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Searching for the elusive mitochondrial longevity signal in C. elegans

Christopher F Bennett[1,2], Haeri Choi[1], and Matt Kaeberlein[1,*]

[1]Department of Pathology; University of Washington; Seattle, WA USA; [2]Molecular and Cellular Biology Program; University of Washington; Seattle, WA USA

There is a growing list of examples where perturbed mitochondrial function is associated with increased longevity, yet the exact mechanisms have remained elusive. This phenomenon was first documented, and has been studied most extensively, in *C. elegans*. One prominent model proposed that lifespan extension resulting from electron transport chain inhibition is due to induction of the mitochondrial unfolded protein response. This model requires revision in light of recent data showing that the mitochondrial unfolded protein response, as defined by the field, is neither necessary nor sufficient for lifespan extension in *C. elegans*. Several additional factors have been proposed to underlie this lifespan extension, which is likely to be multifactorial and complex.

Keywords: aging, ATFS-1, electron transport chain, *hsp-6*, longevity, lifespan, mitochondrial unfolded protein response

*Correspondence to: Matt Kaeberlein; Email: kaeber@u.washington.edu

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Introduction: Mitochondrial Stress and the Mitochondrial Unfolded Protein Response

Inhibition of mitochondrial function was first associated with longevity in *C. elegans* when it was discovered that *clk-1* mutants with reduced coenzyme Q production are long-lived.[1,2] Soon after this, animals defective for function of the electron transport chain complex III component, ISP-1, were also found to have increased lifespan.[3] Genome-wide screens for RNAi clones that extend lifespan then identified dozens of mitochondrial factors that modulate aging, confirming the generality of these observations.[4,5] To date, lifespan extension has been reported following RNAi knockdown of components of complex I, III, IV, and V of the electron transport chain (ETC), as well as mitochondrial ribosomal proteins, proteases, and other mitochondrial proteins.[6]

Much of the work aimed at understanding the relationship between mitochondrial inhibition and lifespan extension has focused on the quality control mechanisms that regulate mitochondrial homeostasis and respond to damaged proteins and complexes. During aging there is a decreased ability of cells to cope with misfolded, damaged, and aggregated proteins.[7,8] This deterioration of protein quality control mechanisms contributes to negative effects on cellular function. Compartment specific responses have evolved to increase expression of proteins the promote proteostasis, including chaperones and proteases, when misfolded proteins are detected. Such compartment specific responses include the cytoplasmic heat shock response, the endoplasmic reticulum unfolded protein response (UPR^{ER}) and the mitochondrial unfolded protein response (UPR^{mt}). Each of these has been proposed to promote increased lifespan in *C. elegans* by preserving protein homeostasis with age.

The UPR^{mt} was first characterized in mammalian cells lacking mtDNA, which results in an imbalance of the nuclear and mitochondrial encoded subunits of the ETC.[9] In this context, it was shown that several nuclear encoded mitochondrial chaperones are coordinately induced, akin to the well-characterized induction of ER chaperones in response to ER stress. In addition to depletion of mitochondrial DNA, targeting an unstable transgenic protein to the mitochondria was also sufficient to activate the UPR^{mt} in mammalian cells.[10]

This pathway has since been extensively studied in *C. elegans*, primarily using GFP reporters for *hsp-6* (mitochondrial Hsp70) and *hsp-60*, which have allowed for the identification of a handful of factors required for their full induction in

response to mitochondrial stress.[11] These include the ubiquitin-like protein UBL-5,[12] the homeobox transcription factor DVE-1, the mitochondrial protease ClpP,[13] the ATP-dependent peptide transporter HAF-1,[14] and the basic-leucine zipper transcription factor ATFS-1.[15]

ATFS-1 is the best-characterized regulator of the UPR^{mt} in *C. elegans*, and has reproducibly been shown to be required for UPR^{mt} induction resulting from a variety of stressors including paraquat, ethidium bromide, and knockdown of and mutations in ETC components.[15,16] ATFS-1 contains both nuclear and mitochondrial localization sequences allowing it to control mitochondrial homeostasis by shuttling to either subcellular compartment. When imported into the mitochondria, it is degraded by the Lon protease in the mitochondrial matrix.[15] Under conditions of mitochondrial stress, ATFS-1 is excluded from the mitochondria and imported into the nucleus where it upregulates expression of mitochondrial stress response factors such as chaperones and proteases. Both *hsp-6* and *hsp-60* are among genes regulated in an ATFS-1-dependent manner.

Dissociating the Mitochondrial UPR from Longevity

The Dillin lab first introduced the hypothesis that the UPR^{mt} promotes longevity during mitochondrial dysfunction with the report that inhibition of ETC function specifically in neurons was sufficient to induce the UPR^{mt} in non-neuronal cells, including intestine, as well as extend lifespan.[17] Cell non-autonomous induction of the UPR^{mt} in this study was shown only using the *hsp-6$_p$::gfp* reporter (**Fig. 1**). Thus, it remains unclear whether this represents a general induction of the UPR^{mt}, induction of only a subset of UPR^{mt} targets, or induction of only the reporter construct. In the same study, the authors concluded that the UPR^{mt} promoted lifespan extension based on their observation that lifespan extension from mutation of *isp-1* required factors previously reported to be involved in UPR^{mt} signaling.[17]

Based on this model, we set out to identify novel longevity factors by performing an RNAi screen for genes that negatively regulate the UPR^{mt}. Specifically, we looked for RNAi clones that induced both the *hsp-6$_p$::gfp* reporter and the *hsp-60$_p$::gfp* reporter. Although conceptually similar screens had been performed previously, we identified dozens of novel genetic modifiers of UPR^{mt} signaling using this approach.[18] We anticipated that a majority of the RNAi clones found to induce the *hsp-6$_p$::gfp* and *hsp-60$_p$::gfp* reporters should also extend lifespan. In contrast to this prediction, about half of the clones tested had no effect or significantly shortened lifespan. This led us to speculate that the UPR^{mt} may not be a direct regulator of longevity.

To further explore the role of the UPR^{mt} in aging, we utilized gain and loss of function alleles of *atfs-1*. As previously reported for RNAi knockdown of the nuclear-encoded mitochondrial metalloprotease *spg-7*,[15] we observed that loss of function in *atfs-1* suppressed induction of the UPR^{mt} following knock-down or mutation of *cco-1* or *isp-1*.[18] This was determined both by reduced expression of GFP reporter for *hsp-6*, as well as endogenous expression of several UPR^{mt} target genes. Despite blocking induction of the UPR^{mt} by these measures, we still observed increased lifespan in every case tested. We also found that 2 gain of function alleles of *atfs-1* failed to extend lifespan relative to wild type N2 animals, despite the fact that these alleles did cause increased expression of UPR^{mt} target genes. Based on these observations, we concluded that the UPR^{mt}, as currently defined by the field, is neither necessary nor sufficient for lifespan extension in *C. elegans*.

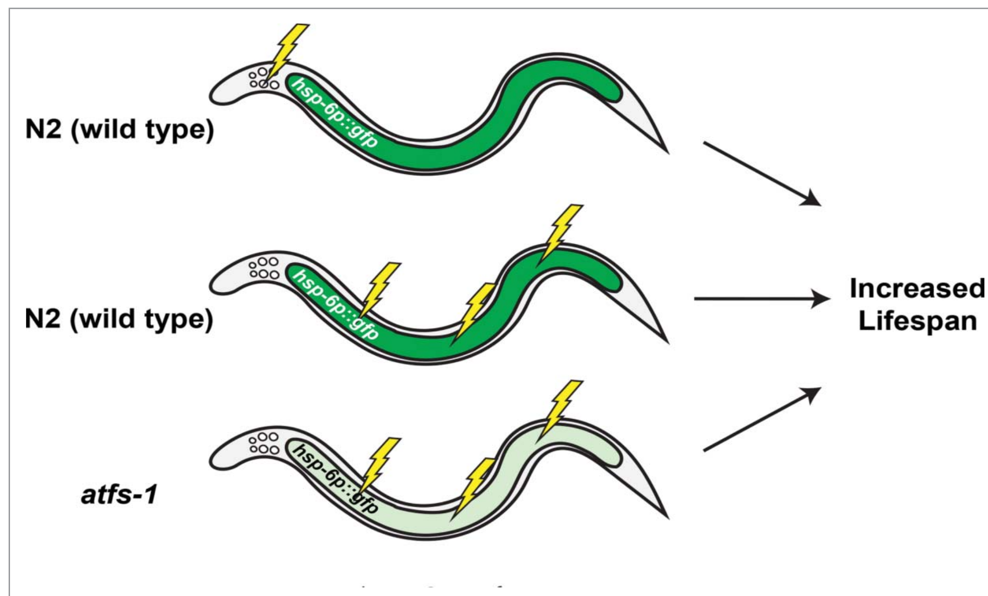
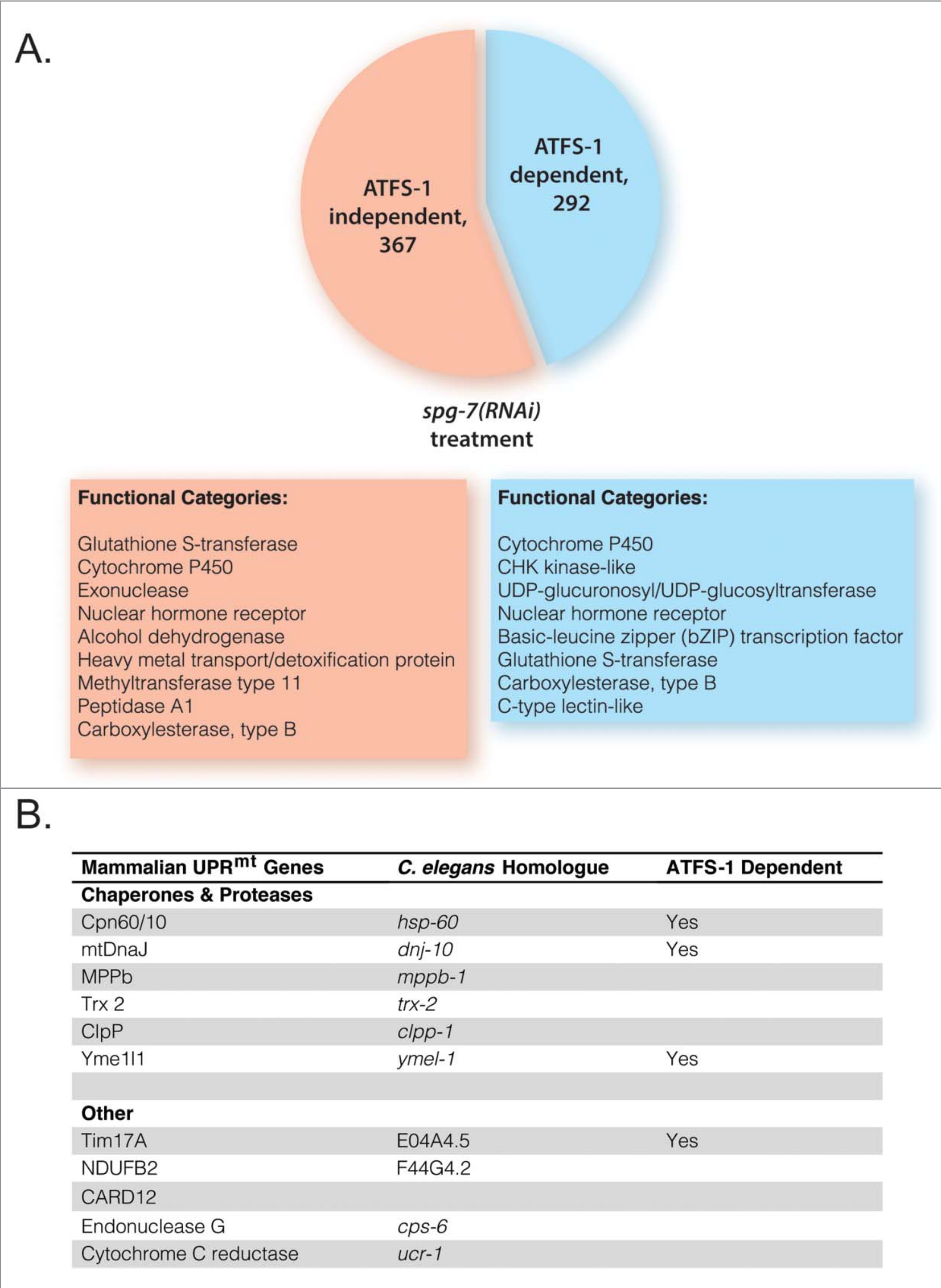


Figure 1. Mitochondrial dysfunction induces the UPR^{mt} in a cell autonomous and non-autonomous fashion, but does not require the UPR^{mt} for longevity. The *C. elegans* UPR^{mt} can be monitored by the transcriptional upregulation of mitochondrial chaperone genes including *hsp-6* and *hsp-60*. Electron transport chain inhibition by RNAi knockdown of *cco-1* (indicated by lightning bolts) in neurons or the intestine is sufficient for lifespan extension in worms and causes obvious induction of the *hsp-6$_p$::gfp* reporter in intestinal cells (Panels 1 and 2). Loss of function in the UPR^{mt} transcription factor *atfs-1* prevents induction of *hsp-6$_p$::gfp* following *cco-1(RNAi)* but does not prevent lifespan extension (Panel 3).

The UPR^{mt} and Aging: Where did Things Go Wrong?

Disproving hypotheses is an important and necessary part of the scientific process. In this case,



Mammalian UPR^{mt} Genes	*C. elegans* Homologue	ATFS-1 Dependent
Chaperones & Proteases		
Cpn60/10	*hsp-60*	Yes
mtDnaJ	*dnj-10*	Yes
MPPb	*mppb-1*	
Trx 2	*trx-2*	
ClpP	*clpp-1*	
Yme1l1	*ymel-1*	Yes
Other		
Tim17A	E04A4.5	Yes
NDUFB2	F44G4.2	
CARD12		
Endonuclease G	*cps-6*	
Cytochrome C reductase	*ucr-1*	

Figure 2. ATFS-1 regulates expression of several functional categories of genes, besides mitochondrial chaperones and proteases. (**A**) ATFS-1 regulates expression of at least 292 genes during mitochondrial stress. These include a multitude of cytochrome P450 enzymes, kinases, and nuclear hormone transcription factors, among others. Based on data from Nargund et al.[15] (**B**) The UPR^{mt} appears to be highly conserved between mammals and worms with respect to target genes that function as mitochondrial chaperones, proteases, and protein import machinery. If a UPR^{mt} gene is known to require ATFS-1 for induction, 'Yes' is marked in the third column. Mammalian UPR^{mt} genes from Aldridge et al.[42]

we did not set out to disprove the model proposed by Durieux et al.[17]; however, in the course of our experiments, it became apparent to us that important details were missing from the initial and subsequent publications in this area. Among those subsequent publications are 2 papers from the Auwerx lab that have propagated this model, arguing the *C. elegans* lifespan extension from knockdown of mitochondrial ribosomal proteins, rapamycin, resveratrol, antibiotics, overexpression of sirtuins, and NAD$^+$ precursors all require the UPR^{mt}.[19,20] As enticing as this idea may be, the data supporting these claims is not entirely consistent, and key controls are missing in some cases.[21]

One major limitation in this area is that the field has not carefully defined the UPR^{mt} in *C. elegans*, which has resulted in the near exclusive use of the *hsp-6$_p$::gfp* reporter to measure this response. Indeed, this was the only measure of UPR^{mt} activity reported in the papers proposing a mechanistic link between the UPR^{mt} and longevity in *C. elegans*.[17,19,20] Aside from the obvious limitations associated with using a single readout for a coordinated stress response involving dozens of genes, this is also a questionable choice given that the *hsp-6* ortholog, mtHsp70, is not generally considered a component of the UPR^{mt} in mammals.[10] The Haynes lab has recently identified numerous genes that are transcriptionally altered in *C. elegans* in response to mitochondrial stress induced by RNAi knockdown of *spg-7*.[15] Induction of the canonical UPR^{mt} genes was dependent upon ATFS-1; however, there were also numerous additional ATFS-1-dependent transcriptional changes as well as genes whose expression was modulated in an ATFS-1-independent manner (**Fig. 2**). It would be quite valuable going forward if some consensus could be reached as to which subset of genes regulated in response to mitochondrial stress make up the UPR^{mt}.

A second limitation of prior studies of the UPR^{mt} in *C. elegans* is the use of genetic modifiers of UPR^{mt} signaling whose function and mechanisms of action are poorly characterized. For example, loss of function alleles or RNAi knockdown of

ubl-5 and *haf-1* have been 2 methods used to argue for UPR^{mt}-mediated effects on longevity.[17,19,20] UBL-5 is an ubiquitin-like protein that interacts with the homeobox protein DVE-1 to promote expression of *hsp-6* and *hsp-60* in response to mitochondrial stress. The yeast ortholog of UBL-5, Hub1, is an essential gene involved in cell cycle regulation and pre-mRNA splicing,[22] and numerous phenotypes have been associated with *ubl-5 (RNAi)* in *C. elegans*, most of which are not obviously linked to its role as a regulator of the UPR^{mt}. Thus it seems likely that UBL-5 has functions in addition to its role as a regulator of *hsp-6* and *hsp-60* expression, which may complicate epistasis studies aimed at exploring its role in the UPR^{mt} and aging. HAF-1 encodes an ATP-Binding Cassette (ABC) transporter in the mitochondrial inner membrane that transports peptide fragments from the mitochondrial matrix.[14] There is some evidence that HAF-1 negatively regulates mitochondrial protein import, including that of ATFS-1, by an unknown mechanism; however, the role of HAF-1 in the UPR^{mt} may be limited to low levels of mitochondrial stress, and HAF-1 has been shown to be dispensable for induction of UPR^{mt} target genes in several cases.[15,16]

With respect to the role of the UPR^{mt} in aging, it is noteworthy that neither HAF-1 nor UBL-5 has been shown to be required for induction of multiple UPR^{mt} target genes under the conditions where lifespan is extended. In one case, Houtkooper et al.[19] did examine expression of the *hsp-6*$_p$*::gfp* reporter in response to *mrps-5 (RNAi)* and *cco-1(RNAi)*, both of which extend lifespan, and found that induction is only partially reduced by either *haf-1* or *ubl-5* knockdown or mutation. Interpretation of these experiments is somewhat further complicated by the use of double RNAi knockdown in some cases without the corresponding control of quantifying the level of knockdown for each targeted gene. Double RNAi knockdown is generally less efficient than the use of single RNAi clones,[23] and properly controlling for this may be especially important in the case of electron transport inhibition, where there is a clear dose response with respect to efficacy of RNAi knockdown and longevity.[24] Unfortunately, there is also currently very little information regarding the effects of *haf-1* and *ubl-5* knockdown or mutation on endogenous expression of UPR^{mt} target genes. Thus, we feel strongly that the current data suggesting that UBL-5 or HAF-1 are sometimes required for lifespan extension cannot be rigorously interpreted due to the absence of necessary controls and lack of understanding regarding how and whether these factors do, indeed, generally regulate the UPR^{mt}.

In contrast to other factors reported to be involved in the UPR^{mt} in *C. elegans*, the function and mode of regulation for ATFS-1 is relatively well characterized. ATFS-1 is critical for induction of the UPR^{mt}, as measured both by GFP reporters and endogenous expression of UPR^{mt} target genes, across many different mitochondrial stressors including paraquat, ethidium bromide, and RNAi depletion of the mitochondrial AAA metalloprotease *spg-7* and subunits of the TIM complex.[14-16] We found that partial deletion and knockdown of *atfs-1* greatly attenuated the UPR^{mt} without causing any overt phenotypes including effects on lifespan. The *atfs-1(tm4525)* allele prevented induction of the *hsp-6*$_p$*::gfp* reporter as well as increased expression of *hsp-6*, *hsp-60*, and *timm-23* following RNAi knockdown of *cco-1* or mutation of *isp-1* without influencing the lifespan extension associated with these interventions.[18] We note that this allele causes a truncated *atfs-1* mRNA to be expressed,[15] so it is possible that residual ATFS-1 activity could be present. Nevertheless we observed a complete block in UPR^{mt} induction, based on expression of 3 different endogenous UPR^{mt} target genes. Taken in combination with the failure of gain of function alleles in ATFS-1 to extend lifespan, as observed both by us and the Pilon lab,[18,25] these data greatly weaken the model that the UPR^{mt} plays a direct role in promoting longevity in *C. elegans*.

Mitochondrial Stress and Aging: Multiple Paths to Longevity?

Although the UPR^{mt} has gained a level of prominence as a potential mediator of longevity due to the high profile papers mentioned above, a host of additional cellular factors have also been implicated in controlling longevity in response to mitochondrial dysfunction. These include GCN-2, CEP-1, HIF-1, CEH-23, TAF-4, AHA-1, CEH-18, JUN-1, NHR-27 and NHR-49.[26-30] To date, however, no universal mechanism has been demonstrated that can account for all, or even most, of the cases where mitochondrial dysfunction is associated with longevity in *C. elegans*. This raises the possibility that there are multiple pathways to longevity mediated by mitochondrial stress (**Fig. 3**), which is supported by studies from the Hekimi lab suggesting that RNAi knockdown of the genes encoding the ETC components *isp-1* or *nuo-6* extends lifespan by a mechanism that is distinct from mutations in those same genes.[31] Along these lines, there is growing evidence for at least 2 distinct general mechanisms by which mitochondrial dysfunction can extend lifespan in *C. elegans*: reactive oxygen species (ROS)-mediated signaling and metabolic adaptation.

One way in which ROS-mediated signaling can extend lifespan is through stabilization of the hypoxia inducible transcription factor HIF-1.[27,32] HIF-1 is typically degraded under normoxic conditions by the proteasome. When oxygen levels are very low, however, HIF-1 becomes stabilized and induces transcription of a suite of genes involved in promoting survival under hypoxia.[33] Interestingly, HIF-1 can also become stabilized under conditions where mitochondrial respiration is inhibited, and HIF-1 is required for lifespan extension from mutations in *isp-1* or *clk-1*.[27] Several studies have shown that stabilization of HIF-1 is sufficient to extend lifespan in animals with normally functioning mitochondria,[34-36] providing additional compelling evidence in support of this model. HIF-1 is only partially required for lifespan extension from RNAi knockdown of electron transport chain components,[27] however, indicating that additional mechanisms must also exist.

Recently, the intrinsic apoptotic pathway has been implicated in ROS-mediated lifespan extension.[37] Lifespan extending mutations in *isp-1* or *nuo-6*, as well as treatment with paraquat, can activate a protective program mediated by CED-3/Casp9, CED-4/Apaf1, and CED-9/Bcl2.

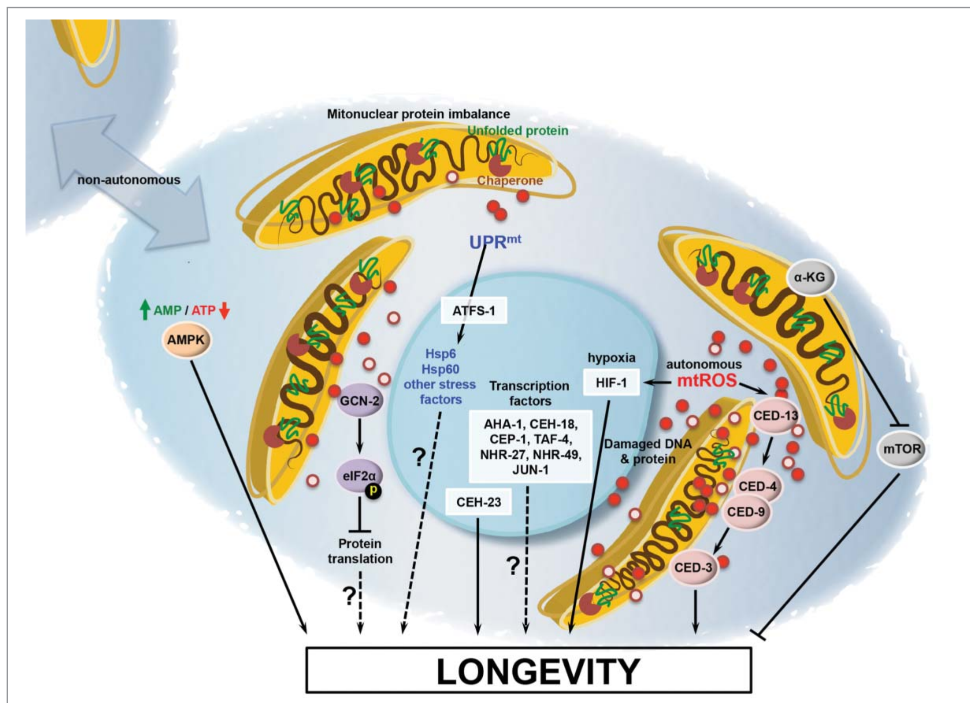


Figure 3. Current models for mitochondrial longevity. Inhibition of ETC function can lead to increased level of ROS (red circles), which can induce lifespan extension. One effect of this ROS production is stabilization of the hypoxia-inducible transcription factor HIF-1 and activation of the intrinsic apoptotic pathway. Protein misfolding in the mitochondria causes induction of the UPR^{mt} and inhibition of translation by the kinase GCN-2. This concerted mechanisms promotes protein homeostasis in the mitochondria. Other factors have also been implicated in lifespan extension following inhibition of mitochondrial function including AMPK, the homebox protein CEH-23, the p53 homolog CEP-1, and other transcription and signaling factors.

Despite the requirement of apoptotic genes, this response does not induce apoptosis. Furthermore, the BH3-only protein CED-13 also functions in this pathway, conceivably upstream of mitochondrial ROS since it is required for full lifespan extension from mutations in *nuo-6* and *isp-1*, but not paraquat. In addition to longevity, the phenotypes of decreased pumping rate, thrashing, and developmental rate from mitochondrial ETC mutations were partially dependent on CED-4 and CED-13. Thus, the intrinsic apoptotic pathway can function to promote organismal health and cellular survival in the presence of mitochondrial dysfunction. The importance of this type of response to mitochondrial dysfunction is plausible, since *C. elegans* is a largely post-mitotic adult organism with a small number of cells that cannot be lost without severe consequence. Therefore, it will be interesting to determine if a similar mechanism exists in higher eukaryotes, perhaps in cell types that have limited regenerative capacity.

Significant metabolic adaptations to mitochondrial stress have also been recently described and there is growing evidence that they are likely to be important for longevity. Metabolomic profiling of mitochondrial mutants revealed that specific α-ketoacids and α-hydroxyacids are produced a high levels by long-lived mutants, due to inhibition of α-ketoacid dehydrogenases.[38] Such metabolites could act as signaling factors or inhibit/activate specific enzymes important for longevity. Along these lines, certain α-ketoacids are known to inhibit the prolyl hydroxylase EGL-9, a negative regulator of HIF-1 stability.[39] It was also recently reported that the α-ketoacid α-ketoglutarate is sufficient to extend lifespan in *C. elegans* by binding to and inhibiting the mitochondrial ATP synthase.[40] This was associated with inhibition of the mechanistic target of rapamycin (mTOR) pathway and induction of autophagy,[40] which has previously been shown to extend lifespan in *C. elegans* as well as several other species.[41] The importance of α-ketoglutarate and the mTOR pathway, if any, in lifespan extension from mitochondrial dysfunction remains to be determined.

Conclusion

The basis for *C. elegans* lifespan extension from mitochondrial stress is still not fully understood and appears to be mediated by multiple downstream processes. Whether these processes act by truly distinct mechanisms or as part of a coordinated metabolic and genetic network, and whether the UPR^{mt} plays any role in such a longevity network, will require further study. It will be important for the field to arrive at some consensus as to what defines the UPR^{mt}. We suggest that endogenous expression of multiple UPR^{mt} target genes, perhaps along with in the currently widely utilized GFP reporters, should minimally be required in future studies of the UPR^{mt} in *C. elegans* and that similar approaches should be used when studying this pathway in other model organisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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