Activation of the mitochondrial unfolded protein response does not predict longevity in *Caenorhabditis elegans*

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Abstract

Recent studies have propagated the model that the mitochondrial unfolded protein response (UPR^{mt}) is causal for lifespan extension from inhibition of the electron transport chain (ETC) in *C. elegans*. Here we report a genome-wide RNAi screen for negative regulators of the UPR^{mt}. Lifespan analysis of nineteen RNAi clones that induce the *hsp-6$_p$::gfp* reporter demonstrate differential effects on longevity. Deletion of *atfs-1*, which is required for induction of the UPR^{mt}, fails to prevent lifespan extension from knockdown of two genes identified in our screen or following knockdown of the ETC gene *cco-1*. RNAi knockdown of *atfs-1* also has no effect on lifespan extension caused by mutation of the ETC gene *isp-1*. Constitutive activation of the UPR^{mt} by gain of function mutations in *atfs-1* fails to extend lifespan. These observations identify several new factors that promote mitochondrial homeostasis and demonstrate that the UPR^{mt}, as currently defined, is neither necessary nor sufficient for lifespan extension.

INTRODUCTION

As the primary source of cellular energy and a major source of damage, mitochondria play an important role in modulating many age-related processes [1,2]. Harman first proposed the free radical theory of aging, which posits that damage resulting from reactive oxygen species (ROS) produced as a by-product of mitochondria metabolism determines the rate of organismal aging [3]. Recent studies have demonstrated that the situation is more complex than originally proposed, however, with important pro-longevity signaling functions of ROS having been established in several species [4].

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The effect of mitochondria on aging has been studied thoroughly in the nematode *Caenorhabditis elegans*, where RNAi knockdown of several mitochondrial electron transport chain (ETC) genes has been shown to extend lifespan [5,6]. Knockdown of ETC genes must occur during a specific stage of development in order to promote longevity [7–9], suggesting that a signal or altered metabolic state is established following ETC disruption during development to modulate adult longevity. The requirement of developmental disruption of ETC function to enhance longevity is distinct from most other longevity interventions, such as reduced insulin/IGF-1-like signaling or dietary restriction (DR), which appear to act on aging primarily during adulthood [10,11]. The effect of ETC knockdown on lifespan is also dose-responsive, such that too much inhibition of ETC function during development can shorten lifespan [8]. A few mutations that impair mitochondrial function have also been shown to extend lifespan in *C. elegans*, including alleles of the coenzyme Q biosynthetic gene *clk-1* [12,13], the Rieske iron-sulfur protein gene *isp-1* [14], and thiamine pyrophosphokinase gene *tpk-1* [15,16].

Several factors have been suggested to directly promote lifespan extension from ETC knockdown and mitochondrial inhibition in *C. elegans*. For example, inhibition of ETC function can lead to increased levels of ROS, which can induce lifespan extension [17,18]. One effect of this ROS production is stabilization and activation of the hypoxia-inducible transcription factor HIF-1, which is also required for full lifespan extension in some mitochondrial mutants and RNAi knockdowns [18]. Stabilization of HIF-1 is sufficient to extend lifespan robustly through a mechanism that is also distinct from insulin/IGF-1-like signaling and DR [19–22], providing a plausible explanation for lifespan extension in these cases. In addition to HIF-1, other factors have also been implicated in lifespan extension following inhibition of mitochondrial function. These include AMP-activated protein kinase (AMPK) [23], the homeobox protein CEH-23 [24], the transcription factor TAF-4 [25], and the p53 homolog CEP-1 [26]. It remains unclear, however, whether any of these putative downstream mechanisms can account for a majority of the lifespan effects seen in the different long-lived models of mitochondrial inhibition.

Recently, induction of the mitochondrial unfolded protein response (UPR^mt) was proposed to directly mediate lifespan extension from ETC inhibition in a cell non-autonomous manner [27]. The UPR^mt is a stress response pathway first characterized in mammalian cells, whereby nuclear encoded mitochondrial chaperones are induced in response to misfolded proteins within the mitochondria or a stoichiometric imbalance of mitochondrial respiratory complexes [28–30]. The *C. elegans* UPR^mt appears similar to that of mammals [27,31–34], where induction of the UPR^mt results in transcriptional up-regulation of the mitochondrial chaperone genes *hsp-6* and *hsp-60*. RNAi knockdown of a subset of ETC components has been shown to induce the UPR^mt in *C. elegans* using GFP reporters for both *hsp-6* and *hsp-60* [27,31]. In addition, the *C. elegans* UPR^mt can only be induced robustly when mitochondrial stress precedes the L3/L4 larval stage transition [27], a critical time period for mitochondrial biogenesis and longevity [8,35]. This signaling pathway is not completely understood, but several factors are reported to be required for full induction of the response, including the HAF-1 peptide exporter [34], the CLPP-1 protease [33], a ubiquitin-like protein UBL-5 [32], and two transcription factors, DVE-1, and ATFS-1 (ZC376.7) [33,34,36,37].

Durieux et al. [27] linked the UPR^{mt} to aging by showing that RNAi knockdown of UPR^{mt} components can suppress the lifespan extension from mutations in *isp-1* or *clk-1*. Most of these knockdowns shortened the lifespan of wild type animals, however, and also suppressed the lifespan extension associated with non-mitochondrial factors such as *daf-2* and *eat-2*, suggesting that the effects were non-specific. In one case, *ubl-5(RNAi)*, the suppression appeared to be specific for *isp-1* and *clk-1*, which was interpreted to indicate that induction of the UPR^{mt} plays a causal role in lifespan extension from ETC inhibition [27]. This model was further supported by experiments showing that *ubl-5(RNAi)* can also partially or completely prevent lifespan extension from knockdown of *cco-1*, which encodes cytochrome c oxidase subunit Vb/COX4, or *mrps-5*, which encodes a mitochondrial ribosomal protein [38].

Based on the report by Durieux et al. [27], we set out to identify additional genetic modifiers of lifespan by screening for RNAi clones that induce the UPR^{mt}. Here we report the identification and validation of 25 previously unreported negative regulators of the UPR^{mt}. Unexpectedly, several RNAi clones that induce the UPR^{mt} shorten lifespan, and among at least a subset of those that extend lifespan, induction of the UPR^{mt} is not required for lifespan extension. Constitutive activation of the UPR^{mt} in the absence of mitochondrial stress also fails to extend lifespan.

RESULTS

A genomic screen for negative regulators of the *UPR^{mt}*

We sought to identify RNAi clones that increase lifespan by screening for induction of fluorescence in animals expressing the *hsp-6$_p$::gfp* reporter following RNAi knockdown of individual genes (Fig. 1a). We identified 95 putative inducers of the UPR^{mt} from the Vidal ORFeome RNAi library (11,511 clones) (Supplementary Table 1, 2). Of these, 39 RNAi clones target subunits of the ETC and 22 target mitochondrial ribosomal subunits or translation factors (Supplementary Table 2). Of the 95 clones identified from this screen, 29 have been previously reported to induce the UPR^{mt} [31,36,38–41], and the remaining 66 are novel.

Since ETC components and mitochondrial ribosomal proteins are known to modulate both the UPR^{mt} and longevity [7,9,42], we chose to further characterize RNAi clones identified from our screen that have not been shown to play a direct role in these processes. After sequence-validation of these RNAi clones, we focused on 34 that reproducibly induced expression of the *hsp-6$_p$::gfp* reporter relative to empty vector RNAi (Fig. 1b). The targeted genes function in mitochondrial protein import, fat storage, sugar metabolism, and other aspects of mitochondrial biology such as mitochondrial fission, protein quality control, and ion transport (Table 1, Supplementary Table 1). Although several of the identified genes have not been shown to modulate mitochondrial function in *C. elegans*, some are homologous to known mitochondrial genes in other species or are expected to localize to the mitochondria based on predicted mitochondrial targeting sequences (Supplementary Table 1). About half (18/34) of the RNAi clones that induced expression of the *hsp-6$_p$::gfp* reporter also significantly induced expression of the mitochondrial *hsp-60$_p$::gfp* reporter, while none of the RNAi clones induced reporters of either the endoplasmic reticulum UPR

(hsp-4_p::gfp) or the cytoplasmic heat shock response (hsp-16.2_p::gfp) to a detectable level, with the exception of $Y38E10A.24$($RNAi$), which modestly increased fluorescence in the hsp-4_p::gfp strain (Supplementary Table 3).

Relationship between the UPR^{mt} and longevity

Based on the model that lifespan extension from ETC inhibition results from induction of the UPR[mt], we predicted that a majority of the clones identified from our screen would extend lifespan. Out of 19 RNAi clones tested, ten significantly increased lifespan ($p < 0.05$, Wilcoxon rank-sum, Table 1), of which seven increased lifespan by more than ten percent (Fig. 2a–g). Six other RNAi clones significantly decreased mean lifespan (Fig. 3a–f). No correlation between UPR[mt] induction and lifespan was detected for knockdowns that significantly affected lifespan (Figs 2h, 3g, 4). Notably, all of the clones that significantly reduced lifespan correspond to worm homologs of proteins important for transport of mitochondrial-localized proteins into the mitochondria. These included $tomm$-22, a component of the TOM complex which functions as a translocase in the outer mitochondrial membrane and $E04A4.5$ (TIM17), $T09B4.9$ (TIM44), $F45G2.8$ (TIM16), $F15D3.7$ (TIM23), and dnj-21 (TIM14), which function in the TIM23 complex that transports proteins into the inner membrane and the matrix [43,44]. The RNAi clones that significantly increased lifespan appear to correspond to functionally diverse proteins. These include LETM-1, a mitochondrial transmembrane protein involved in potassium homeostasis that is associated with seizures in Wolf-Hirschhorn syndrome patients, LPD-9, a protein involved in fat storage, and $Y24D9A.8$, which is orthologous to human TALDO1 encoding transaldolase, an enzyme of the pentose phosphate pathway.

ATFS-1 is required for UPR^{mt} induction but not longevity

We next set out to more directly characterize the role of the UPR[mt] in lifespan extension from knockdown of two of the novel longevity clones identified from our screen: $letm$-1 and $Y24D9A.8$ (transaldolase). We first considered using the haf-$1(ok705)$ mutant for epistasis experiments, but found that haf-1 was not required for induction of the UPR[mt] caused by RNAi knockdown of cco-1 or by RNAi knockdown of the gene encoding the mitochondrial prohibitin phb-2 (Supplementary Fig. 1). Therefore, we utilized the $atfs$-$1(tm4525)$ mutation, which has been shown to prevent induction of the UPR[mt] following knockdown of mitochondrial AAA-protease spg-7, knockdown of mitochondrial import machinery, and ethidium bromide treatment [36]. In the absence of mitochondrial stress, ATFS-1 is imported into the mitochondria where it is degraded; when mitochondria are dysfunctional, import and degradation of ATFS-1 is impaired, and it relocalizes to the nucleus to induce expression of UPR[mt] genes, including hsp-6 and hsp-60 [36]. Based on the model that the UPR[mt] plays a causal role in lifespan extension, we reasoned that RNAi of either $letm$-1 or $Y24D9A.8$ (transaldolase) should have no effect on lifespan in the $atfs$-$1(tm4525)$ background. Unexpectedly, deletion of $atfs$-1 failed to significantly attenuate the lifespan extension from RNAi knockdown in either case (Fig. 5a,b).

In order to determine whether the lack of effect from $atfs$-1 deletion on lifespan extension is specific to these newly identified UPR[mt]-inducing clones, we asked whether knockdown of cco-1, which is already known to extend lifespan [7–9], was dependent on $atfs$-1. To rule out

the possibility that high temperature (25°C) could influence the outcome by modulating expression of UPR[mt] genes independently of the mitochondrial stress, we performed subsequent experiments at 20°C in addition to 25°C. Once again, deletion of *atfs-1* failed to significantly attenuate lifespan extension from *cco-1(RNAi)* at both temperatures (Fig. 5c,d). The presence of the *atfs-1(tm4525)* allele was verified by PCR (Supplementary Fig. 2), and deletion of *atfs-1* prevented induction of both *hsp-6~p~::gfp* reporter, as well as three different endogenous UPR[mt] genes, *hsp-6*, *hsp-60*, and *timm-23*, following *cco-1(RNAi)* (Fig. 5e,f).

In order to further test the generality of our observations, we treated long-lived *isp-1(qm150)* mutant animals with *atfs-1(RNAi)*. As has been previously reported [36], we confirmed that *atfs-1(RNAi)* delayed the development of *isp-1* mutant animals (Supplementary Fig. 3). Even when this delay is taken into account, mutation of *isp-1* significantly increased the lifespan of animals treated with *atfs-1(RNAi)* comparably to animals treated with empty vector RNAi (Fig. 6a), despite the fact that *atfs-1(RNAi)* completely prevented induction of GFP in *isp-1(qm150)* animals expressing the *hsp-6~p~::gfp* reporter (Fig. 6b).

Stabilization of ATFS-1 does not extend lifespan

Recently, constitutively active alleles of ATFS-1 have been described which result in induction of the UPR[mt] in the absence of exogenous mitochondrial stress [45]. To determine whether constitutive activation of the UPR[mt] is sufficient to extend lifespan in the absence of ETC inhibition, we measured the lifespans of strains carrying either the *atfs-1(et17)* or *atfs-1(et18)* alleles. In both cases, lifespan was significantly reduced at 20°C, rather than extended (Fig. 7a,b). Similar results were obtained at 25°C, although the reduction in lifespan was attenuated at the higher temperature (Fig. 7c,d). The endogenous UPR[mt] targets *hsp-6*, *hsp-60*, and *timm-23* were significantly induced in the *atfs-1(et17)* and *atfs-1(et18)* strains, relative to N2 (Fig. 7e), demonstrating constitutive activation of the UPR[mt] in these animals.

DISCUSSION

The model that the UPR[mt] plays a causal role in lifespan extension has become widely accepted, despite the correlative nature of the supporting data and the lack of evidence that induction of the UPR[mt] is sufficient to increase lifespan. In this study, we identified 95 gene knockdowns that induce the UPR[mt], 66 of which are novel, and found that, at least for the subset tested here, there was no correlation between lifespan extension and the degree of UPR[mt] induction. Furthermore, we found that the UPR[mt] is neither necessary nor sufficient for lifespan extension by utilizing both null and gain of function mutations in the UPR[mt] transcription factor ATFS-1.

As with prior studies of mitochondrial function in *C. elegans*, the studies described here are limited to mutations and RNAi knockdowns that only partially impair ETC function, since complete absence of ETC function is incompatible with life. Thus, we anticipate that additional genetic modifiers of the UPR[mt], which may have resulted in lethality or early larval arrest in our RNAi screen, remain to be identified. This is consistent with prior studies indicating that RNAi knockdown of some mitochondrial factors results in severe lifespan

reduction or lethality when the RNAi is undiluted, but increase lifespan when the RNAi is diluted or initiated post-developmentally [8,26,42].

The best evidence that the UPR[mt] directly promotes longevity comes from epistasis experiments reporting that knockdown of *ubl-5* or *haf-1*, which are required for full induction of the UPR[mt], prevents lifespan extension from *isp-1(qm150)* or RNAi knock-down of either *cco-1* or *mrps-5* [27,38]. One major caveat to these experiments, however, is that neither *ubl-5(RNAi)* nor *haf-1(RNAi)* actually prevented induction of the UPR[mt] in these experiments. For example, in the study from Houtkooper et al. [38], double knock-down of *mrps-5* and *haf-1* still resulted in an approximately 15-fold induction of the *hsp-6$_p$::gfp* reporter relative to control animals, suggesting that the UPR[mt] is, at best, attenuated. Furthermore, no analysis of endogenous expression UPR[mt] targets was reported in these studies. In addition, we found that *haf-1* is not required for induction of the UPR[mt] caused by knockdown of two additional mitochondrial genes: *cco-1* and *phb-2*. In the case of *ubl-5*, which encodes an ubiquitin-like peptide homologous to the yeast splicing factor Hub1 [46], interpretation is further complicated by the fact that this protein likely has numerous functions beyond modulating the UPR[mt].

For these reasons, we felt that loss of *atfs-1*, which has no known function outside of the UPR[mt], might provide a useful alternative test of this model. In contrast to knockdown of either *haf-1* or *ubl-5*, we were able to demonstrate that deletion or RNAi knockdown of *atfs-1* largely or completely abolishes induction of both the *hsp-6$_p$::gfp* reporter and endogenous expression of three different UPR[mt] components (*hsp-6*, *hsp-60*, and *timm-23*), while having minimal effect on lifespan extension from multiple forms of mitochondrial stress, including mutation of *isp-1*, RNAi knockdown of *cco-1*, and RNAi knock-down of two new aging genes identified in this study: *letm-1* and *Y24D9A.8*.

Our observations that longevity is reduced by constitutive activation of ATFS-1 or by a subset of RNAi clones related to mitochondrial protein import (*tomm-22*, *E04A4.5*, *T09B4.9*, *F45G2.8*, *F15D3.7*, and *dnj-21*), further argue against a direct causal link between the UPR[mt] and longevity, and demonstrate that activation of the UPR[mt] is not sufficient to enhance longevity. This is also consistent with prior reports showing that knockdown of the gene encoding the mitochondrial prohibitin *phb-2* induces the UPR[mt] in both yeast and worms while simultaneously shortening lifespan in both organisms [47,48]. In the case of prohibitin deficiency, the lifespan defects can be suppressed by a reduction in cytoplasmic translation in yeast or by deletion of the S6 kinase homolog *rsks-1* in worms, which both attenuate the UPR[mt]. It will be of interest to determine whether loss of *rsks-1* or deletion of *atfs-1* can prevent lifespan shortening in response to knockdown of mitochondrial protein import machinery.

Although we reach a different conclusion with respect to the role of the UPR[mt] in longevity than has been previously suggested, the data presented in this study are generally consistent with the prior data from Durieux et al. [27] and Houtkooper et al. [38]. Our data also do not address the "mitokine" mechanism proposed by Durieux et al. [27], whereby a neuronal signal is transmitted to distal cells to induce the UPR[mt] in a cell non-autonomous manner. By utilizing both RNAi and mutant alleles of *atfs-1*, however, we are able to exclude the

possibility that the presence (or absence) of neuronal UPR^{mt} signaling (knockdown by RNAi feeding is generally less efficient in neurons) could yield different outcomes with respect to lifespan.

It is also important to consider that the UPR^{mt} is still relatively poorly characterized and imprecisely defined. Nearly every study of the UPR^{mt} in *C. elegans* has utilized either the *hsp-6$_p$::gfp* reporter or the *hsp-60$_p$::gfp* reporter, with analysis of endogenous expression of *hsp-6*, *hsp-60*, or other UPR^{mt} targets rarely performed. Our data indicate that some RNAi clones that robustly induce expression of *hsp-6$_p$::gfp* do not strongly induce expression of *hsp-60$_p$::gfp* (Supplementary Table 3). Whether this represents a biologically relevant difference in regulation of *hsp-6* and *hsp-60* or simply reflects differential sensitivities of the two reporters will require further study, but it does point out the possibility that there may be substantial differences in regulation of individual UPR^{mt} targets, depending on the type of mitochondrial stress and the resulting signals from the mitochondria to the nucleus. For this reason, we chose to directly quantify mRNA levels for three mitoUPR-regulated genes, in addition to utilizing these GFP reporter strains. In each case, similar changes in expression were observed. Our data therefore dissociate the mitoUPR, as commonly defined in the field, from longevity; however, it remains possible that additional, as yet unknown, UPR^{mt} targets that are regulated independently of ATFS-1 could influence lifespan directly. Thus, although we do not completely rule out a causal role for the UPR^{mt} in aging, our data strongly suggest that if the UPR^{mt} does play any direct longevity-promoting role it is subtle, variable, and highly context-dependent. Indeed, our interpretation of the entire body of data is that a stronger case can be made that the UPR^{mt} generally suppresses longevity, and that inhibition of ETC function increases lifespan *in spite of*, rather than *because of*, the UPR^{mt}.

Our observation that the lifespan-extending RNAi clones which also induce the UPR^{mt} appear to encompass a range of mitochondrial and cytoplasmic functions suggests that the mechanisms by which mitochondrial perturbations modulate aging in *C. elegans* may extend beyond reduced ETC function or inhibition of mitochondrial translation. The fact that each of these RNAi clones also induces the UPR^{mt} indicates that they are likely perturbing mitochondrial homeostasis, or at least inducing a signal of mitochondrial stress. It will be important to understand how each of these factors is influencing mitochondrial function, particularly in those cases where there is no obvious link to mitochondria, such as transaldolase deficiency. In this regard, it is interesting to note that altered expression of pentose phosphate pathway enzymes has been associated with lifespan extension from calorie restriction in rhesus monkeys [49], and transaldolase deficiency in mice causes loss of mitochondrial membrane potential and morphology in spermatozoa [50].

In addition to the UPR^{mt}, several cellular pathways and factors have been proposed to mediate enhanced longevity following mitochondrial stress in *C. elegans*. As with prior studies on the UPR^{mt}, however, each of these factors (e.g. HIF-1, CEH-23, AMP kinase, p53/cep-1, TAF-4) have only been tested relative to one or a few long-lived mutants or RNAi clones. For now, it remains an open question as to whether a single mechanism will ultimately explain all of the various mitochondrial longevity interventions, or if multiple overlapping but distinct mechanisms contribute. Given the central role of mitochondrial function in metabolism, nutrient response, and energetics, it is perhaps not surprising that

the role of mitochondria in aging is complex and challenging to understand solely through genetic analyses.

Taken together with prior studies, the observations reported here demonstrate that the relationship between the UPR^mt and aging is significantly more complex than currently appreciated. At best, induction of the UPR^mt is only weakly correlated with longevity, since many RNAi clones that induce the UPR^mt either have no effect on lifespan or reduce lifespan, and induction of the UPR^mt in the absence of mitochondrial stress fails to increase lifespan. Of the additional factors previously implicated in mitochondrial longevity, it remains unclear whether any represent a unifying mechanism. Despite the mechanistic uncertainties, however, the identification here of several novel RNAi clones that induce the UPR^mt, at least eight of which also extend lifespan, further emphasizes the central importance of this pathway and suggests that this will be a fertile area for future research and insights into the fundamental mechanisms of mitochondrial quality control and aging.

METHODS

Strains

NQ887 *(isp-1(qm150))*, QC117 (*atfs-1(et17)*), QC118 (*atfs-1(et18)*), SJ4100 (zcIs13[*hsp-6$_p$::gfp*]), SJ4058 (zcIs9[*hsp-60$_p$::gfp*]), CL2070 (dvIs[*hsp-16.2$_p$::gfp*]), and SJ4005 (zcIs4[*hsp-4$_p$::gfp*]) were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN). The *atfs-1(tm4525)* was obtained from the National BioResource Project (Tokyo, Japan) and backcrossed to our lab N2 strain twice.

RNAi Screen and Fluorescence Microscopy

All fluorescence microscopy was performed using a Zeiss SteREO Lumar. V12 microscope (Thornwood, NY, USA). The RNAi screen was performed using the Vidal RNAi library [51] and *hsp-6$_p$::gfp* worms. RNAi bacteria were inoculated into LB^Amp media and GFP reporter worms were hypochlorite treated and hatched in M9 overnight. The next day, RNAi bacteria were induced with 4mM IPTG for 1 hour, pelleted, and resuspended in liquid NGM supplemented with ampicillin and IPTG. RNAi bacteria were then added to 96-well plates with ~30 L1 reporter worms per well. After 3 days, GFP fluorescence was assessed manually by scoring photos as weak induction, strong induction, or no induction.

For validation of RNAi clones and quantification of different GFP reporter expression, GFP fluorescence of individual worms was quantified using IMAGEJ (Rasband, 1997–2012). Relative fluorescence to empty-vector was calculated and fluorescence data was pooled across three independent experiments. A Student's t-test was used to calculate significance with a Bonferonni correction to account for multiple comparisons. Synchronized eggs were grown on RNAi bacteria at 20°C, unless otherwise stated. After 3 days, worms were assayed for GFP expression by fluorescence microscopy. For the *hsp-16.2$_p$::gfp* and *hsp-4$_p$::gfp* reporters, expression was induced by treatment with 4+ hour 30°C heat shock and tunicamycin respectively. At least two independent experiments, with approximately 10 animals per condition per experiment, performed on different days from different RNAi cultures, was obtained for each reporter with similar results.

Lifespan Analyses

Lifespans were performed as described previously [52]. Synchronized eggs obtained by treating adult animals with hypochlorite were grown on NGM plates containing 4 mM IPTG, 25 μg ml^{-1} carbenicillin and seeded with HT115(DE3) RNAi bacteria. At larval stage 4, worms were transferred to plates with 50 μM FUDR to prevent hatching of progeny. Lifespans were performed at 25°C for the lifespan screen and at 20°C and 25°C in other experiments as described in the text and figures. Cohorts were examined every 1–3 days using tactile stimulation to verify viability of animals. Animals lost due to foraging were not included in the analysis. All lifespan analyses were replicated using independent cohorts on different dates with replicate statistics provided in Supplementary Materials (Supplementary Data 1, 2). *P*-values were calculated using the Wilcoxon rank-sum test. GraphPad Prism was used for Pearson's correlation statistics of *hsp-6$_p$::gfp* induction versus lifespan.

qRT-PCR

RNA was isolated from L4 worms using a TRIzol (Life Technologies) chloroform extraction and cDNA was prepared according to the SuperScript III First Strand Synthesis protocol (Life Technologies). qRT-PCR was used to measure the expression levels of *hsp-6*, *hsp-60*, *timm-23* (primers in Supplementary Table 4) and normalization controls *pmp-3* and *cdc-42* (TaqMan® Gene Expression Assays, Life Technologies). The relative standard curve method was used to calculate gene expression.

Supplementary Material

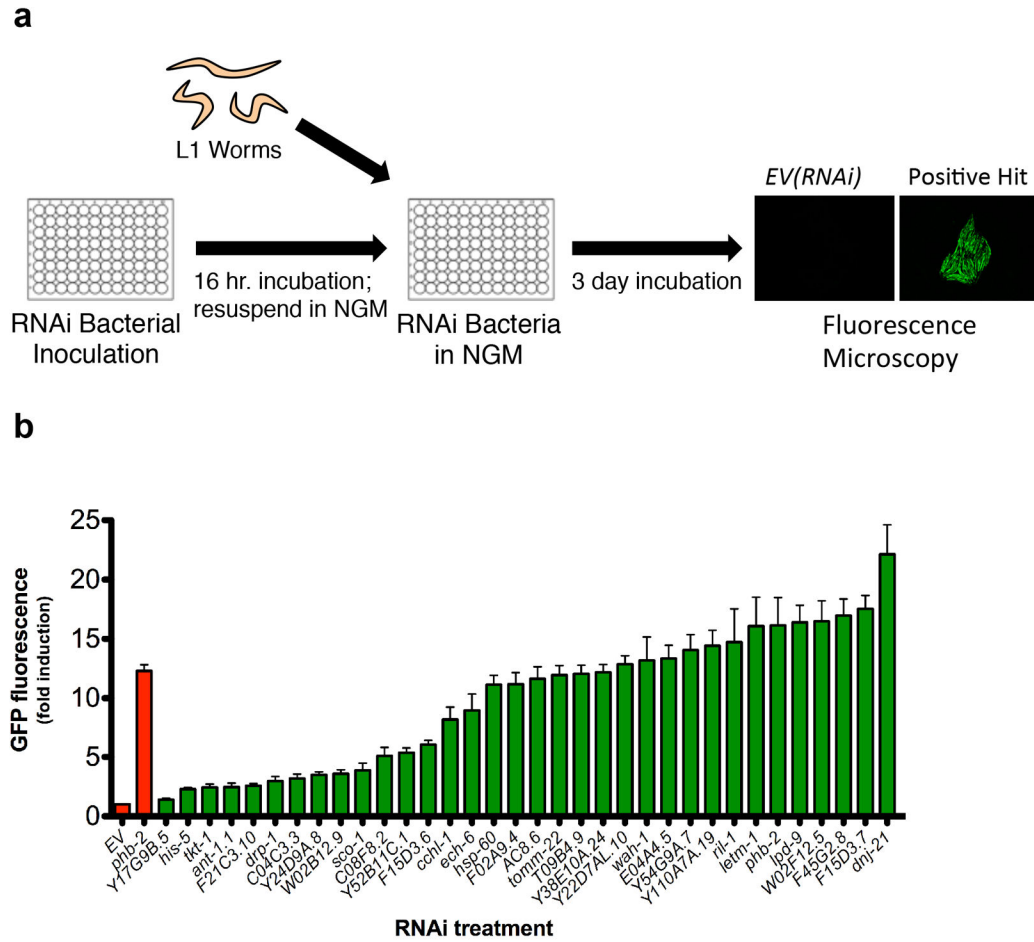Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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a

L1 Worms

RNAi Bacterial Inoculation

16 hr. incubation; resuspend in NGM

RNAi Bacteria in NGM

3 day incubation

EV(RNAi) Positive Hit

Fluorescence Microscopy

b

Figure 1. A genome-wide RNAi screen for negative regulators of the mitochondrial unfolded protein response

(a) RNAi bacteria were grown overnight in 96 well plates, while *hsp-6_p::gfp* animals were synchronized at L1 larval stage. The next day, RNAi bacteria was induced with IPTG, resuspended in liquid NGM, and added to reporter animals in 96-well plates. Animals were allowed to develop for three days and GFP was measured by fluorescent microscopy. (b) *hsp-6_p::gfp* induction was quantified for 34 RNAi clones corresponding to positive hits that were not annotated as functioning in the ETC or mitochondrial translation. Validation included sequencing each RNAi clone and GFP quantification of individual animals grown at 20°C. GFP fluorescence is the mean fluorescence relative to *EV(RNAi)* (N=3 independent experiments, error bars indicate SEM).
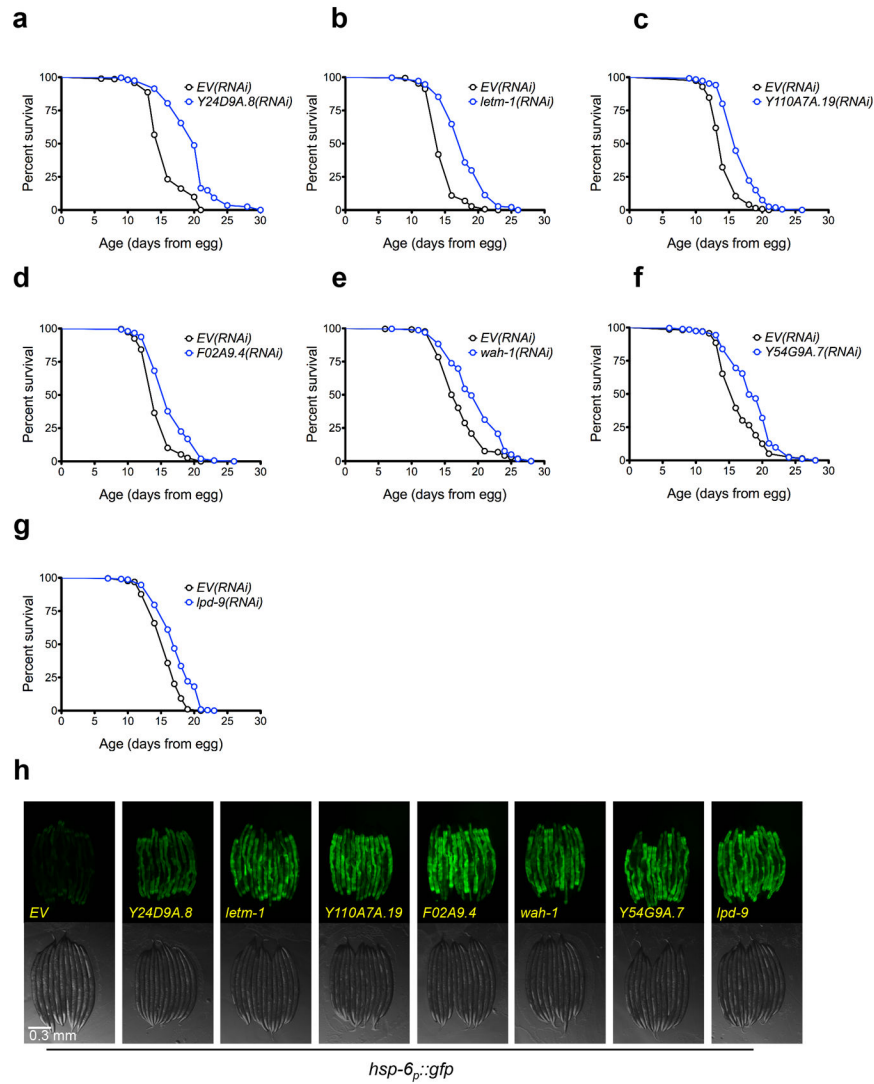
Figure 2. Several UPR^mt inducing RNAi clones extend lifespan

(a) N2 fed *EV(RNAi)* (mean 15.8 ± .2 days, n = 284), N2 fed *Y24D9A.8(RNAi)* (mean 19.8 ± .2 days, n = 316, p < 0.0001). (b) N2 fed *EV(RNAi)* (mean 15.0 ± .1 days, n = 320), N2 fed *letm-1(RNAi)* (mean 18.1 ± .2 days, n = 318, p < 0.0001). (c) N2 fed *EV(RNAi)* (mean 14.3 ± .1 days, n = 267), N2 fed *Y110A7A.19(RNAi)* (mean 16.8 ± .1 days, n = 360, p < 0.0001). (d) N2 fed *EV(RNAi)* (mean 14.6 ± .1 days, n = 266), N2 fed *F02A9.4(RNAi)* (mean 16.6 ± .2 days, n = 302, p < 0.0001). (e) N2 fed *EV(RNAi)* (mean 17.4 ± .2 days, n = 264), N2 fed *wah-1(RNAi)* (mean 19.6 ± .2 days, n = 301, p < 0.0001). (f) N2 fed *EV(RNAi)* (mean 16.5 ± .2 days, n = 279), N2 fed *Y54G9A.7(RNAi)* (mean 18.5 ± .2 days, n = 266, p < 0.0001). (g) N2 fed *EV(RNAi)* (mean 15.7 ± .1 days, n = 293), N2 fed *lpd-9(RNAi)* (mean 17.3 ± .2 days, n = 226, p < 0.0001). (h) The *hsp-6p::gfp* reporter is induced by knockdown of longevity conferring RNAi clones. *hsp-6p::gfp* worms were placed onto RNAi bacteria from egg and GFP measurements were taken three days later. Scale bar, 0.3 mm. Lifespans experiments in this figure were performed at 25°C. N=3 independent experiments, with pooled data shown. Lifespans are indicated as mean +/− SEM and *p*-values were calculated using Wilcoxon

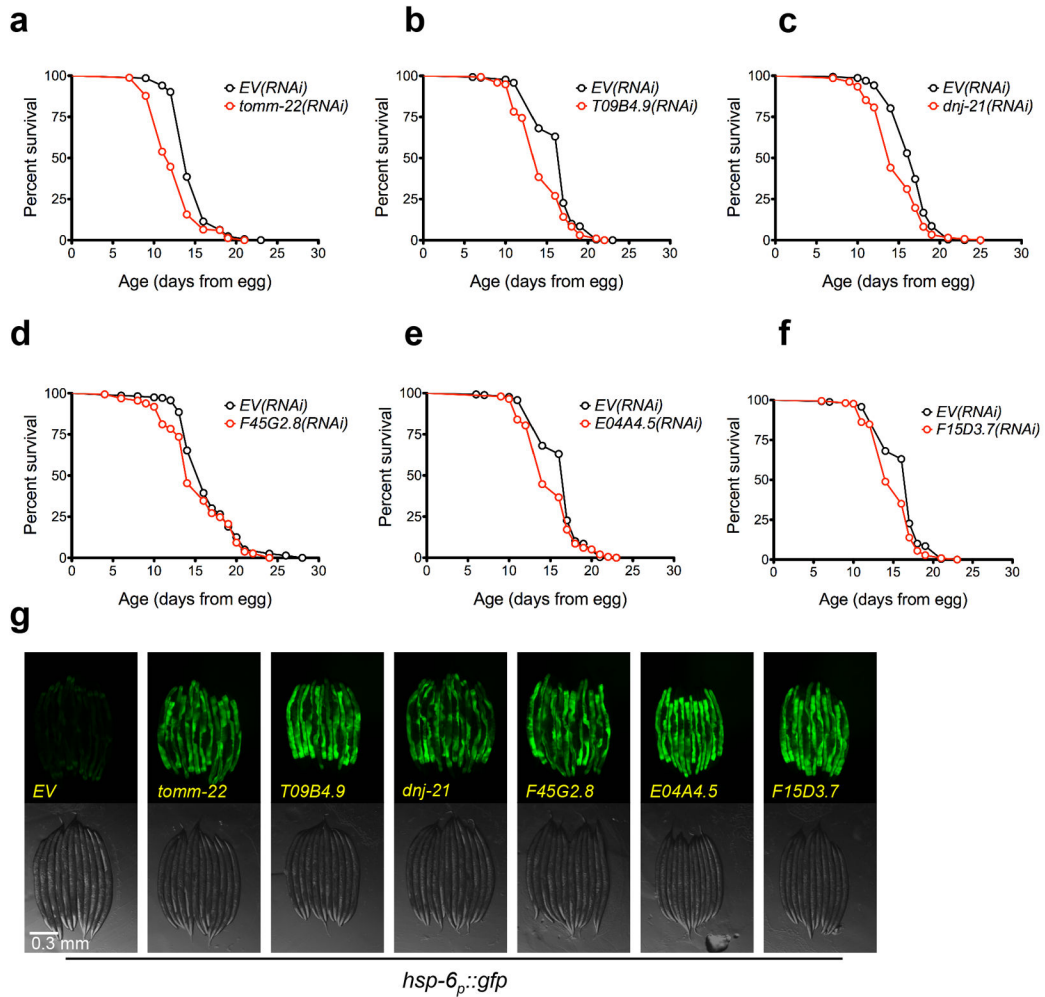rank-sum test. Data by individual experiment and statistical analysis provided in Supplementary Materials (Supplementary Data 1).
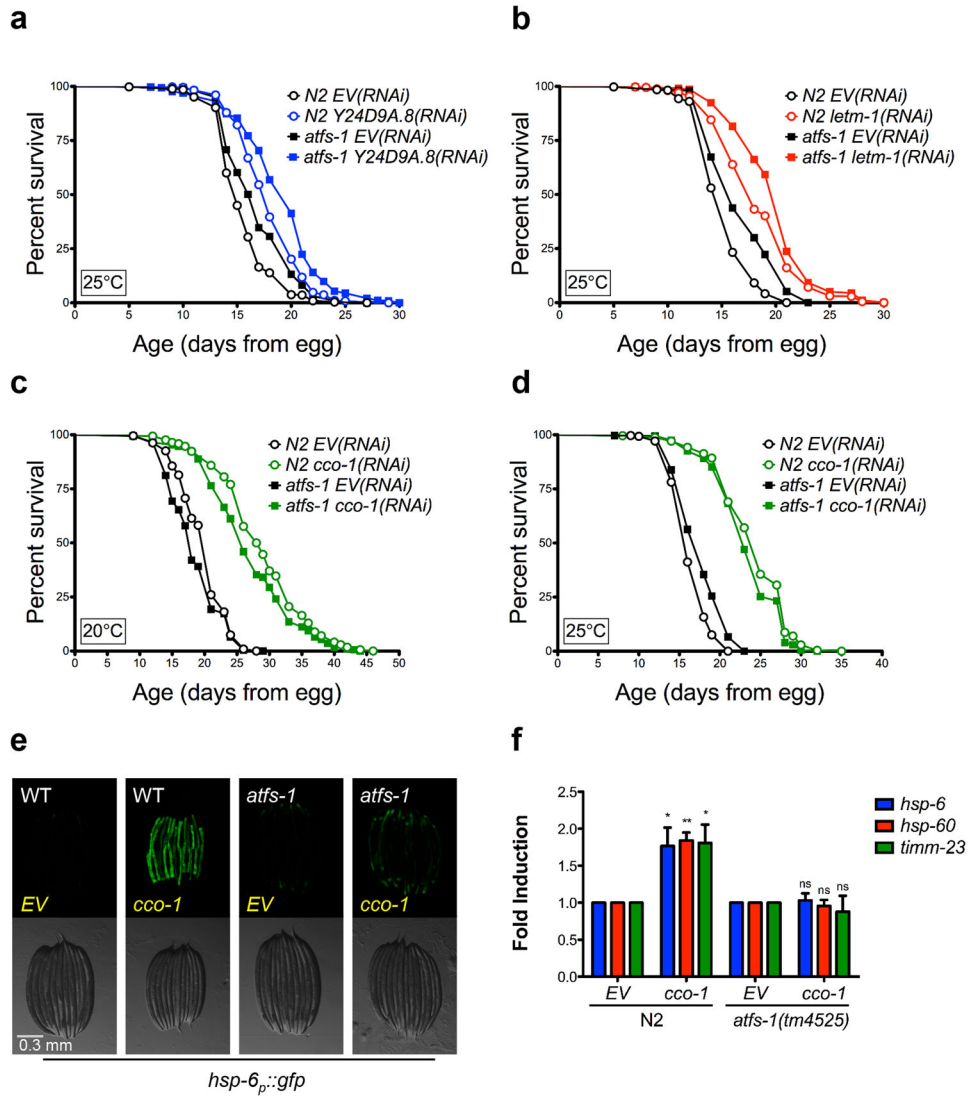
Figure 3. Several UPR^mt inducing RNAi clones shorten lifespan

(a) N2 fed *EV(RNAi)* (mean 14.8 ± .1 days, n = 335), N2 fed *tomm-22(RNAi)* (mean 12.7 ± .2 days, n = 327, p < 0.0001). (b) N2 fed *EV(RNAi)* (mean 16.3 ± .2 days, n = 260), N2 fed *T09B4.9(RNAi)* (mean 14.5 ± .2 days, n = 289, p < 0.0001). (c) N2 fed *EV(RNAi)* (mean 16.7 ± .1 days, n = 293), N2 fed *dnj-21(RNAi)* (mean 14.9 ± .2 days, n = 270, p < 0.0001). (d) N2 fed *EV(RNAi)* (mean 16.5 ± .2 days, n = 279), N2 fed *F45G2.8(RNAi)* (mean 15.2 ± .2 days, n = 291, p < 0.0001). (e) N2 fed *EV(RNAi)* (mean 16.3 ± .2 days, n = 260), N2 fed *E04A4.5(RNAi)* (mean 15.1 ± .2 days, n = 199, p < 0.0001). (f) N2 fed *EV(RNAi)* (mean 16.3 ± .2 days, n = 260), N2 fed *F15D3.7(RNAi)* (mean 15.1 ± .2 days, n = 217, p < 0.0001). (g) The *hsp-6_p::gfp* reporter is induced by knockdown of RNAi clones that reduce lifespan. *hsp-6_p::gfp* worms were placed onto RNAi bacteria from egg and grown at 25°C. GFP measurements were taken three days later. Scale bar, 0.3 mm. Lifespan experiments in this figure were performed at 25°C. N=3 independent experiments, with pooled data shown. Lifespans are indicated as mean +/− SEM and *p*-values were calculated using Wilcoxon rank-sum test. Data by individual experiment and statistical analysis provided in Supplementary Materials (Supplementary Data 1).

a

b

c
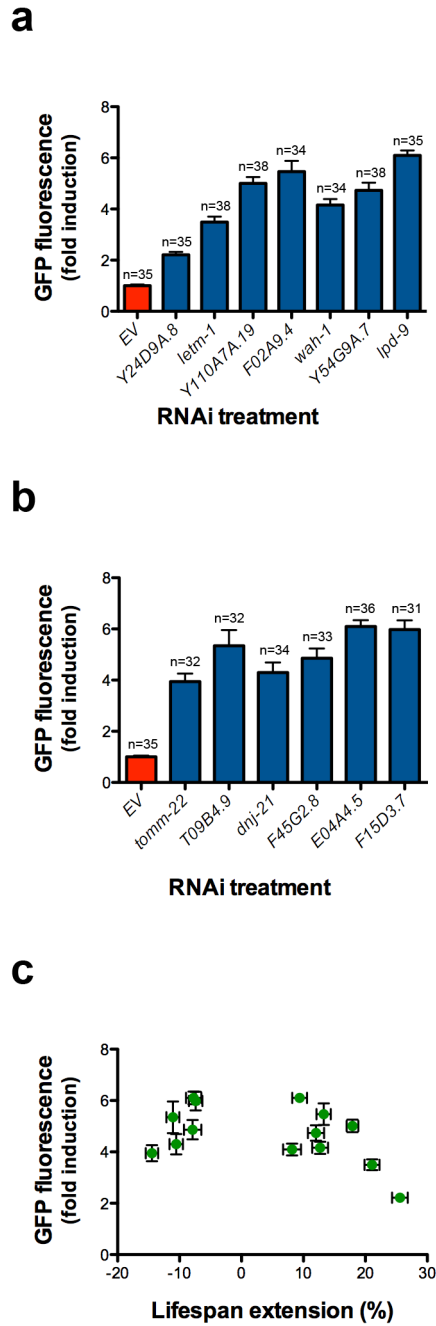
Figure 4. Induction of *hsp-6p::gfp* is not correlated with lifespan extension

hsp-6p::gfp induction from three separate experiments performed at 25°C for (a) long-lived RNAi clones and (b) short-lived RNAi clones. GFP fluorescence is the average fluorescence of an individual worm relative to *EV(RNAi)* and error bars indicate SEM. (c) *hsp-6p::gfp* induction is not significantly positively correlated with lifespan extension. The Pearson's correlation R^2 is 0.1912 and *p*-value is 0.12. Error bars indicate SEM for GFP fluorescence and % mean lifespan extension relative to *EV(RNAi)*.

Figure 5. UPR^mt inducing RNAi clones do not require *atfs-1* for lifespan extension
(a) *Y24D9A.8(RNAi)* lifespan extension is not dependent on *atfs-1*. N2 fed *EV(RNAi)* (mean 15.7 ± .1 days, n = 535), N2 fed *Y24D9A.8(RNAi)* (mean 18.1 ± .1 days, n = 456, p < 0.0001), *atfs-1(tm425)* fed *EV(RNAi)* (mean 17 ± .2 days, n = 417), *atfs-1(tm4525)* fed *Y24D9A.8(RNAi)* (19.2 ± .2 days, n = 334, p < 0.0001). (b) *letm-1(RNAi)* lifespan extension is not dependent on *atfs-1*. N2 fed *EV(RNAi)* (mean 15.5 ± .1, n = 460), N2 fed *letm-1(RNAi)* (mean 18.6 ± .2, n = 421, p < 0.0001), *atfs-1(tm425)* fed *EV(RNAi)* (mean 17 ± .2, n = 386), *atfs-1(tm4525)* fed *letm-1(RNAi)* (mean 20.1 ± .2, n = 358, p < 0.0001). (c) The long lifespan of *cco-1(RNAi)* is not dependent on *atfs-1* at 20°C. N2 fed *EV(RNAi)* (mean 19.9 ± .2, n = 215), N2 fed *cco-1(RNAi)* (mean 28.9 ± .5, n = 170, p < 0.0001), *atfs-1(tm425)* fed *EV(RNAi)* (mean 18.6 ± .3, n = 202), *atfs-1(tm4525)* fed *cco-1(RNAi)* (mean 26.9 ± .5, n = 170, p < 0.0001). (d) The long lifespan of *cco-1(RNAi)* is not dependent on *atfs-1* at 25°C. N2 fed *EV(RNAi)* (mean 16.6 ± .1, n = 279), N2 fed *cco-1(RNAi)* (mean 24.2 ± .3, n = 242, p < 0.0001), *atfs-1(tm425)* fed *EV(RNAi)* (mean 17.8 ± .2, n = 302), *atfs-1(tm4525)* fed *cco-1(RNAi)* (mean 23.5 ± .3, n = 202, p < 0.0001). (e) *hsp-6p::gfp*

induction by *cco-1(RNAi)* is attenuated in the *atfs-1(tm4525)* mutant. Scale bar, 0.3 mm. (f) Induction of UPR^{mt} targets *hsp-6*, *hsp-60*, and *timm-23* does not occur in the *atfs-1(tm4525)* mutant. N2 and *atfs-1(tm4525)* worms were grown on *cco-1(RNAi)* from egg at 20°C and harvested at L4. Gene expression was normalized to *EV(RNAi)*. (N=4, Error bars represent SEM, p* < 0.05, p** < 0.01), p > 0.05 is not significant (ns), student's t-test). Lifespan experiments in this figure represent pooled data, are indicated as mean +/− SEM, and *p*-values were calculated using Wilcoxon rank-sum test. Data by individual experiment and statistical analysis provided in Supplementary Materials (Supplementary Data 2).
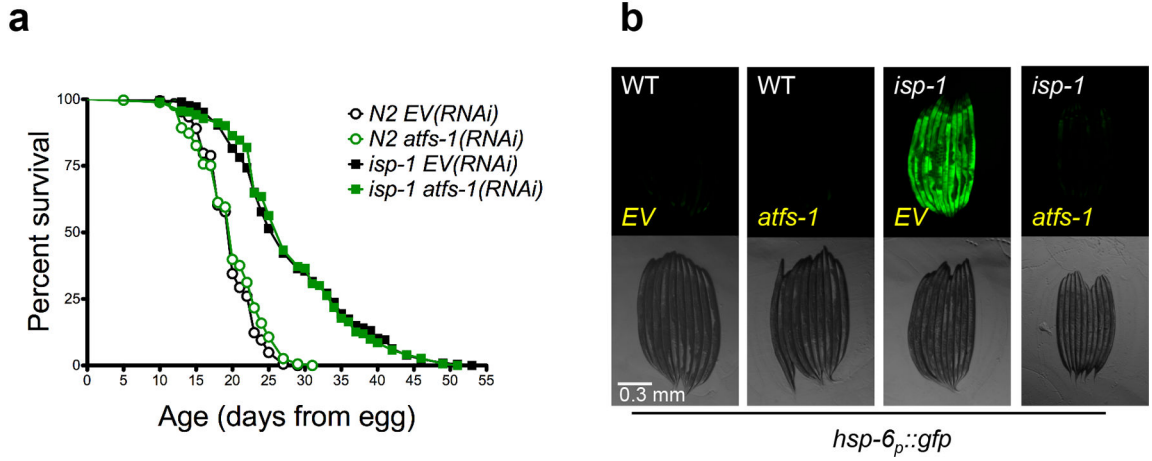
Figure 6. The UPR^{mt} is not required for *isp-1(qm150)* longevity
(a) Knockdown of *atfs-1* does not attenuate the longevity of complex III mutant *isp-1(qm150)*. N2 fed *EV(RNAi)* (mean 19.7 ± .2, n = 365), N2 fed *atfs-1(RNAi)* (mean 20 ± .2 days, n = 346, p = 0.399), *isp1(qm150)* fed *EV(RNAi)* (mean 28.2 ± .6 days, n = 206), *isp-1(qm150)* fed *atfs-1(RNAi)* (mean 28.3 ± .5 days, n = 293, p = 0.612). (b) *hsp-6$_p$::gfp* induction by *isp-1(qm150)* mutation is attenuated by *atfs-1(RNAi)*. Scale bar, 0.3 mm. Lifespan experiments in this figure represent pooled data, are indicated as mean +/− SEM, and *p*-values were calculated using Wilcoxon rank-sum test. Data by individual experiment and statistical analysis provided in Supplementary Materials (Supplementary Data 2).
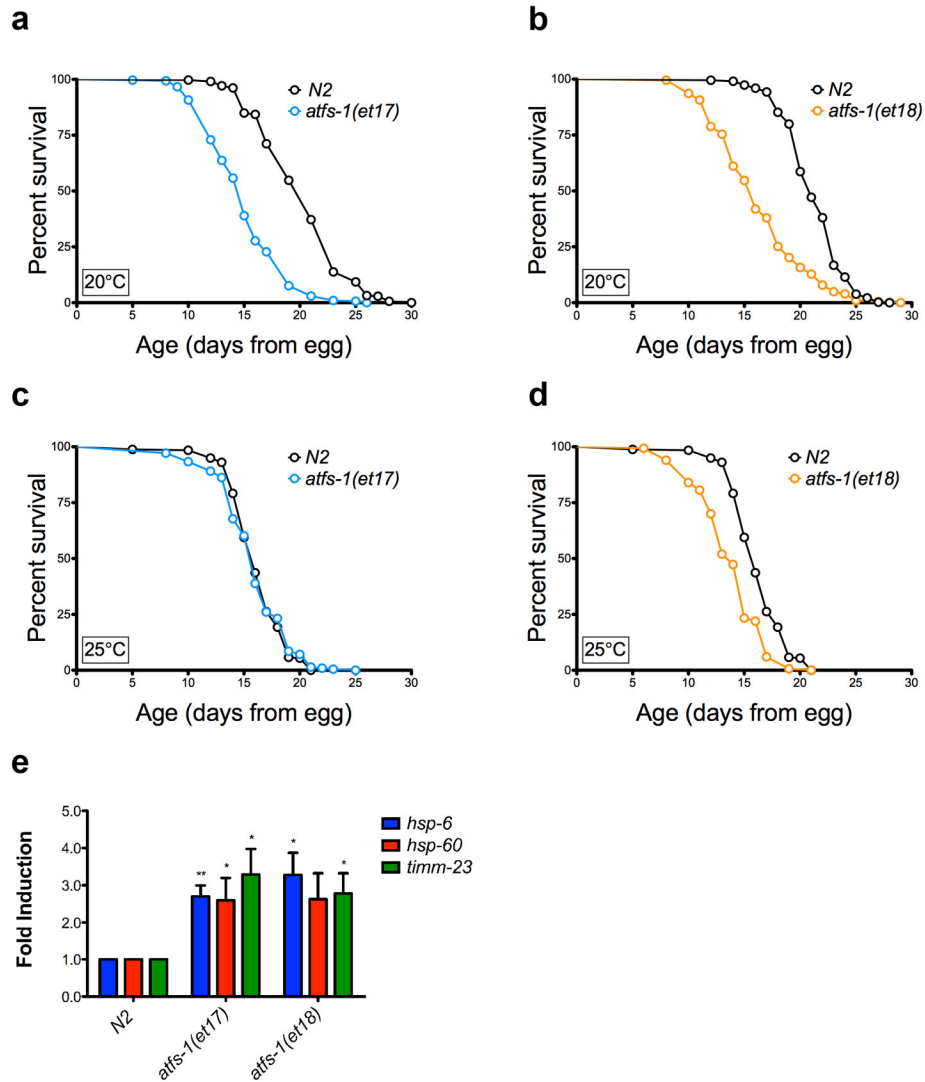
Figure 7. The UPR^{mt} is not sufficient for lifespan extension

(a) Constitutive UPR^{mt} mutation *atfs-1(et17)* shortens lifespan at 20°C. N2 fed HT115 bacteria (mean 20.3 ± .2 days, n = 312), *atfs-1(et17)* fed HT115 bacteria (mean 15 ± .2, n = 303, p < 0.0001). (b) Constitutive UPR^{mt} mutation *atfs-1(et18)* shortens lifespan at 20°C. N2 fed HT115 bacteria (mean 21.3 ± .1, n = 418), *atfs-1(et18)* fed HT115 bacteria (mean 16.3 ± .3 days, n = 203, p < 0.0001). (c) *atfs-1(et17)* mutation does not affect lifespan at 25°C. N2 fed HT115 bacteria (mean = 16.2 ± .15 days), *atfs-1(et17)* fed HT115 bacteria (mean = 15.9 ± .21 days, p = 0.31). (d) *atfs-1(et18)* mutation shortens lifespan at 25°C. N2 fed HT115 bacteria (mean = 16.2 ± .15 days), *atfs-1(et18)* fed HT115 bacteria (mean = 13.8 ± .13 days, p < 0.0001). (e) Constitutive UPR^{mt} mutations *atfs-1(et17)* and *atfs-1(et18)* induce expression of *hsp-6*, *hsp-60*, and *timm-23*. Gene expression was normalized to N2 fed *EV(RNAi)*. (N=4, Error bars represent SEM, p* < 0.05, p** < 0.01, student's t-test). The gene expression data for *hsp-60* in the *atfs-1(et18) allele* is trending significant, with *p* = 0.05. Lifespan experiments in this figure represent pooled data, are indicated as mean +/− SEM, and *p*-values were calculated using Wilcoxon rank-sum test. Data by individual

experiment and statistical analysis provided in Supplementary Materials (Supplementary Data 2).

Table 1

Effects of 19 UPR^{mt} regulators on lifespan.

Condition	Gene Function/Domain	Effect on Mean Lifespan		
		(% EV)	p	n
Y24D9A.8(RNAi)	Transaldolase	25.6	7.9E-42	316/284
letm-1(RNAi)	Ca2+-binding transmembrane protein, LETM1/MRS7	21.1	2.7E-39	318/320
Y110A7A.19(RNAi)	Pentatricopeptide repeat-containing protein 3	17.9	1.5E-34	360/267
F02A9.4(RNAi)	Methylcrotonoyl-Coenzyme A carboxylase 2, beta subunit	13.3	1.5E-16	302/266
wah-1(RNAi)	Programmed cell death 8, AIF homologue	12.7	7.1E-13	301/264
Y54G9A.7(RNAi)	Unknown	12.0	3.6E-13	266/279
lpd-9(RNAi)	Unknown	10.2	1.1E-11	226/293
W02F12.5(RNAi)	Dihydrolipoamide succinyltransferase (2-oxoglutarate dehydrogenase, E2 subunit)	8.2	7.0E-04	237/242
Y22D7AL.10(RNAi)	HSP10 homologue	5.5	4.8E-05	309/335
F15D3.6(RNAi)	UPS2/UPS3 homologue	4.8	1.6E-03	371/323
C04C3.3(RNAi)	Pyruvate dehydrogenase E1, beta subunit	−0.1	5.6E-01	302/279
ech-6(RNAi)	Enoyl-CoA hydratase	−1.1	9.4E-01	306/296
hsp-60(RNAi)	HSP60 homologue	−4.0	8.6E-02	187/282
F15D3.7(RNAi)	Translocase of inner mitochondrial membrane complex, subunit TIM23	−7.4	1.3E-07	217/260
E04A4.5(RNAi)	Translocase of inner mitochondrial membrane complex, subunit TIM17	−7.7	4.4E-07	199/260
F45G2.8(RNAi)	Translocase of inner mitochondrial membrane complex, subunit TIM16	−7.9	8.8E-05	291/279
dnj-21(RNAi)	Translocase of inner mitochondrial membrane complex, subunit TIM14	−10.5	4.5E-14	270/293
T09B4.9(RNAi)	Translocase of inner mitochondrial membrane complex, subunit TIM44	−11.1	2.4E-14	289/260
tomm-22(RNAi)	Translocase of outer mitochondrial membrane complex, subunit TOM22	−14.5	9.9E-28	327/335

RNAi knockdown of the shown genes significantly induced expression of the $hsp-6_p::gfp$ reporter. The effect on mean lifespan from each RNAi clone is shown relative to empty vector (EV) treated animals. p-values are shown for a Wilcoxon Rank-Sum comparison to EV control. n is number of animals for a certain RNAi treatment compared to EV control.