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

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Recent studies have propagated the model that the mitochondrial unfolded protein response (UPRmt) is causal for lifespan extension from inhibition of the electron transport chain (ETC) in *Caenorhabditis elegans*. Here we report a genome-wide RNAi screen for negative regulators of the UPRmt. Lifespan analysis of nineteen RNAi clones that induce the *hsp-6p::gfp* reporter demonstrate differential effects on longevity. Deletion of *atfs-1*, which is required for induction of the UPRmt, 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 UPRmt by gain of function mutations in *atfs-1* fails to extend lifespan. These observations identify several new factors that promote mitochondrial homoeostasis and demonstrate that the UPRmt, as currently defined, is neither necessary nor sufficient for lifespan extension.
As the primary source of cellular energy and a major source of damage, mitochondria play an important role in modulating many age-related processes\textsuperscript{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 mitochondrial metabolism determines the rate of organismal aging\textsuperscript{3}. Recent studies have demonstrated that the situation is more complex than originally proposed, however, with important pro-longevity signalling functions of ROS having been established in several species\textsuperscript{4}. 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\textsuperscript{5,6}. Knockdown of ETC genes must occur during a specific stage of development to promote longevity\textsuperscript{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 shortening of the L3/L4 larval stage transition\textsuperscript{27}, a critical time period for mitochondrial biogenesis and longevity\textsuperscript{8,9}. 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\textsuperscript{10,11}. 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\textsuperscript{12}. Stabilization of HIF-1 is sufficient to extend lifespan robustly through a mechanism that is distinct from insulin/IGF-1-like signalling or dietary restriction\textsuperscript{13-16}, 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\textsuperscript{23}, the homeobox protein CEH-23 (ref. 24), the transcription factor TAF-4 (ref. 25) and the p53 homologue CEP-1 (ref. 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\textsuperscript{mt}) was proposed to directly mediate lifespan extension from ETC inhibition in a cell non-autonomous manner\textsuperscript{27}. The UPR\textsuperscript{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\textsuperscript{28-30}. The C. elegans UPR\textsuperscript{mt} appears similar to that of mammals\textsuperscript{27,31-34}, where induction of the UPR\textsuperscript{mt} results in transcriptional upregulation 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\textsuperscript{mt} in C. elegans using GFP reporters for both hsp-6 and hsp-60 (refs 27,31). In addition, the C. elegans UPR\textsuperscript{mt} can only be induced robustly when mitochondrial stress precedes the L3/L4 larval stage transition\textsuperscript{27}, a critical time period for mitochondrial biogenesis and longevity\textsuperscript{8,35}. This signalling 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\textsuperscript{33}, the CLPP-1 protease\textsuperscript{33}, a ubiquitin-like protein UBL-5 (ref. 32) and two transcription factors, DVE-1 and ATFS-1 (ZC376.7)\textsuperscript{33,34,36,37}.

Durieux et al.\textsuperscript{33} linked the UPR\textsuperscript{mt} to aging by showing that RNAi knockdown of UPR\textsuperscript{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\textsuperscript{mt} plays a causal role in lifespan extension from ETC inhibition\textsuperscript{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 nrasp-5, which encodes a mitochondrial ribosomal protein\textsuperscript{38}.

On the basis of the report by Durieux et al.\textsuperscript{37}, we set out to identify additional genetic modifiers of lifespan by screening for RNAi clones that induce the UPR\textsuperscript{mt}. Here we report the identification and validation of 25 previously unreported negative regulators of the UPR\textsuperscript{mt}. Unexpectedly, several RNAi clones that induce the UPR\textsuperscript{mt} shorten lifespan, and among at least a subset of those that extend lifespan, induction of the UPR\textsuperscript{mt} is not required for lifespan extension. Constitutive activation of the UPR\textsuperscript{mt} in the absence of mitochondrial stress also fails to extend lifespan.

### Results

A genomic screen for negative regulators of the UPR\textsuperscript{mt}. We sought to identify RNAi clones that increase lifespan by screening for induction of fluorescence in animals expressing the hsp-6::gfp reporter following RNAi knockdown of individual genes (Fig. 1a). We identified 95 putative inducers of the UPR\textsuperscript{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\textsuperscript{mt}\textsuperscript{31,36,38-41}, and the remaining 66 are novel.

Since ETC components and mitochondrial ribosomal proteins are known to modulate both the UPR\textsuperscript{mt} and longevity\textsuperscript{39,42}, 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::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 mitochondrial on the basis of predicted mitochondrial targeting sequences (Supplementary Table 1). About half (18/34) of the RNAi clones that induced expression of the hsp-6::gfp reporter also significantly induced expression of the mitochondrial hsp-60::gfp reporter, while none of the RNAi clones induced reporters of either the endoplasmic reticulum UPR (hsp-4::gfp) or the cytoplasmic heat shock response (hsp-16.2::gfp) to a detectable level, with the exception of Y38E10A.24(RNAi), which modestly increased fluorescence in the hsp-4::gfp strain (Supplementary Table 3).
Relationship between the UPR\textsuperscript{mt} and longevity. On the basis of the model that lifespan extension from ETC inhibition results from induction of the UPR\textsuperscript{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\textsuperscript{mt} induction and lifespan was detected for knock-downs that significantly affected lifespan (Figs 2h,3g and 4).

Table 1 | Effects of 19 UPR\textsuperscript{mt} regulators on lifespan.

| Condition | Gene function/Domain | Effect on mean lifespan ($\%$ EV) | $P$-value | $n$ |
|-----------|----------------------|----------------------------------|----------|-----|
| Y24D9A.8(RNAi) | Transaldolase | 25.6 | 7.9E–42 | 316/284 |
| letm-1(RNAi) | Ca\textsuperscript{2+}-binding transmembrane protein, LEMT-1/MRS7 | 21.1 | 2.7E–39 | 318/320 |
| Y1007A.19(RNAi) | Pentatricopeptide repeat-containing protein 3 | 17.9 | 1.5E–34 | 360/267 |
| F0249.4(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) | Dihydrodiolopimide 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 |
| CD04C3.3(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) | HSP-60 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 |
| E04A3.3(RNAi) | Translocase of inner mitochondrial membrane complex, subunit TIM17 | – 7.7 | 4.4E–07 | 199/260 |
| F15D2.8(RNAi) | Translocase of inner mitochondrial membrane complex, subunit TIM16 | – 7.9 | 8.8E–05 | 291/279 |
| dnr-21(RNAi) | Translocase of inner mitochondrial membrane complex, subunit TIM14 | – 10.5 | 4.5E–14 | 270/293 |
| T0984.9(RNAi) | Translocase of inner mitochondrial membrane complex, subunit TIM44 | – 11.1 | 2.4E–14 | 289/260 |
| tomn-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-6p::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 with EV control; $n$ is number of animals for a certain RNAi treatment compared with EV control.
Notably, all of the clones that significantly reduced lifespan correspond to worm homologues of proteins important for transport of mitochondrial-localized proteins into the mitochondria. These included *fomt-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. 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\textsuperscript{mt} induction but not longevity.**

We next set out to more directly characterize the role of the UPR\textsuperscript{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\textsuperscript{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 *atsf-1*(tm4525) mutation, which has been shown to prevent induction of the UPR\textsuperscript{mt} following knockdown of mitochondrial AAA-protease *spg-7*, knockdown of mitochondrial import machinery, and etidium bromide treatment\textsuperscript{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 relocates to the nucleus to induce expression of UPR\textsuperscript{mt} genes, including *hsp-6* and *hsp-60* (ref. 36). On the basis of the model that the UPR\textsuperscript{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 *atsf-1*(tm4525) background. Unexpectedly, deletion of *atsf-1* failed to significantly attenuate the lifespan extension from RNAi knockdown in either case (Fig. 5a,b).

To determine whether the lack of effect from *atsf-1* deletion on lifespan extension is specific to these newly identified UPR\textsuperscript{mt}-inducing clones, we asked whether knockdown of *cco-1*, which is
already known to extend lifespan\(^7\)–\(^9\), was dependent on \textit{atfs-1}. To rule out the possibility that high temperature (25 °C) could influence the outcome by modulating expression of UPR\(\text{mt}\) genes independently of the mitochondrial stress, we performed subsequent experiments at 20 °C in addition to 25 °C. Once again, deletion of \textit{ats-1} failed to significantly attenuate lifespan extension from \textit{cco-1} (RNAi) at both temperatures (Fig. 5c,d). The presence of the \textit{ats-1}(\textit{tm}4525) allele was verified by PCR (Supplementary Fig. 2), and deletion of \textit{ats-1} prevented induction of both \textit{hsp-60} and \textit{isp-1} (Fig. 5c,d). The endogenous UPR\(\text{mt}\) targets \textit{hsp-6} and \textit{isp-1} were significantly induced in the \textit{cco-1} (RNAi) animals expressing \textit{timm-23}, as well as three different constitutively active alleles of \textit{ATFS-1} have been described which constitutively activate the UPR\(\text{mt}\) in these animals. Stabilization of \textit{ATFS-1} does not extend lifespan. Recently, constitutively active alleles of \textit{ATFS-1} have been described which result in induction of the UPR\(\text{mt}\) in the absence of exogenous mitochondrial stress\(^{45}\). To determine whether constitutive activation of the UPR\(\text{mt}\) is sufficient to extend lifespan in the absence of ETC inhibition, we measured the lifespans of strains carrying either the \textit{ats-1}(\textit{et}17) or \textit{ats-1}(\textit{et}18) 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\(\text{mt}\) targets \textit{hsp-6}, \textit{isp-1} and \textit{timm-23} were significantly induced in the \textit{ats-1}(\textit{et}17) and \textit{ats-1}(\textit{et}18) strains, relative to N2 (Fig. 7e), demonstrating constitutive activation of the UPR\(\text{mt}\) in these animals.

**Discussion**

The model that the UPR\(\text{mt}\) 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\(\text{mt}\) is sufficient to increase lifespan. In this study, we identified 95 gene knockdowns that induce the UPR\(\text{mt}\),
Pearson's correlation is not significantly positively correlated with lifespan extension. The studies described here are limited to mutations and RNAi 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 (ref. 46), interpretation is further complicated by the fact that this protein likely has numerous functions beyond modulating the UPRmt.

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 UPRmt and longevity, and demonstrate that activation of the UPRmt 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 UPRmt in both yeast and worms while simultaneously shortening lifespan in both organisms. 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 homologue rsk-1 in worms, which both attenuate the UPRmt. It will be of interest to determine whether loss of rsk-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 UPRmt in longevity than has been previously suggested, the data presented in this study are generally consistent with the prior data from Durieux et al. and Houtkooper et al. Our data also do not address the “mitokine” mechanism proposed by Durieux et al., whereby a neuronal signal is transmitted to distal cells to induce the UPRmt in a cell non-autonomous manner. By utilizing both RNAi and mutant alleles knockdown of both attenuation of the UPRmt. It will be of interest to determine whether loss of atfs-1 or deletion of atfs-1 can prevent lifespan shortening in response to knockdown of mitochondrial protein import machinery.

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 UPRmt, 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-developentially.

The best evidence that the UPRmt directly promotes longevity comes from epistasis experiments reporting that knockdown of ubl-5 or haf-1, which are required for full induction of the UPRmt, prevents lifespan extension from isw-1(gmt150) or RNAi knockdown of either cco-1 or mrps-5 (refs 27,38). One major caveat to these experiments, however, is that neither ubl-5(RNAi) nor haf-1(RNAi) actually prevented induction of the UPRmt in these experiments. For example, in the study from Houtkooper et al., double knockdown of mrps-5 and haf-1 still resulted in an approximately 15-fold induction of the hsp-6p::gfp reporter relative to control animals, suggesting that the UPRmt is, at best, attenuated. Furthermore, no analysis of endogenous expression UPRmt targets was reported in these studies. In addition, we found that haf-1 is not required for induction of the UPRmt 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 (ref. 46), interpretation is further complicated by the fact that this protein likely has numerous functions beyond modulating the UPRmt.

For these reasons, we felt that loss of atfs-1, which has no known function outside of the UPRmt, 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-6p::gfp reporter and endogenous expression of three different UPRmt components (hsp-6, hsp-60 and timm-23), while having minimal effect on lifespan extension from multiple forms of mitochondrial stress, including mutation of isw-1, RNAi knockdown of cco-1 and RNAi knockdown 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 UPRmt and longevity, and demonstrate that activation of the UPRmt 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 UPRmt in both yeast and worms while simultaneously shortening lifespan in both organisms. 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 homologue rsk-1 in worms, which both attenuate the UPRmt. It will be of interest to determine whether loss of rsk-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 UPRmt in longevity than has been previously suggested, the data presented in this study are generally consistent with the prior data from Durieux et al. and Houtkooper et al. Our data also do not address the “mitokine” mechanism proposed by Durieux et al., whereby a neuronal signal is transmitted to distal cells to induce the UPRmt 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 UPRmt signalling (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\textsuperscript{mt} is still relatively poorly characterized and imprecisely defined. Nearly every study of the UPR\textsuperscript{mt} in *C. elegans* has utilized either the *hsp-6p::gfp* reporter or the *hsp-60p::gfp* reporter, with analysis of endogenous expression of *hsp-6, hsp-60* or other UPR\textsuperscript{mt} targets rarely performed. Our data indicate that some RNAi clones that robustly induce expression of *hsp-6p::gfp* do not strongly induce expression of *hsp-60p::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\textsuperscript{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 UPR\textsuperscript{mt}-regulated genes, in addition to utilizing these GFP reporter strains. In each case, similar changes in expression were observed. Our data therefore dissociate the UPR\textsuperscript{mt}, as commonly defined in the field, from longevity; however, it remains possible that additional, as yet unknown, UPR\textsuperscript{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\textsuperscript{mt} in aging, our data strongly suggest that if the UPR\textsuperscript{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\textsuperscript{mt} generally suppresses longevity, and that inhibition of ETC function increases lifespan in spite of, rather than because of, the UPR\textsuperscript{mt}. 

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**Figure 5 | UPR\textsuperscript{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 ± 0.1 days, *n* = 535), N2 fed *Y24D9A.8(RNAi)* (mean 18.1 ± 0.1 days, *n* = 456, *P* < 0.0001), *atfs-1(tm425)* fed EV(RNAi) (mean 17 ± 0.2 days, *n* = 417), *atfs-1(tm4525)* fed *Y24D9A.8(RNAi) (19.2 ± 0.2 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 ± 0.1, *n* = 460), N2 fed *letm-1(RNAi)* (mean 18.6 ± 0.2, *n* = 421, *P* < 0.0001), *atfs-1(tm425)* fed EV(RNAi) (mean 17 ± 0.2, *n* = 386), *atfs-1(tm4525)* fed *letm-1(RNAi)* (mean 20.1 ± 0.2, *n* = 358, *P* < 0.0001). (c) The long lifespan of *cco-1::gfp* is not dependent on atfs-1 at 20°C. N2 fed EV(RNAi) (mean 19.9 ± 0.2, *n* = 215), N2 fed *cco-1::gfp* (mean 28.9 ± 0.5, *n* = 170, *P* < 0.0001), *atfs-1(tm425)* fed EV(RNAi) (mean 18.6 ± 0.3, *n* = 202), *atfs-1(tm4525)* fed *cco-1::gfp* (mean 26.9 ± 0.5, *n* = 170, *P* < 0.0001). (d) The long lifespan of *cco-1::gfp* is not dependent on atfs-1 at 25°C. N2 fed EV(RNAi) (mean 16.6 ± 0.1, *n* = 279), N2 fed *cco-1::gfp* (mean 24.2 ± 0.3, *n* = 242, *P* < 0.0001), *atfs-1(tm425)* fed EV(RNAi) (mean 17.8 ± 0.2, *n* = 302), *atfs-1(tm4525)* fed *cco-1::gfp* (mean 23.5 ± 0.3, *n* = 202, *P* < 0.0001). (e) *hsp-6p::gfp* induction by *cco-1::gfp* is attenuated in the *atfs-1(tm4525)* mutant. Scale bar, 0.3 mm. (f) Induction of UPR\textsuperscript{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::gfp* from egg at 20°C and harvested at L4. Gene expression was normalized to EV(RNAi). (n = 4, Error bars represent s.e.m., *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 ± s.e.m., and *P*-values were calculated using Wilcoxon rank-sum test. Data by individual experiment and statistical analysis provided in Supplementary Materials (Supplementary Data 2). WT, wild type.
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 homoeostasis, 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 (for example, 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. NQ878 (isp-1(qm150)), QC117 (af-1(e171)), QC118 (af-1(e118)), SI4100 (zcs130(hsp-6::gfp)), SI4058 (zcs190(hsp-60::gfp)), CL2070 (dvlA1(hsp-16.2::gfp)) and SJ4005 (zcs404(hsp-4::gfp)) were obtained from the Caenorhabditis Genetics Centre (Minneapolis, MN, USA). The atf-1(erm525) 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 library51 and hsp-6::gfp worms. RNAi bacteria were inoculated into LB media and GFP reporter worms were hypochlorite treated and heated in M9 overnight. The next day, RNAi bacteria were induced with 4 mM isopropyl-β-D-thiogalactoside (IPTG) for 1 hour, pelleted and resuspended in liquid nematode growth media 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 worms 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 vector was calculated and fluorescence data were pooled across three independent experiments. A Student’s t-test was used to calculate significance with a Bonferroni 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::gfp and hsp-4::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 previously52. Synchronized eggs obtained by treating adult animals with hypochlorite were grown on nematode growth media 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 5-fluoro-2-deoxyuridine 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 Table S2 and normalized controls jpm-3 and cdc-42 (TagMan Gene Expression Assays, Life Technologies). The relative standard curve method was used to calculate gene expression.

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