdown.

(A) *hsp-4p::GFP* UPR-ER reporter worms were placed on *rpc-1* RNAi and control RNAi plates containing 10 μ g/ml of tunicamycin at the L4 larval stage, and GFP fluorescence was assessed on days 2, 5, and 8 of adulthood. Representative images of 3 biological repeats, each with a sample size of 10 – 15 worms are shown. Images were taken at 10X magnification.

(B) *hsp-4p::GFP* UPR-ER reporter worms were treated as above, and GFP fluorescence was measured on days 2, 5, and 8 of adulthood using fluorescent microscopy. Relative fluorescence of worms on plates containing *rpc-1* RNAi + 10 μ g/ml tunicamycin and control RNAi + 10 μ g/ml tunicamycin is shown, with error bars indicating standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: n/s = not significant. Representative data of 3 biological repeats, each with a sample size of 10 – 15 worms are shown.

3.3 Pol III knockdown requires *xbp-1* and *atf-4* to mediate activation of the UPR

All three known UPR pathways, IRE1/*ire-1*, PERK/*pek-1*, and ATF6/*atf-6*, are well conserved in *C. elegans*. These pathways facilitate a response to ER stress in part by activating downstream transcription factors that translocate into the nucleus and upregulate genes to return homeostasis to the ER (Figure 5). We postulated that examining these transcription factors would give us an idea of the involvement of each arm in UPR-ER activation modulated by Pol III inhibition. Previous studies in the worm have shown that the *ire-1* signalling cascade leads to the activation of the transcription factor *xbp-1*, which facilitates the upregulation of most stress-response-related UPR genes. In contrast, *pek-1* and *atf-6* signalling and their corresponding transcription factors, *atf-4* and *atf-6*, mainly act as complementary pathways (Shen *et al.*, 2005). Thus we hypothesised that the induction in *hsp-4* seen when Pol III is inhibited is facilitated by the *ire-1/xbp-1* signalling cascade and examined this pathway first.

To determine the role the *ire-1* pathway plays during *hsp-4* activation in response to Pol III inhibition, we focused on the downstream transcription factor *xbp-1*. The SJ17 (*xbp-1(-);[hsp-4p::GFP]*) *C. elegans* strain contains worms with a nonsense mutation in the *xbp-1* gene as well as having GFP tagged to the *hsp-4* transcriptional promoter. This strain allowed us to examine the role of *xbp-1* in modulating a UPR-ER response by measuring *hsp-4* activation. Using the *xbp-1* mutant (SJ17) strain and the standardised method of causing Pol III inhibition via RNAi (Figure 8), we first noticed an apparent lack of induction in the *hsp-4p::GFP* reporter across all periods of adulthood in worms feeding on *rpc-1* RNAi and control RNAi (Figure 11A). This effect was observed across all repeats, and the contrast is accentuated when compared to *hsp-4p::GFP* reporter worms with functioning *xbp-1* (Figure 9A). Statistical analysis determined this observation to be accurate and found that mutants with *xbp-1* compromised could not activate *hsp-4p::GFP* to the same level as worms with functioning

xbp-1 (Figure 11C). This was statistically true in *xbp-1* mutants (SJ17) experiencing partial inhibition of Pol III and *xbp-1* mutants (SJ17) with Pol III intact (Figure 11C). Furthermore, *xbp-1* mutants (SJ17) with Pol III inhibited did not instigate increased induction of *hsp-4p::GFP* when compared to the control *xbp-1* mutants (SJ17). These results confirm the work done in previous studies showing that *xbp-1* is required to modulate a UPR-ER response (Wang, Chen and Ouyang, 2011; Taylor and Dillin, 2013c; Imanikia, Özbey, *et al.*, 2019) and also show that *xbp-1* is required for Pol III mediated regulation of *hsp-4*/UPR-ER.

Similarly to *ire-1/xbp-1*, *pek-1* has been shown to be required for UPR activation and provides adaptive functions upon ER stress (Shen *et al.*, 2001). During the stress response, the transcription factor *atf-4* acts downstream of *pek-1* and becomes preferentially translated, avoiding impaired translation via eIF2 α phosphorylation; a process that has been shown to modulate longevity in *C. elegans* (Statzer *et al.*, 2022). Therefore, to determine the role of *pek-1*-branch signalling on *hsp-4* induction in response to Pol III inhibition, we examined the downstream target, transcription factor *atf-4*. Using the transgenic *atf-4*;[*hsp-4p::GFP*] mutant strain (described in section 2.2 and Figure 7) and the standardised method of causing Pol III inhibition via RNAi (Figure 8), we examined the role *atf-4* plays in *hsp-4* induction with regards to Pol III knockdown. Similarly to *xbp-1* mutants, *atf-4* mutants showed repeatedly visible reductions in *hsp-4p::GFP* induction across all points of adulthood (Figure 11B) compared to worms with functioning *atf-4* (Figure 9A). Furthermore, statistical analysis of fluorescence levels confirmed observations and determined that mutants with *atf-4* compromised could not activate the *hsp-4p::GFP* reporter to the same level as worms with functioning *atf-4* (Figure 11D). Also, there was no significant difference in *hsp-4p::GFP* induction between *atf-4* mutants feeding *rpc-1* RNAi and *atf-4* mutants feeding on control RNAi (Figure 11D), suggesting that the lack of

atf-4 is preventing the induction of *hsp-4p::GFP* effect that is seen when Pol III is inhibited; suggesting that *atf-4* is also required for this response.

After determining that *xbp-1* is required for Pol III-mediated induction of *hsp-4p::GFP*, we were surprised to find that *atf-4* is also necessary even when *xbp-1* is fully functional. Having been shown multiple times to modulate the UPR-ER response, it was not surprising that compromising *xbp-1* led to reduced induction of the UPR-ER even when Pol III is inhibited. However, we thought that the removal of *atf-4* could still facilitate a strong UPR-ER response through the *ire-1/xbp-1* signalling pathway. This was determined to be false and suggests that both *xbp-1* and *atf-4* are required for Pol III-mediated activation of the UPR-ER.

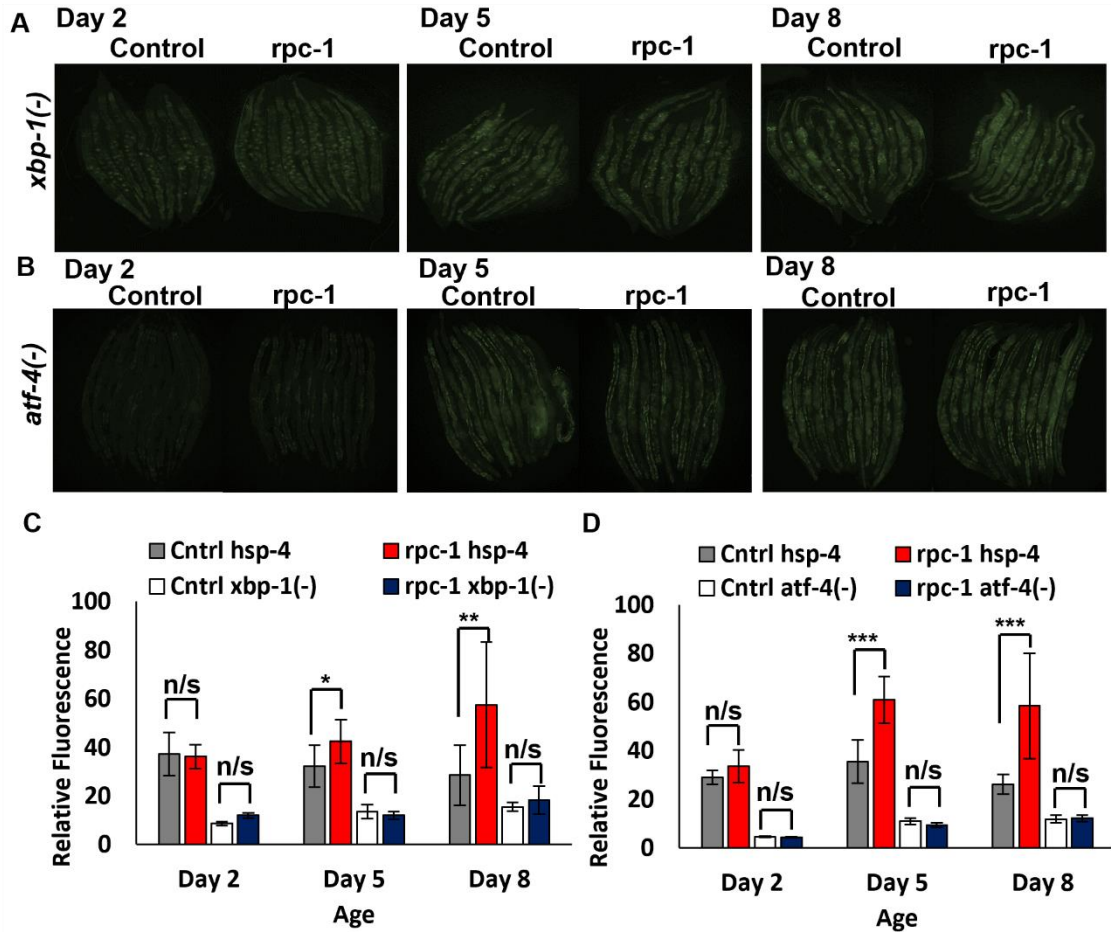


Figure 11. Pol III knockdown requires *xbp-1* and *atf-4* to mediate activation of the UPR.

(A) *xbp-1(-);[hsp-4p::GFP]* (SJ17) worms were placed on *rpc-1* RNAi and control RNAi plates at the L4 larval stage, and GFP fluorescence was assessed on days 2, 5, and 8 of adulthood. Representative images of 3 biological repeats, each with a sample size of 10 – 15 worms are shown. Images were taken at 10X magnification.

(B) *atf-4(-);[hsp-4p::GFP]* worms were placed on *rpc-1* RNAi and control RNAi plates at the L4 larval stage, and GFP fluorescence was assessed on days 2, 5, and 8 of adulthood. Representative images of 3 biological repeats, each with a sample size of 10 – 15 worms are shown. Images were taken at 10X magnification.

(C) *xbp-1(-);[hsp-4p::GFP]* (SJ17) and *hsp-4p::GFP* worms were treated as described in Figure 11A, and GFP fluorescence was measured on days 2, 5, and 8 of adulthood using fluorescent microscopy. The relative fluorescence of SJ17 worms on *rpc-1* RNAi and control RNAi is shown, as well as the relative fluorescence of *hsp-4p::GFP* worms on control RNAi and *rpc-1* RNAi for comparison. Error bars indicate standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: * $p < 0.05$, ** $p < 0.005$, n/s = not significant. Representative data of 3 biological repeats, each with a sample size of 10 – 15 worms are shown.

(D) *atf-4(-);[hsp-4p::GFP]* (strain name) and *hsp-4p::GFP* worms were treated as described in Figure 11B, and GFP fluorescence was measured on days 2, 5, and 8 of adulthood using fluorescent microscopy. The relative fluorescence of *atf-4* mutant worms on *rpc-1* RNAi and control RNAi is shown, as well as the relative fluorescence of *hsp-4p::GFP* worms on control RNAi and *rpc-1* RNAi for comparison. Error bars indicate standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: *** $p < 0.0005$, n/s = not significant. Representative data of 3 biological repeats, each with a sample size of 10 – 15 worms are shown.

3.4 Pol III effects are specific to the UPR-ER

The mitochondria have their own adaptive stress response to cope with the accumulation of misfolded proteins, termed the mitochondrial unfolded protein response (UPR-MT). Conceptually similar to the UPR-ER, activation of the UPR-MT regulates nuclear transcripts encoding mitochondrial chaperones and proteases to maintain mitochondrial proteostasis (Yoneda *et al.*, 2004). Activation of the UPR-MT in *C. elegans* has also been shown to promote longevity and is well reviewed by (Bennett *et al.*, 2014). Therefore, to determine if Pol III-mediated induction was UPR-ER specific, we needed to explore the effect of Pol III inhibition on the UPR-MT and decipher if this mechanism contributed to improved longevity.

Previous studies have shown that activation of the UPR-MT in *C. elegans* functions similarly to that of mammals and leads to activation of the mitochondrial chaperone genes *hsp-6* and *hsp-60* (Yoneda *et al.*, 2004; Benedetti *et al.*, 2006; Haynes *et al.*, 2007; Durieux, Wolff and Dillin, 2011). Thus, to establish the relationship between Pol III inhibition and the UPR-MT, we used the well-characterised UPR-MT reporter, *hsp-60::GFP*, which is induced explicitly by manipulations that activate the UPR-MT (Yoneda *et al.*, 2004).

Using the *hsp-60::GFP* UPR-MT reporter strain (SJ4058) and the standardised method of causing Pol III inhibition via RNAi (Figure 8), we could explore the interaction between Pol III and the UPR-MT in *C. elegans*. Visual analysis of initial fluorescence levels determined no clear modulation of the *hsp-60::GFP* reporter by Pol III at any point of adulthood (Figure 12A). There was also no apparent change in the physical condition of the SJ4058 worms feeding on *rpc-1* RNAi compared to those feeding on control RNAi. These effects were continuously observed in every biological repeat. Finally, statistical

analysis confirmed that Pol III inhibition did not modulate a significant *hsp-60::GFP* response at any point in adulthood (Figure 12B).

This indicates that the UPR-MT is not activated by Pol III inhibition in *C. elegans* and that induction of the UPR-MT is not contributing to Pol III-mediated longevity. However, UPR-MT activation in *C. elegans* also induces protein chaperone gene *hsp-6*; thus, it will be necessary to study the effects of Pol III inhibition on *hsp-6* induction to validate these results.

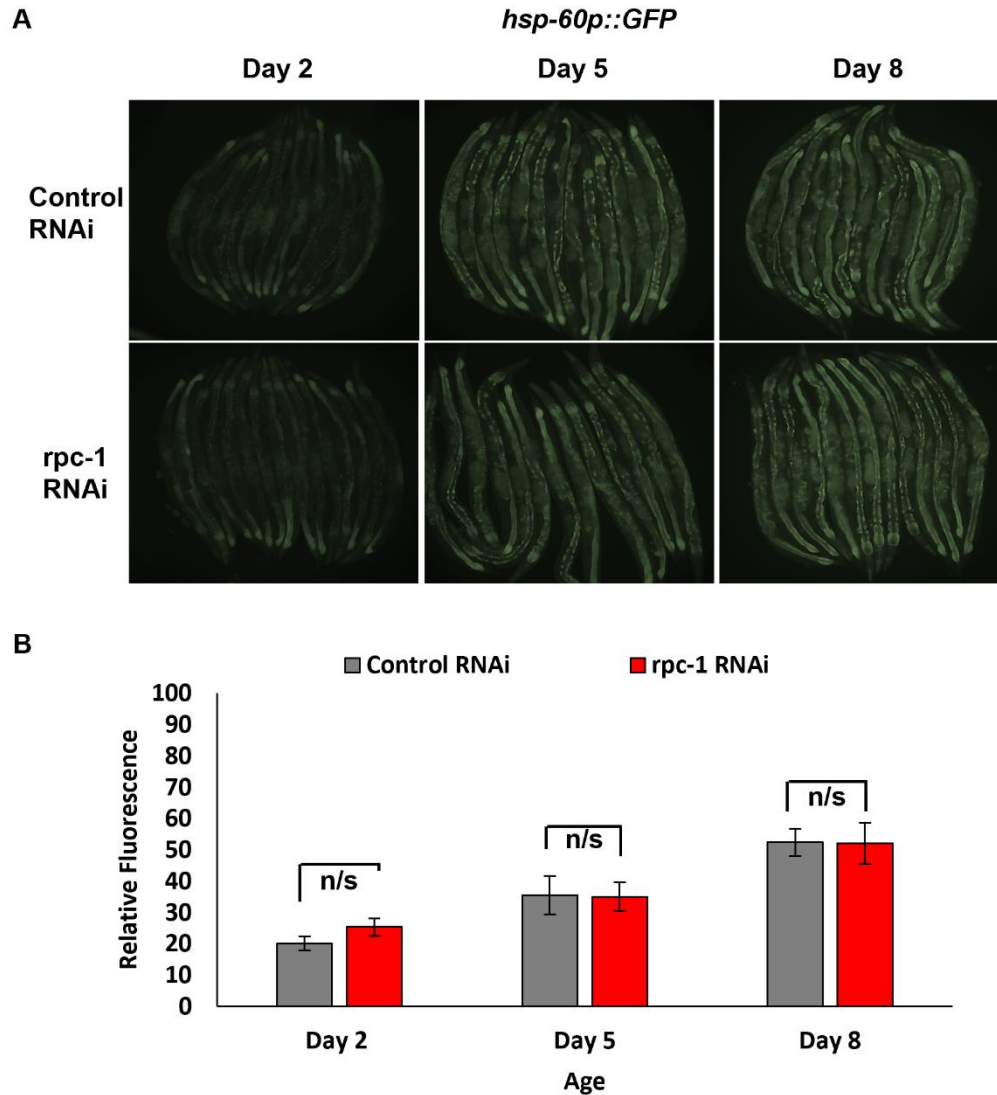


Figure 12. Pol III effects are specific to the UPR-ER.

(A) *hsp-60p::GFP* UPR-MT reporter worms were placed on *rpc-1* RNAi and control RNAi plates at the L4 larval stage, and GFP fluorescence was assessed on days 2, 5, and 8 of adulthood. Representative images of 3 biological repeats, each with a sample size of 10 – 15 worms are shown. Images were taken at 10X magnification.

(B) *hsp-60p::GFP* UPR-MT reporter worms were treated as above, and GFP fluorescence was measured on days 2, 5, and 8 of adulthood using fluorescent microscopy. Relative fluorescence of worms on *rpc-1* RNAi and control RNAi is shown, with error bars indicating standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: n/s = not significant. Representative data of 3 biological repeats are shown.

Discussion

Cellular processes face inevitable deterioration as we age, dampening the ability to maintain proteostasis and further accentuating ageing and disease. Since the UPR-ER is shown to become attenuated with age and its activation in late life extends longevity, as well as previous work in the lab suggesting that Pol III manages increased proteotoxicity to promote survivability; we hypothesised that Pol III inhibition activates the UPR-ER to cope with proteotoxic stress, contributing to improved lifespan.

Consistent with this hypothesis, we report that inhibition of Pol III activates protein chaperone *hsp-4*, leading to improved ER stress resistance. At the same time, Pol III does not induce mitochondrial chaperone *hsp-60*, suggesting that the UPR-MT is not involved in Pol III-mediated longevity. However, it is unclear if this mechanism leads to enhanced survivability in the presence of tunicamycin. Possible explanations include: the inhibition of protein glycosylation effect caused by tunicamycin is by itself such a robust UPR-ER inducer that it might be challenging to differentiate changes in *hsp-4* induction caused by Pol III inhibition. I would suggest repeating the experiment using a lower concentration of tunicamycin to enable better differentiation of *hsp-4* activity. At the same time, the improved physical condition and increased survivability seen in worms with Pol III compromised compared to worms with fully functioning Pol III when exposed to tunicamycin suggests Pol III knockdown is having a protective effect against excessive ER stress; since there appears to be no apparent difference in UPR-ER activity, this suggests Pol III is modulating a protective response to tunicamycin via a different mechanism.

At this time, we do not yet understand the fundamental mechanism behind Pol III-mediated induction of *hsp-4*. However, we report that *hsp-4* activation via Pol III knockdown requires the transcription factors *xbp-1* and *atf-4*, suggesting that this mechanism depends on at least the *ire-1* and *pek-1* arms

of the UPR-ER response in *C. elegans*. *xbp-1* and *atf-4* regulate genes involved in resolving ER stress and have been shown separately to modulate longevity (Imanikia, Sheng, *et al.*, 2019; Statzer *et al.*, 2022). For example, conditions that reduce mRNA translation increase *atf-4* expression leading to an *atf-4*-dependent extension in lifespan (Statzer *et al.*, 2022). This represented a potential link with Pol III mediated longevity; Pol III targets play an essential role in the translational machinery; therefore, by inhibiting Pol III, we postulated that there could be a global reduction in mRNA translation that activated *atf-4* to promote genes involved in ER stress resistance and longevity. However, Pol III inhibition was not seen to increase *atf-4* levels when total worm fluorescence was measured (Supplementary Figure 1). To confirm, it will be necessary to perform further experimentation, such as a western blot to measure ATF-4 protein levels, examine the expression of downstream target hydrogen sulfide, and study the lifespan effects of Pol III inhibition on *atf-4* mutants.

Similarly, activation of *xbp-1* is lost with age, and constitutive expression of the spliced *xbp-1s* in later life promotes proteostasis and longevity by modulating lipid metabolism (Taylor and Dillin, 2013c; Imanikia, Sheng, *et al.*, 2019). It is then possible that Pol III inhibition promotes *xbp-1* splicing in middle and old age via an unknown mechanism and the ensuing UPR-ER activation contributes to longevity. Thus to test this theory, we will need to determine if Pol III mediates lipid metabolism and the lifespan effect of Pol III inhibition on *xbp-1* mutants. Regardless, mechanistically, it would remain unclear how exactly Pol III inhibition would cause increased *xbp-1* splicing.

Our data suggest that Pol III inhibition facilitates the activation of the UPR-ER to promote longevity and that induction is dependent on downstream transcription factors *xbp-1* and *atf-4* (Figure 13). To form a clearer picture of the role of the UPR-ER in longevity, future studies should focus on the last arm of the pathway, *atf-6*. Much like *xbp-1*, and *atf-4*, the transcription factor, *atf-6*, has been shown to modulate lifespan via the regulation of calcium homeostasis (Burkewitz *et al.*, 2020). Ascertaining

the role of *atf-6* concerning Pol III mediated activation of the UPR-ER will hopefully bring us closer to understanding the definitive mechanism contributing to longevity.

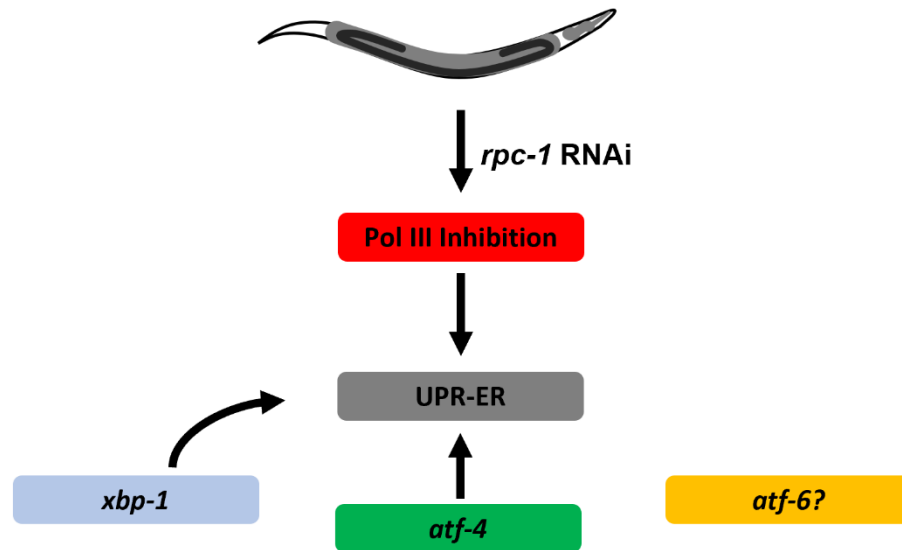


Figure 13. Pol III inhibition induces UPR-ER activation and is dependent *xbp-1* and *atf-4*.

Pol III inhibition via *rpc-1* RNAi activates the UPR-ER in *C. elegans*, and induction is dependent on downstream transcription factors from two of the three signalling pathways: *ire-1/xbp-1* and *pek-1/atf-4*. The role of *atf-6* remains unknown.

Chapter 4: Defining the link between Pol III function and the UPR-ER

Introduction

Previous work from the Tullet, Alic, and Selman labs employed RNA-seq to examine cellular RNA content and determine the state of the transcriptome upon Pol III inhibition. Interestingly, RNA-seq data determined that Pol III downregulates the expression of tRNAs and other small RNAs (Supplementary Figure 3). Among the tRNAs, the aminoacyl-tRNA synthetases (ARSs) play critical roles in the protein synthesis machinery and are implicated in a host of other non-canonical functions (Gomez and Ibba, 2020). Notably, several studies on ARSs across multiple model organisms have shown they can also modulate longevity (Lee *et al.*, 2013; Niehues *et al.*, 2015; Oh *et al.*, 2016; Son *et al.*, 2017). Thus, we decided to target the ARSs genes to determine if they are implicated in Pol III-mediated longevity in *C. elegans*. We hypothesised that disturbing the expression of ARSs would essentially lead to reduced protein synthesis, making the burden of translation more manageable into late adulthood and promoting longevity. However, ARSs' play a multi-faceted role in modulating cellular activity aside from protein synthesis and may promote longevity via a different mechanism.

Having concluded that Pol III inhibition activates the UPR-ER by inducing *hsp-4p::GFP* levels, we wanted to ascertain if there was a link between dysregulated ARSs expression and UPR-ER induction. While mutations that silenced the expression of ARSs genes *lars-1*, *lars-2*, and *yars-2* have been shown to promote longevity in worms, there has not so far been a clear link with the UPR-ER (Lee *et al.*, 2003; Nakamura *et al.*, 2016; Son *et al.*, 2017). Thus, we were unsure as to a potential mechanism pertaining to how ARSs gene inhibition could lead to UPR-ER upregulation; a likely hypothesis was that perturbations in ARSs genes could lead to reduced protein synthesis and activate *atf-4* to induce the UPR-ER and promote longevity similarly to the study by (Statzer *et al.*, 2022). However, any link is likely to present a completely novel mechanism.

Results

4.1: Modifying aminoacyl tRNA synthetase expression alters HSP-4::GFP expression

To determine the relationship between ARSs gene inhibition and the UPR-ER; a screen was performed that observed the effects of RNAi-targeted knockdown (Figure 14) of 18 of the 20 known ARSs genes and tRNA-splicing ligase *rtcb-1* on *hsp-4p::GFP* UPR-ER reporter worms (Table 2). RNA ligation alters RNA sequence, structure, and coding potential; *rtcb-1* has previously been shown to ligate *xbp-1* mRNA during *ire-1*-branch UPR signalling and ensures a proper UPR-ER stress response and was therefore also included in this screen (Kosmaczewski *et al.*, 2014). The initial screen clearly showed varied *hsp-4p::GFP* expression in many worms experiencing knockdown of different ARSs genes (Figure 14). Statistical analysis of fluorescence levels confirmed varied *hsp-4p::GFP* expression and determined that RNAi-targeted knockdown of the genes *tars-1* and *cars-1* significantly reduced *hsp-4p::GFP* expression while targeting genes *yars-2*, *hars-1*, and *gars-1* significantly induced *hsp-4p::GFP* expression (Figure 15A). A second assay targeting the genes that experienced significant reduction or induction confirmed altered *hsp-4p::GFP* expression in response to ARSs knockdown. *tars-1* and *cars-1* were again determined statistically to significantly reduce *hsp-4p::GFP* expression, while *gars-1* induced *hsp-4p::GFP* levels (Figure 15B).

Interestingly our data suggest that knockdown of different ARSs genes can control UPR-ER activation by modulating *hsp-4* expression. Specifically, RNAi-induced knockdown of *gars-1* appears to induce a UPR-ER response. In contrast, knockdown of *tars-1* and *cars-1* facilitates a reduced UPR-ER, suggesting these two genes might be required for UPR activation via an unknown mechanism. Regardless, there is a possibility that our data show a novel mechanism for Pol III-mediated UPR-ER control.

Gene name	Gene encoding	ARSs Class	Location	Human Orthologue
<i>aars-1</i>	alanyl aminoacyl tRNA synthetase	IIc	Mitochondrion	AARS2
<i>aars-2</i>	alanyl aminoacyl tRNA synthetase	IIc	Cytoplasm	AARS1
<i>cars-1</i>	cysteinyl aminoacyl tRNA synthetase	Ia	Cytoplasm	CARS1
<i>cars-2</i> pseudo-gene	cysteinyl aminoacyl tRNA synthetase	N/A	N/A	N/A
<i>dars-1</i>	aspartyl(D) aminoacyl tRNA synthetase	IIb	Cytoplasm	DARS1
<i>dars-2</i>	aspartyl(D) aminoacyl tRNA synthetase	IIb	Mitochondrion	DARS2
<i>ears-1</i>	glutamyl(E) aminoacyl tRNA synthetase	Ib	Cytoplasm	EPRS-1
<i>ears-2</i>	glutamyl(E) aminoacyl tRNA synthetase	Ib	Mitochondrion	EARS2
<i>fars-1</i>	phenylalanyl aminoacyl tRNA synthetase	IIc	Cytoplasm	FARSA
<i>fars-2</i>	phenylalanyl aminoacyl tRNA synthetase	IIc	Mitochondrion	FARS2
<i>fars-3</i>	phenylalanyl aminoacyl tRNA synthetase	IIc	Cytoplasm	FARSB
<i>gars-1</i>	glycyl aminoacyl tRNA synthetase	IIa	Mitochondrion	GARS1
<i>hars-1</i>	histidyl aminoacyl tRNA synthetase	IIa	Cytoplasm, Mitochondrion	HARS1, HARS2
<i>iars-1</i>	isoleucyl aminoacyl tRNA synthetase	Ia	Cytoplasm	IARS1
<i>iars-2</i>	isoleucyl aminoacyl tRNA synthetase	Ia	Mitochondrion	IARS2
<i>kars-1</i>	lysyl(K) aminoacyl tRNA synthetase	IIb	Cytoplasm, Mitochondrion	KARS1
<i>lars-1</i>	leucyl aminoacyl tRNA synthetase	Ia	Cytoplasm	LARS1
<i>lars-2</i>	leucyl aminoacyl tRNA synthetase	Ia	Mitochondrion	LARS2
<i>mars-1</i>	methionyl Aminoacyl tRNA Synthetase	Ia	Cytoplasm	MARS1
<i>nars-1</i>	asparaginyl(N) aminoacyl tRNA synthetase	IIb	Cytoplasm	NARS1
<i>nars-2</i>	asparaginyl(N) aminoacyl tRNA synthetase	IIb	Mitochondrion	NARS2
<i>pars-1</i>	prolyl aminoacyl tRNA synthetase	IIa	Cytoplasm	EPRS1
<i>pars-2</i>	prolyl aminoacyl tRNA synthetase	IIa	Mitochondrion	PARS2
<i>qars-1</i>	glutamyl(Q) aminoacyl tRNA synthetase	Ib	Cytoplasm	QARS1
<i>rars-1</i>	arginyl(R) aminoacyl tRNA synthetase	Ia	Cytoplasm	RARS1
<i>rars-2</i>	arginyl(R) aminoacyl tRNA synthetase	Ia	Mitochondrion	RARS2
<i>sars-1</i>	seryl aminoacyl tRNA synthetase	IIb	Cytoplasm	SARS1
<i>sars-2</i>	seryl aminoacyl tRNA synthetase	IIb	Mitochondrion	SARS2
<i>tars-1</i>	threonyl aminoacyl tRNA synthetase	IIa	Cytoplasm	TARS1, TARS2
<i>vars-1</i>	valyl aminoacyl tRNA synthetase	Ia	Cytoplasm	VARS2
<i>wars-1</i>	tryptophanyl(W) aminoacyl tRNA synthetase	Ic	Cytoplasm	WARS1
<i>yars-1</i>	tyrosinyl(Y) aminoacyl tRNA synthetase	Ic	Cytoplasm	YARS1
<i>yars-2</i>	tyrosinyl(Y) aminoacyl tRNA synthetase	Ic	Cytoplasm, Mitochondrion	YARS2
<i>rtcb-1</i>	RTCB (tRNA-splicing ligase) homolog	N/A	Cytoplasm, Nucleus	RTCB (RNA 2',3'-cyclic phosphate and 5'-OH ligase)

Table 2. Known ARSs genes in *C. elegans*.

The gene *rtcb-1* was tested in the screen shown in Figure 14 and is shown in the table but is not considered an ARSs gene. The rows highlighted in red were not tested in the screen shown in Figure 14 due to lack of availability.

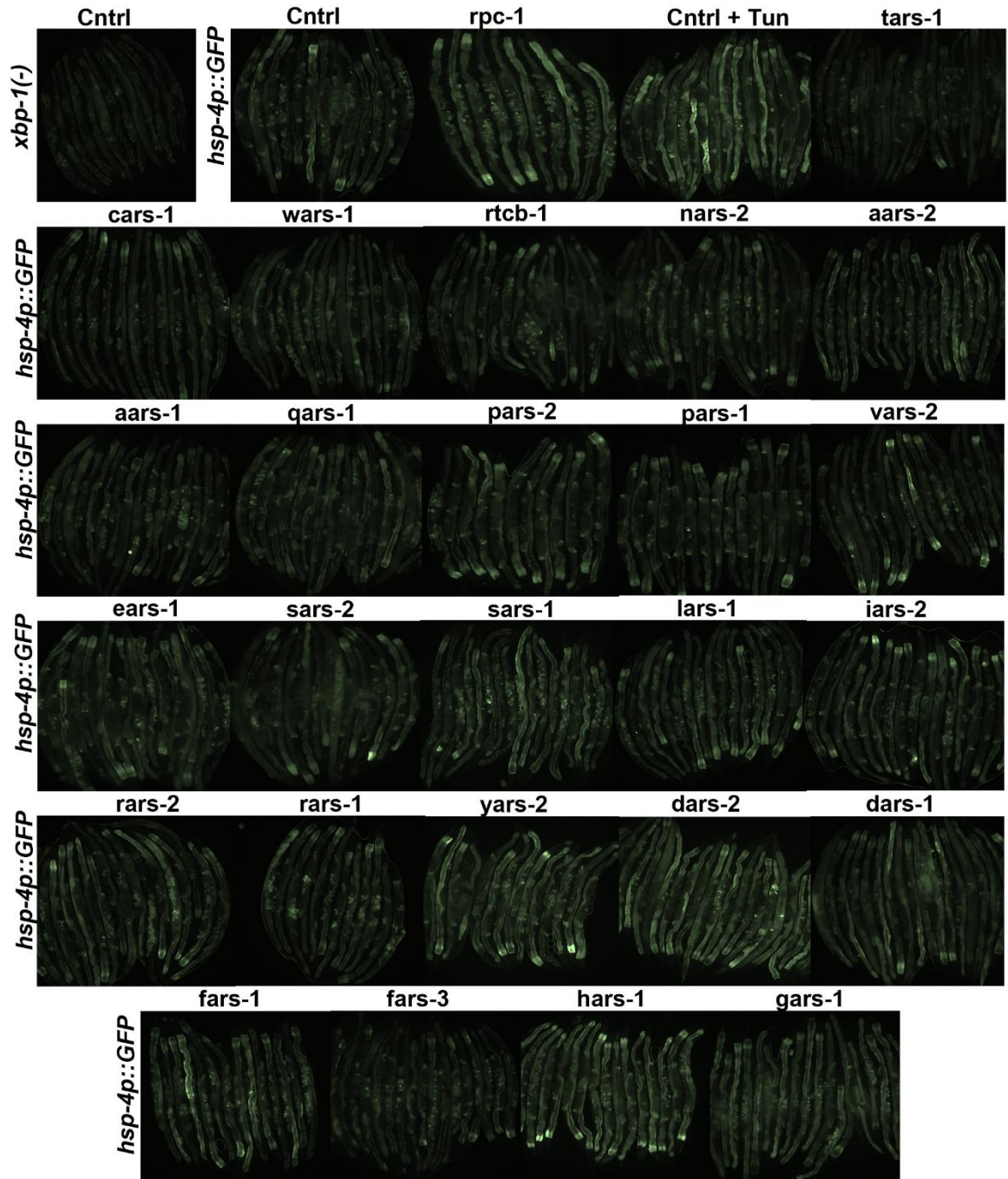


Figure 14. Aminoacyl tRNA synthetase targeted RNAi alters *hsp-4p::GFP* expression (Fluorescence)

hsp-4p::GFP UPR-ER reporter worms were grown to the L4 larval stage and placed on plates containing RNAi targeting 18 of the 20 known ARSs genes and tRNA-splicing ligase *rtcb-1* (Table 2). *xbp-1(-);[hsp-4p::GFP]* (SJ17) worms were placed on control RNAi and used as a negative control; *hsp-4p::GFP* worms were placed on control RNAi containing 10 µg/ml tunicamycin and were used as a positive control; *hsp-4p::GFP* worms on Control RNAi were used as control; *hsp-4p::GFP* worms on *rpc-1* RNAi were used to compare to Pol III-mediated upregulation of *hsp-4p::GFP*. GFP fluorescence was assessed on day 5 of adulthood. Sample size of 10 – 15 worms are shown. Images were taken at 10X magnification.

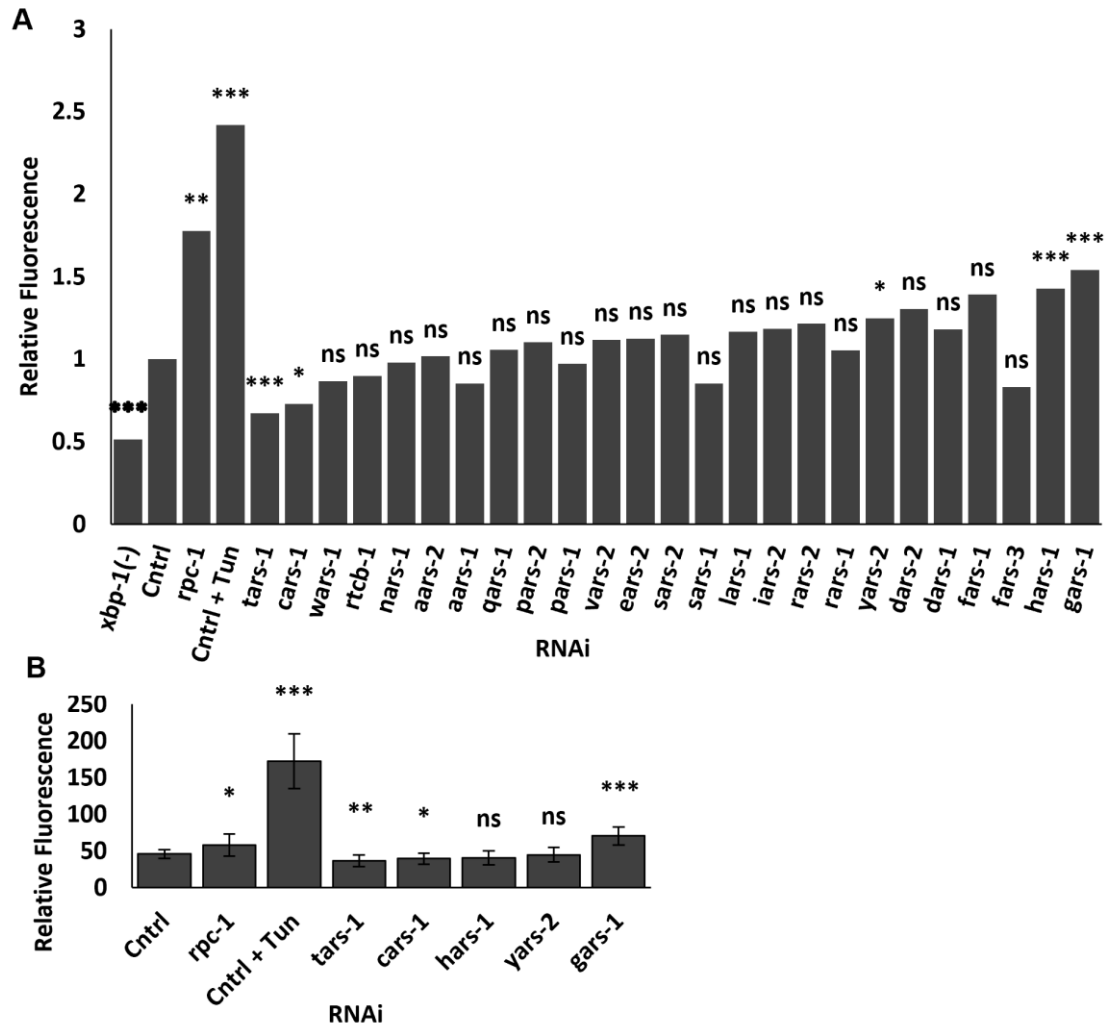


Figure 15. Aminoacyl tRNA synthetase targeted RNAi alters *hsp-4p::GFP* expression (Statistical analysis)

(A) *xbp-1(-);[hsp-4p::GFP]* (SJ17), and *hsp-4p::GFP* UPR-ER reporter worms were treated as described in Figure 14, and GFP fluorescence was measured on day 5 of adulthood using fluorescent microscopy. Relative fluorescence of worms on RNAi plates targeting 18 of the 20 known ARSs genes and tRNA-splicing ligase *rtcb-1* is shown. All controls are also shown. Relative fluorescence was calculated by standardising fluorescence levels to *hsp-4p::GFP* worms on control RNAi. Error bars indicate standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, n/s = not significant.

(B) The experiment described in Figure 14 was repeated using *hsp-4p::GFP* UPR-ER reporter worms and ARSs RNAi that was found to significantly interact with the regulation of *hsp-4p::GFP* in Figure 15A (*tars-1*, *cars-1*, *hars-1*, *yars-2*, and *gars-1*). GFP fluorescence was measured on day 5 of adulthood using fluorescent microscopy. Relative fluorescence of worms on *tars-1* RNAi, *cars-1* RNAi, *hars-1* RNAi, *yars-2* RNAi, and *gars-1* RNAi is shown. Error bars indicate standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, n/s = not significant.

Discussion

Our results suggest that ARSs genes *tars-1*, *cars-1*, and *gars-1* are involved in modulating a UPR-ER response in *C. elegans*. Most ARSs are evolutionary conserved across species, with their primary function facilitating cellular protein synthesis. However, they also show functional adaptability and are involved in a wide range of cellular mechanisms, such as RNA-protein interaction, protein-protein interactions, enzyme catalysis, and cell signalling events. Therefore, while the knockdown of these genes is likely to hinder the protein synthesis machinery, it is also expected to cause a change in other cellular interactions. Therefore, it is difficult to ascertain the mechanism by which these genes interact with the UPR-ER.

The gene *tars-1* encodes a predicted cytoplasmic threonyl-tRNA synthetase that enables threonine-tRNA ligase activity. In *C. elegans*, *tars-1* has been shown to regulate survival after hypoxic cellular injury; worms with loss of function mutations in the *tars-1* gene and RNAi targeted knockdown of *tars-1* in wild-type worms cause a hypoxia resistant phenotype (Itani *et al.*, 2021). This mechanism is thought to be linked with a global reduction in translation due to decreased ribosome biogenesis as a cause of threonyl-tRNA decline (Itani *et al.*, 2021). Therefore our results are contradictory to what might be expected. We initially hypothesised that a global reduction in translation could lead to the induction of the UPR-ER in a similar mechanism to the one described by Statzer *et al.*, 2022; however, our data suggest the opposite and knockdown of *tars-1* seemed to prevent UPR-ER activation.

Knockdown of the gene *cars-1* had a similar effect, though not as significant as *tars-1*. *cars-1* encodes a predicted cytoplasmic cysteinyl-tRNA synthetase that catalyses cysteine-tRNA ligase activity and is required for protein biosynthesis. There isn't much work directly addressing *cars-1* in *C. elegans*, and studies in the human orthologue CARS1 have found increased levels in certain cancers (Nie *et al.*,

2021). Regardless, as seen with *tars-1*, knockdown of *cars-1* and the remaining ARSs genes could potentially reduce global protein synthesis.

What then defines this interaction with the UPR-ER response? Interestingly, recent studies have found that YARS1 responds to prolonged cellular stress and, through cytosol-nucleus translocation, initiates a diverse, protective response to restore cellular homeostasis that could also involve interactions with other ARSs (Fu *et al.*, 2012; Wei *et al.*, 2014, 2020; Sajish and Schimmel, 2015). Mechanistically this is achieved by suppressing genes involved in protein synthesis and reducing global translation downstream of eIF2 α and ATF-4 responses (Wei *et al.*, 2020). Also, removing nucleus-bound YARS1 leads to protein accumulation and eventually apoptosis (Wei *et al.*, 2020). This mechanism potentially explains the activation of the UPR-ER that is seen with the knockdown of specific ARSs genes as instances of misfolded proteins are likely to increase with a higher protein load and therefore activate the UPR to manage ER stress. However, with *gars-1* specifically, this exact mechanism is unlikely. The glycyl-tRNA synthetase it encodes for is predicted to be located in the mitochondrion and, therefore, would activate the UPR-ER via a different pathway.

The function of ARSs genes beyond protein synthesis is becoming ever-apparent, and our data further suggests their possible involvement in the UPR-ER stress response. This concept, however, remains at an early stage of development, and future experimentation is needed to properly determine the mechanism. A possible experimental design to examine if *tars-1* and *cars-1* are required for UPR-ER activation is to measure the response of RNAi targeted knockdown of *tars-1* and *cars-1* in *hsp-4p::GFP* UPR-ER reporter worms when exposed to tunicamycin; reduced *hsp-4p::GFP* fluorescence would suggest they are required.

Concluding remarks

This thesis explored the relationship between Pol III and the UPR-ER. Firstly, Pol III was seen to be involved in modulating the UPR-ER. Specifically, Pol III inhibition induced *hsp-4p::GFP* expression in *C. elegans* in middle and late adulthood. The protein chaperone *hsp-4* is directly activated by the signalling pathways that comprise the UPR-ER and aids in resolving toxic stress by facilitating the proper folding of proteins in the ER. Therefore, we can assume that Pol III inhibition activates the UPR-ER in *C. elegans* and contributes to improved longevity. However, we cannot assume that improved proteostasis via *hsp-4* upregulation is the direct method causing improved longevity; UPR-ER-mediated increased lifespan in *C. elegans* can also be facilitated through metabolic changes caused by improved lipophagy (Daniele *et al.*, 2020). Lack of significant induction in *hsp-4p::GFP*, but improved survivability in Pol III-inhibited worms exposed to the ER-stress-inducing pharmacological reagent tunicamycin, accentuates the notion that knockdown of Pol III is causing a separate protective effect aside from improved proteostasis.

Pol III-mediated induction in *hsp-4* is also dependent on the transcription factors *xbp-1* and *atf-4*. *xbp-1* has been shown to regulate *hsp-4* expression and is also required for improved lipophagy (Taylor and Dillin, 2013c; Daniele *et al.*, 2020). This suggests that Pol III inhibition could cause an interaction that acts on *xbp-1* to activate the UPR-ER and modulate longevity via improved proteostasis and/or improved lipophagy. The effect Pol III inhibition has on metabolic changes is an important area for future research.

Trying to decipher how *atf-4* is involved in regulating Pol III-mediated induction of *hsp-4* is quite tricky. In *C. elegans*, *atf-4* has not been shown to modulate *hsp-4* directly, and in mammalian cells, ATF4 governs multiple signalling pathways, such as autophagy, oxidative stress, inflammation, and translation (Pitale, Gorbatyuk and Gorbatyuk, 2017). The multifaceted nature of ATF4 implicates it in

various potential longevity mechanisms. Regardless, a possibility is that Pol III inhibition is leading to a reduction in global translation that is activating *atf-4* and hydrogen sulfide signalling to promote longevity, similarly to what is seen in the study by (Statzer *et al.*, 2022); induction of *hsp-4* could be facilitated as a by-product of *atf-4* activation but might not be the mechanism directly causing longevity. The interaction between Pol III inhibition and *atf-4*-mediated UPR-ER activation needs to be studied further.

There is the possibility of a novel mechanism for Pol III-mediated control of longevity via an *xbp-1* and *atf-4*-dependent UPR-ER induction. However, exactly how Pol III interacts with these transcription factors to mediate activation of the UPR-ER and contribute to longevity remains unknown.

Secondly, Pol III has a range of tRNA and other small RNA targets that are downregulated upon Pol III inhibition (Supplementary Figure 3). The function of many small Pol III RNA targets remains unknown, and how they might interact to modulate the UPR-ER needs to be explored further. However, the small RNAi screen on the group of tRNAs named the ARSs, determined that their inhibition can modulate *hsp-4::GFP* expression. This suggests that ARSs are involved in directing a UPR-ER response. Our data present an initial link between ARSs and UPR-ER control but does not explain the molecular mechanisms underpinning this interaction. ARSs' primary function pertains to their role in protein synthesis, but a growing list implicates their involvement in other cellular activities. Therefore, their inhibition is likely to affect different cellular pathways aside from reduced protein synthesis, which may contribute to UPR-ER induction and longevity.

Overall, this study uncovers a novel regulatory mechanism that links RNA Pol III and the UPR-ER in *C. elegans*. Further research into the molecular mechanisms underpinning this relationship will provide insight into regulation of the mammalian UPR-ER stress response and facilitate the formation of new treatments to battle age-related diseases, particularly those involving protein misfolding.

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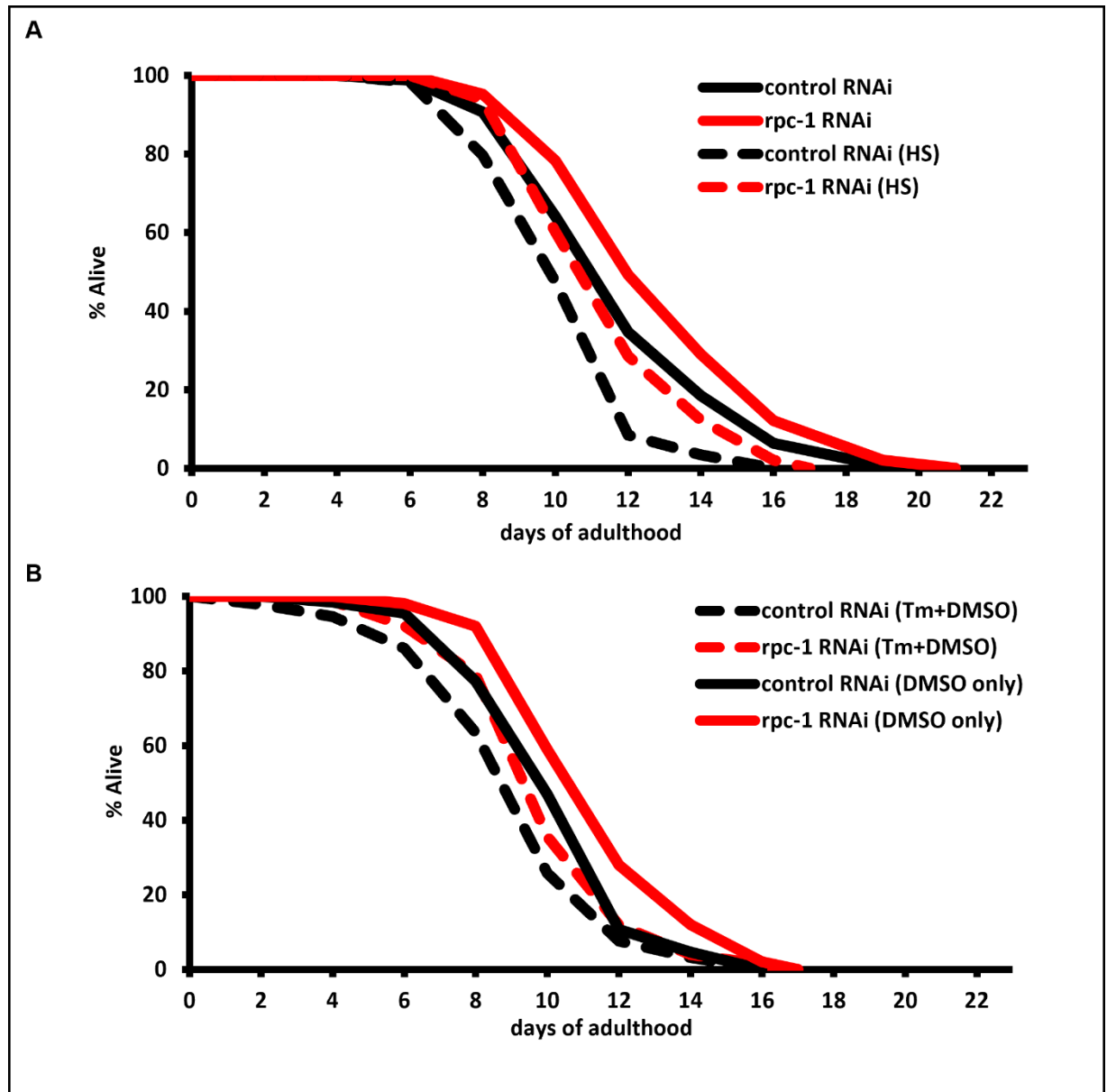
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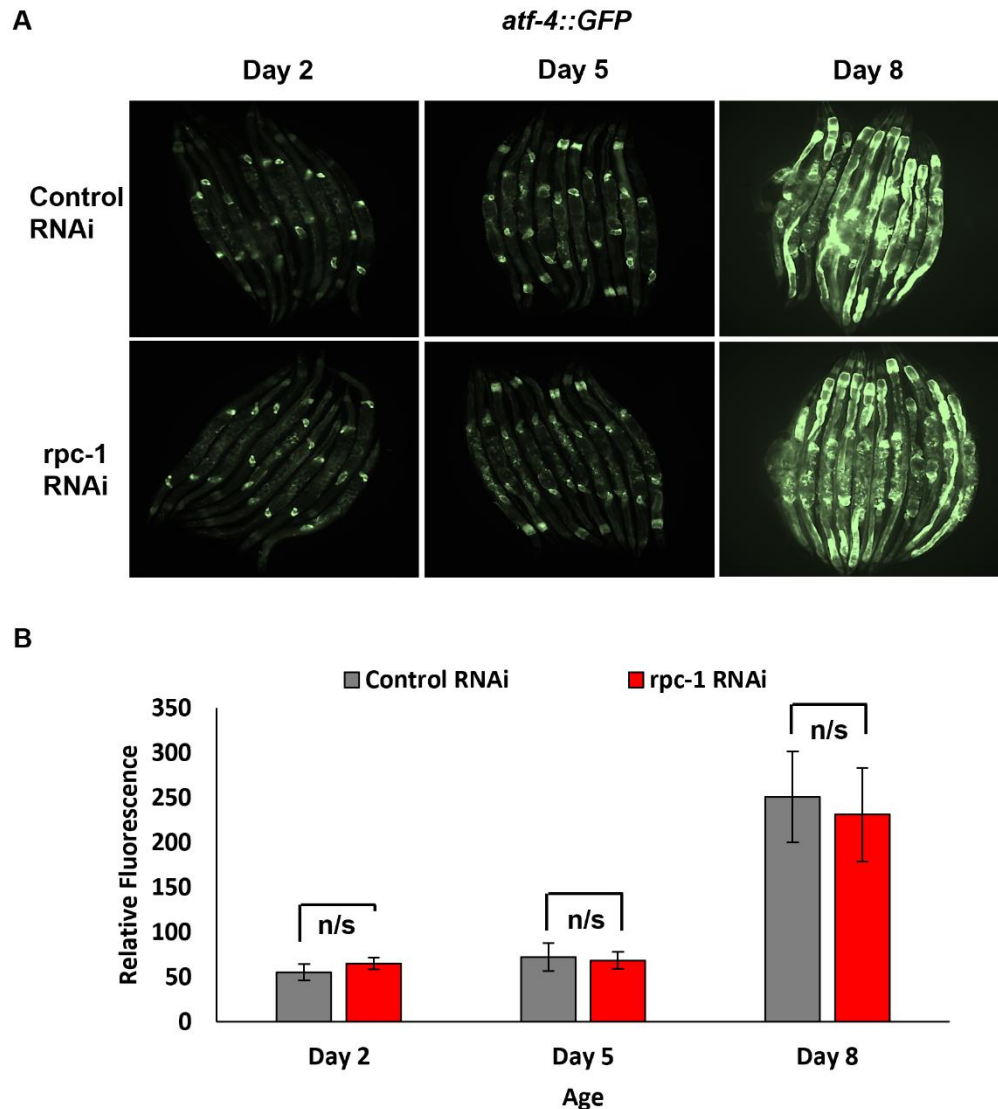
Supplementary Figures



Supplementary Figure 1. Pol III knockdown improves proteostasis.

(A) RNAi mediated knockdown of *rpc-1* from L4 larval stage improves proteostasis. N2 (WT) worms were given a heat-shock at 30 °C for 3 hours and scored until the population was dead. P-values: control RNAi vs *rpc-1* RNAi = 0.0025; control RNAi vs *rpc-1* RNAi (HS) = 0.0015. Log-Rank Mantel Cox Test.

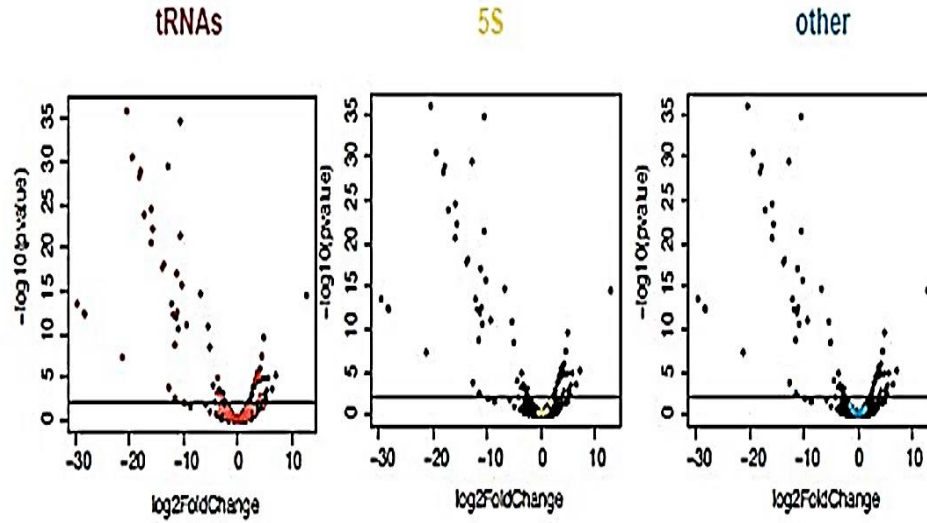
(B) RNAi mediated knockdown of *rpc-1* in from L4 larval stage leads to enhanced resistance to tunicamycin in N2 (WT) worms. DMSO was used as control. P-values. Control RNAi (Tm+DMSO) vs. *rpc-1* RNAi (Tm+DMSO)=0.0521; control RNAi (DMSO only) vs. *rpc-1* (DMSO only)= 0.0043. Log-Rank Mantel Cox Test.



Supplementary Figure 2. Pol III inhibition does not induce *atf-4::GFP*.

(A) *atf-4::GFP* worms were placed on *rpc-1* RNAi and control RNAi plates at the L4 larval stage, and GFP fluorescence was assessed on days 2, 5, and 8 of adulthood. Representative images of 1 biological repeat, with a sample size of 10 – 11 worms is shown. Images were taken at 10X magnification.

(B) *atf-4::GFP* worms were treated as above, and GFP fluorescence was measured on days 2, 5, and 8 of adulthood using fluorescent microscopy. Relative fluorescence of worms on *rpc-1* RNAi and control RNAi is shown, with error bars indicating standard deviation. Welch's t-test (two-tailed distribution, homoscedastic t-test) was used to assess statistical significance: n/s = not significant. Representative data of 1 experiment is shown.



Supplementary Figure 3. Downregulating RNA Polymerase III in adulthood leads to downregulation of multiple tRNAs.

(A) Multiple tRNAs, 5S rRNAs and other small RNAs were found to be significantly downregulated in small RNA-seq on day 2 N2 adult worms.