The UPR^{mt} Preserves Mitochondrial Import to Extend Lifespan

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Author Contribution

N.X. and A.D. conceived the study. J.D. and H-E.K. planed and performed the lifespan analysis. S.W. helped develop the protocol for the import assay. N.X. carried out the rest of the experiments with the help of C.Y., and analyzed the data with input from A.D., H-E.K. and S.W. N.X. took the lead in writing the manuscript. All authors discussed the results and contributed to the final manuscript. A.D. was in charge of the overall direction. H-E.K. supervised the project performed at the University of Texas, Health Sciences Center, Houston, TX.

1 Summary

2 The mitochondrial unfolded protein response (UPR^{mt}) is dedicated to promote mitochondrial proteostasis and is linked to extreme longevity in worms, flies, and mice. The key regulator of this 3 4 process is the transcription factor, ATFS-1. In the absence of mitochondrial stress, ATFS-1 is transported to the mitochondria and degraded. During conditions of mitochondrial stress, ATFS-1 5 is excluded from the mitochondria and enters the nucleus to regulate the expression of UPR^{mt} genes. 6 7 However, there exists a dichotomy in regards to induction of the UPR^{mt} and mitochondrial import. The repair proteins synthesized as a direct result of UPR^{mt} activation must be transported into 8 damaged mitochondria that had previously excluded ATFS-1 due to reduced import efficiency. To 9 10 address this conundrum, we analyzed the role of the import machinery under conditions where the 11 UPR^{mt} was induced. Using *in vitro* biochemical assays of mitochondrial import and *in vivo* analysis 12 of mitochondrial proteins, we surprisingly find that the efficiency of mitochondrial import 13 increases when the UPR^{mt} is activated in an ATFS-1 dependent manner, even though membrane 14 potential is reduced. The import machinery is upregulated at the transcription and translation level, 15 and intact import machinery is essential for UPR^{mt}-mediated increase and lifespan extension. With age, import capacity decreases, and activation of the UPR^{mt} delays this decline and increases 16 longevity. Finally, we find that ATFS-1 has a significantly weaker mitochondrial targeting 17 18 sequence (MTS), allowing for dynamic subcellular localization during the initial stages of UPR^{mt} activation. 19

20 Introduction

21 During the course of eukaryotic evolution and the development of sequestered organelles, communication events co-evolved to allow proper homeostasis within the cell that encompassed 22 23 master coordination by the nucleus. Such events have been discovered to include the unfolded protein response of the endoplasmic reticulum (UPR^{ER}), the mitochondria (UPR^{mt}), and the 24 cytoplasmic heat shock response (HSR)^{1–3}. The primary mode of action of each of these stress 25 26 pathways is the sensation of organelle-specific stress that is then communicated to the nucleus for 27 transcriptional induction of the proper repair machinery to restore homeostasis within each organelle. Genes encoding compartment-specific chaperones are the sentinel transcriptional 28 29 targets of such responses.

Mitochondria pose the most challenging organelle to coordinate stress responsiveness with the 30 nucleus for several reasons. One, the mitochondrion is encapsulated within a double membrane 31 32 system, the inner and outer membrane, thus creating a unique physical barrier that must be 33 accommodated to signal from the mitochondrion to the nucleus. Two, the mitochondrion is 34 composed of more than 1,000 different proteins, encoded within two distinct genomes, that comprise some of the largest complexes found in eukaryotic cells ^{4,5}. Three, the by-products of 35 oxidative respiration, superoxide radicals, pose a constant challenge to mitochondrial integrity 6 . 36 37 Four, import of proteins into the mitochondrion requires energy in the form of membrane potential and ATP created by the electron transport chain ⁴, and each cell can contain hundreds of 38 39 mitochondria that fuse and divide at any given time to change the cellular mitochondrial landscape. Taken together, monitoring the integrity of the mitochondrion and all of its various protein 40

41 complexes with the ability to communicate to the nucleus to ensure the integrity of this organelle42 is extremely complex.

Mitochondria can orchestrate and coordinate a wide number of different stress-response 43 44 mechanisms under various cellular and subcellular perturbations. Such responses include the UPR^{mt}, mitophagy, and programmed cell death. In response to mild mitochondrial stress, the 45 UPR^{mt}, a specific transcriptional stress response system that is mediated by ATFS-1, DVE-1, UBL-46 47 5, LIN-65, MET-2, and PHF-8, is activated to increase the production of mitochondrial localized chaperones and proteases to help relieve the stress ^{7–11}. One of the major contributors to this 48 response is ATFS-1. During mitochondrial stress, mitochondrial import efficiency is compromised, 49 50 presumably due to depolarization of the mitochondrial membrane potential, which results in the 51 inefficient import of the mitochondrial localized protein, ATFS-1. When ATFS-1 is not 52 successfully imported into the mitochondria for degradation by mitochondrial proteases, it instead 53 traffics to the nucleus, where it functions as a transcription factor, which coordinates with DVE-1, 54 UBL-5 MET-2 and LIN-65 to induce the expression of mitochondrial chaperones and other genes 55 required for repair of damaged mitochondria ^{7–11}. Inherent within this model is the balance that must be maintained between the membrane potential, import machinery, and the ability to induce 56 the UPR^{mt}. However, the link between membrane potential, mitochondrial import, and the UPR^{mt} 57 58 is largely unexplored.

59 With the current model of ATFS-1 localization dynamics during stress exists a dichotomy in 60 regards to induction of the UPR^{mt} and mitochondrial import. The repair proteins synthesized as a 61 direct result of UPR^{mt} activation by ATFS-1 must be transported into damaged mitochondria that 62 must have a depolarized membrane potential preventing the import of ATFS-1 into the

63 mitochondria, providing entry of ATFS-1 into the nucleus. If ATFS-1 is unable to enter the mitochondria during stress, how then are other proteins allowed entry, especially mitochondria 64 with reduced membrane potential? Could there be coordination to increase mitochondrial import 65 efficiency once the UPR^{mt} is induced? Is the mitochondrial import machinery a distinct branch of 66 the UPR^{mt} to overcome the lack of integrity of damaged mitochondria? To address these questions, 67 we analyzed the role of the import machinery under conditions where the UPR^{mt} was induced. 68 Using in vitro biochemical assays of mitochondrial import and in vivo analysis of mitochondrially 69 localized proteins, we find the efficiency of mitochondrial import increases when the UPR^{mt} is 70 activated. More surprisingly, the increased import due to UPR^{mt} induction occurs when the 71 72 mitochondrial membrane potential is decreased. Finally, we find that the induction of the import machinery is essential for UPR^{mt}-mediated lifespan extension, and ectopic induction of the UPR^{mt} 73 preserves import into late life. 74

75 Results

76 Assessing mitochondrial import capacity from C. elegans mitochondria

All but a few mitochondrial proteins are transcribed from the nuclear genome and imported posttranslationally through the translocase of the outer/inner membrane (TOM/TIM) complex ^{4,12}. Mitochondrial protein import depends on the mitochondrial membrane potential ($\Delta\Psi$), ATP, and is under the direction of mitochondrial targeting sequences (MTS), which is cleaved after import ¹³. To measure the efficiency of mitochondrial import, we adapted and validated a method in which substrate proteins are synthesized in an *in vitro* transcription/translation reaction, and subsequently imported into isolated mitochondria ^{14,15} (Fig. 1a). We used a model import substrate, su9-DHFR,

84 in which the MTS of subunit 9 of the mitochondrial ATP synthase from Neurospora is fused to a fragment of the cytosolic protein dihydrofolate reductase (DHFR) from mice ¹⁶. An ATP 85 regeneration system¹⁷ was applied to increase the efficiency of import. Importantly, a DHFR 86 87 antibody can readily detect the fusion protein being imported (Fig. 1b, c, Extended Data Fig. 1a), as indicated by: 1) the change in size from a precursor protein to a mature DHFR; 2) the absence 88 of mature DHFR upon disruption of membrane potential ($\Delta \Psi$); 3) the absence of precursor protein 89 90 upon proteinase K treatment; and 4) the accumulation of a mature DHFR in a time-dependent 91 manner. Mitochondrial import efficiency of su9-DHFR was reduced by 30-40% from 92 mitochondrial preparations isolated from animals upon knocking down essential components of 93 the TOM/TIM complex, tomm-20 or timm-17, via RNAi, further validating the sensitivity and 94 fidelity of the assay (Fig. 1d, e, Extended Data Fig. 1b).

95 A critical difference in import capacity among germ and somatic cells in *C. elegans*.

To determine whether induction of the UPR^{mt} had any impact upon mitochondrial import 96 97 efficiency, we first induced the UPR^{mt} in wild-type N2 worms with RNAi against cytochrome c oxidase-1 subunit (cco-1/cox-5B), a component of the electron transport chain complex IV, and 98 found that import efficiency was reduced in mitochondrial preparations isolated from whole 99 100 animals (Extended Data Fig. 1c-e). This finding appears to be consistent with the current model of 101 UPR^{mt} induction and ATFS-1 exclusion. However, we must note that worms treated with *cco-1* 102 RNAi have reduced fecundity, notably due to a significantly underdeveloped germline ¹⁸. The 103 soma of C. elegans is post-mitotic, containing only 959 cells; however, the germline is expansive 104 and mitotic, containing both syncytial and cellularized cells. In fact, the development of the female germline accounts for the vast majority of mtDNA amplification in C. elegans¹⁹. As the germline 105

develops, rapid mitochondrial expansion is essential, and this could be a major component of the
mitochondrial import activity found when entire animals are used to isolate the mitochondria;
hence the reduced import efficiency of UPR^{mt} in animals treated with *cco-1* RNAi could be
explained by the reduced germline found in these animals.

110 We, therefore, tested whether germline cells differ in import competency relative to post-mitotic cells. To this end, we used three temperature-sensitive sterile strains that differentially affect the 111 112 development of the germline at the restrictive temperature. In particular, CB4037 glp-1(e2141ts) ²⁰ and SS104 glp-4(bn2ts) ²¹ are sterile strains that lack the majority of the germline, whereas 113 CF512 fer-15(b26ts); fem-1(hc17)²² is sterile due to the conversion of sperm into oocytes. Of note, 114 115 the sterile strain CF512 was used as the control to ensure that any difference we observed was not 116 due to the contribution of import activity from the offspring. Comparison of the import of 117 substrates into mitochondria isolated from the three sterile strains, we found that the germline-118 deficient mutants (glp-1 and glp-4) were strikingly less import-competent than the sperm-deficient, 119 oocyte proficient, CF512 strain, at the restrictive temperature (Extended Data Fig. 1f-h). In 120 particular, the *glp-1* strain CB4037 is 80% lower than strain CF512 in import. In contrast, we observed no significant difference between these strains when raised at the permissive temperature, 121 15°C (Extended Data Fig. 1i-k), which allows normal germline development. Additionally, 122 123 temporal shifting of *glp-1(e1241ts*) mutant animals from 15°C to 25°C during development allows limited germline development, with earlier shifts resulting in fewer germ cells, and later shifts 124 having a near-complete complement of germ cells. By shifting at a series of time points, we found 125 126 that import capacity was positively correlated with the number of germline cells present in the animals (Extended Data Fig. 11-n). In contrast, the CF512 strain, which has a normal female 127

germline at both permissive and restrictive temperatures, shows higher import competency at 25°C
(Extended Data Fig. 10-q). These results suggest that germline mitochondria are highly importcompetent in comparison to post-mitotic, somatic cells in *C. elegans*.

131 Mitochondrial import is enhanced upon UPR^{mt} induction

To understand if and how mitochondrial import is regulated upon mitochondrial stress in somatic 132 cells, we induced UPR^{mt} in *C. elegans* and examined the import capacity of isolated mitochondria 133 134 from animals lacking a germline. Considering the remarkable discrepancy in import capacity we 135 found between somatic tissue and the germline, we genetically ablated the germline using glp-1(e2141ts) mutant animals ¹⁸, to only assay mitochondria from somatic tissues where the UPR^{mt} 136 137 impacts longevity and health 23,24 . We found that mitochondria isolated from glp-1(e2141ts) 138 mutant animals treated with cco-1 RNAi, import capacity was elevated 2 to 2.5 times that of age-139 matched, mock RNAi control treated animals (Fig. 2a, b, Extended Data Fig. 2a).

140 Struck by the robust increase in mitochondrial import efficiency conferred by *cco-1* RNAi in 141 animals composed only of somatic cells, we next asked whether the increased import efficiency 142 was a common response to mitochondrial stress. SPG-7 is an AAA protease involved in quality 143 control of mitochondrial membrane proteins, as well as the assembly of protein complexes on the mitochondrial inner membrane²⁵. MRPS-5 is a mitochondrial ribosome protein²³. Knockdown of 144 either spg-7 or mrps-5, via RNAi, induces the UPR^{mt} and leads to lifespan extension of worms 145 composed of post-mitotic, somatic cells ^{23,26}. We found that mitochondrial import was also 146 147 significantly enhanced upon either spg-7 RNAi (Fig. 2c, d, Extended Data Fig. 2b) or mrps-5 RNAi (Extended Data Fig. 2c-e). Taken together, both results suggest that the activation of the UPR^{mt} is 148 149 widely associated with enhanced mitochondrial import.

150 To test whether the enhancement of import depends on the activation of UPR^{mt}, we introduced *dve-1* RNAi into animals together with *cco-1* RNAi. DVE-1 is a transcription factor that mediates 151 the activation of stress responsive genes upon UPR^{mt 10}. dve-1 RNAi partially suppressed the 152 153 induction of *hsp-6p::gfp* reporter ²⁶, indicating that knockdown of *dve-1* partially blocks the downstream effect of UPR^{mt} induced by cco-1 RNAi (Extended Data Fig. 2f). We found that 154 155 knockdown of *dve-1* resulted in marked suppression of import capacity that had been enhanced by *cco-1* knockdown (Fig. 2a, b), suggesting that the induction of the UPR^{mt} is essential for increased 156 157 import efficiency. Importantly, consistent with earlier findings ^{7,27}, ATFS-1 is required for the induction of UPR^{mt}, as indicated by the *hsp-6p::gfp* reporter (Extended Data Fig. 2g). When *atfs-*158 159 I RNAi was introduced into animals together with spg-7 RNAi, the enhancement of import was also suppressed (Fig. 2c, d). Taken together, these results indicate that the UPR^{mt} indeed mediates 160 161 the upregulation of mitochondrial import.

162 Mitochondrial import machinery is upregulated upon UPR^{mt} induction.

The UPR^{mt} promotes mitochondrial protein homeostasis through signaling to the nucleus to induce 163 164 the transcription of mitochondrial localized stress-responsive genes. Most of the protein products 165 of the upregulated genes must then be imported into mitochondria to restore proteostasis. The proteins that constitute the import machinery. TIM/TOM complexes, are exclusively encoded by 166 nuclear genes. Therefore, it is conceivable that the UPR^{mt} may enhance import through 167 168 upregulating the expression of the TIM/TOM complex components. Indeed, the import machinery components *timm-17* and *timm-23* were upregulated upon UPR^{mt} induction by *spg-7* RNAi⁷. 169 170 Similarly, *timm-23* was also found to be moderately upregulated upon *cco-1* RNAi treatment⁸. However, when we examined the RNA-sequencing data of animals treated with cco-1 RNAi, we 171

found no significant change in the transcription of most other import machinery components ^{8,9}. 172 As N2 worms were used in this RNA-seq analysis, the loss of germline caused by cco-1 173 174 knockdown during development may counteract any effect in somatic mitochondria caused by 175 cco-1 RNAi. To examine the transcriptional regulation of import machinery in somatic tissue, we tested germline-deficient *glp-1(e2141ts*) mutant animals for the transcription of a series of genes 176 177 encoding the mitochondrial import machinery. Comparing the synchronized and age-matched worms, we found that transcription of the TIM/TOM genes upon cco-1 RNAi treatment was 178 179 consistently higher than the mock RNAi control. Genes encoding TOM complex proteins, 180 including tomm-20, tomm-22, and tomm-40, were enhanced one to two-fold. Core components of 181 the TIM complex, timm-17 and timm-23, were upregulated 3 and 7-fold, respectively (Fig. 2e). 182 The transcription level of *timm-17* and *timm-23* at steady state appeared to be lower than other 183 TIM/TOM component, whereas, their transcription was elevated to levels higher than other 184 components upon UPR^{mt} activation. This is consistent with previous findings, which suggests that 185 TIM23 protein might be the rate-limiting factor in mitochondrial import ²⁸. Following the passage 186 through the inner membrane pore formed by TIM17 and TIM23, the precursor proteins are pulled 187 into the matrix by TIM44 and mtHSP70 (HSP-6). mtHSP70 also facilitates the proper folding of 188 imported proteins, which are subsequently processed by MPP proteins, proteases that cleave off the mitochondrial targeting sequences (MTS)²⁹. We found that *hsp-6*, *tin-44*, as well as *mppa-1* 189 190 and mppb-1, which are C. elegans homologs of mammalian mtHsp70, Tim44, and Mpp, 191 respectively, were also upregulated by cco-1 RNAi. Therefore, the entire repertoire of the import 192 machinery appears to be transcriptionally induced by activation of the UPR^{mt} in somatic cells. 193 Similarly, the TIM/TOM import machinery was also upregulated upon spg-7 RNAi (Extended Data Fig. 2h). Additionally, the *cco-1* induced upregulation of import machinery genes was
suppressed by RNAi against *dve-1* (Fig. 2e).

To further verify the regulation of import machinery, we generated an antibody against the *C*. *elegans* mitochondrial translocase protein TIMM-23 and verified its specificity with RNAi knockdown of *timm-23*. When comparing the endogenous level of TIMM-23, we found that the TIMM-23 protein level is indeed enhanced when the UPR^{mt} is activated (Fig. 2f, g).

200 Given that mitochondrial protein import requires membrane potential ($\Delta \Psi$), we examined if $\Delta \Psi$ is enhanced upon UPR^{mt} activation. Surprisingly, in early Day 1 adult, the same stage when enhanced 201 202 mitochondrial import was detected, we did not observe stronger TMRE staining, a marker of $\Delta \Psi$, 203 in worms with activated UPR^{mt} (Fig. 2h, i). On the contrary, membrane potential was found to be reversely correlated with the activation of the UPR^{mt}. In particular, knockdown of *cco-1* induced 204 the UPR^{mt} more robustly than knockdown of *mrps-5*, as indicated by the level of *hsp-6p::gfp* 205 206 reporter, whereas membrane potential is lower with *cco-1* knockdown (Fig. 2h, i). This is 207 consistent with the model that mitochondrial stress leads to membrane depolarization. Together, 208 these findings suggest that the enhancement of mitochondrial import we observed in the in vitro 209 import assay is regulated through increased transcriptional regulation of import machinery, rather 210 than enhanced membrane potential. Furthermore, and most surprisingly, these results indicate that 211 aspects of mitochondrial import can be decoupled from membrane potential.

212 The mitochondrial targeting sequence of ATFS-1 is less import competent.

213 Presumably, the enhancement of import would allow efficient translocation of the repair proteins into the mitochondria to restore their proper function. However, previous findings suggest that 214 215 import is compromised upon mitochondrial stress, thereby allowing ATFS-1 to translocate into 216 the nucleus, where it activates the transcription of stress response genes². Is the import of ATFS-217 1 differentially regulated? To interrogate this possibility, we made a chimeric protein with the predicted mitochondrial targeting sequence of ATFS-1 (N terminal 73 amino acids)⁷ fused with 218 219 DHFR (ATFS1-DHFR). We found that upon the induction of the UPR^{mt}, the import of ATFS1-220 DHFR is also upregulated (Fig. 3a, b, Extended Data Fig. 3a). Though the regulation of ATFS-1 221 import shows the same trend, we observed that the import of ATFS1-DHFR is less robust as 222 compared to su9-DHFR (Fig. 3a). Indeed, when we compared the import of the two fusion proteins 223 side by side, we found that mitochondrial import directed by the MTS of ATFS-1 is significantly 224 less robust (Fig. 3a, c, Extended Data Fig. 3a). Our finding is consistent with the model that ATFS-225 1 has a weak MTS, which allows it to sense modest mitochondrial dysfunction and membrane depolarization to send a stress signal to the nucleus by translocation ³⁰. Point mutations in the MTS 226 227 of *atfs-1*, such as *et15* or *et18*, display constitutively active UPR^{mt 33}, presumably due to lack of 228 mitochondrial import of ATFS-1 and relocation to the nucleus. We introduced the *et15* and *et18* 229 mutations into the MTS of the ATFS1-DHFR construct and used them in the *in vitro* import assay. 230 We found that the mitochondrial targeting capacity deteriorated with both mutations in the MTS 231 of atfs-1 (Fig. 3d, Extended Data Fig. 3b).

232 The lifespan extension caused by UPR^{mt} induction requires intact TIM/TOM complexes.

Our findings suggest that the mitochondrial import machinery is upregulated at the transcriptional
 level upon UPR^{mt} induction, thereby elevating import capacity and allowing stress-responsive

proteins to translocate into the mitochondrial matrix and restore proteostasis. One such stressresponsive proteins is the mitochondrial chaperone mtHSP70(HSP-6), which is transcriptionally
upregulated upon the activation of UPR^{mt}. Analyzing the subcellular fractionation of mitochondria
from UPR^{mt} induced animals, we find increased levels of HSP-6 in the mitochondrial fraction (Fig.
4a, b, extended data Fig. 4a), suggesting that import *in vivo* is maintained at a level that allows
efficient mitochondrial translocation of the elevated level of stress-responsive proteins, despite the
reduction of membrane potential (Fig. 2h, i).

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243 The next question we asked was whether the improvement of import is a necessary element for 244 UPR^{mt}-induced longevity. To test this, we used double RNAi to knockdown import activity in 245 long-lived *cco-1*-deficient animals. We found that when worms were treated with *tomm-22* RNAi 246 simultaneously with *cco-1* RNAi, both the enhancement of import (Fig. 4e, f, Extended Data Fig. 247 4b) and the extension of lifespan was largely suppressed, whereas tomm-22 RNAi had a minimal effect on lifespan in wild-type animals (Fig. 4c, d). Similarly, treating worms with timm-17 RNAi 248 249 also partially suppressed the lifespan extension (Extended Data Fig. 4c). Taken together, these results indicate that intact import machinery is essential for UPR^{mt}-induced lifespan extension. 250

251 Induction of the UPR^{mt} maintains mitochondrial import during aging

Intrigued by the finding that enhanced import efficiency was required for UPR^{mt}-mediated longevity, we asked how import efficiency might play a role in normal aging. As organisms age mitochondria gradually depolarize and the membrane potential, the major driving force of import, declines ²². We tested import efficiency across mitochondria isolated from aging cohorts of *C*. *elegans*. Indeed, we confirmed that mitochondrial import declines dramatically as the animals age

257 (Extended Data Fig. 4d-f). Three age groups (Days 1, 5, and 9) were chosen to represent worms in the process of aging. We observed a more than one-half reduction in the import efficiency of su9-258 259 DHFR from Day 1 to Day 5, and no further decline from Day 5 to Day 9. This finding suggests a 260 catastrophic loss in import capacity early in the aging process. Similar age-associated decline in import was observed in both glp-1(e2141ts) worms, which lack a germline (Fig. 4g, h, Extended 261 262 Data Fig. g), and CF512 *fer-15(b26ts); fem-1(hc17)* worms, which have intact female germline (Extended Data Fig. 4d-f), suggesting that import in somatic tissue and germline are both affected 263 264 by aging.

Treating worms with RNAi against *cco-1* delayed the age-associated decline of import. For example, import remained significant on day 5 in *cco-1* RNAi treated worms, whereas import in mock-RNAi control worms was barely detectable (Fig. 4g, h, Extended Data Fig. 4g). The transcriptional level of TIM/TOM import machinery also remained higher in older worms under *cco-1* RNAi treatment (Extended Data Fig. 4h).

As lifespan extension is a common effect of UPR^{mt} activation, it is intriguing to know whether the 270 271 enhancement in mitochondrial import capacity we observed is a secondary effect of delayed aging 272 or more specific to UPR^{mt} induced forms of longevity. *glp-1(e2141ts)* mutant worms, in which 273 germline deficiency induces longevity, display highly compromised import capacity (Extended 274 Data Fig. 1f-n), thus arguing against a causative effect of delayed aging on increasing 275 mitochondrial import. We also tested another major pathway that regulates lifespan, the 276 insulin/IGF1 signaling pathway mediated by the insulin/IGF1 receptor, *daf-2* in worms. We found 277 that import capacity in day 1 adult worms was not affected by either daf-2 RNAi or the daf-2(e1370) mutation (Extended Data Fig. 2j-l and m-o), despite the dramatic increase in lifespan in 278

- these strains. In addition, upon knockdown of *daf-2*, we did not observe an increase in import
- 280 machinery, either at the transcription level (Extended Data Fig. 2i) or protein level (Fig. 2f, g).
- 281 Similarly, the mitochondrial chaperone HSP-6 is not enhanced with *daf-2* RNAi (Fig. 4a, b). Taken
- together, these findings indicate that the enhancement of import in somatic cells is unique to UPR^{mt}
- activation, and the aging process itself reduces import that can be combated by UPR^{mt} activation.

284 Discussion

Metazoans have evolved various defense mechanisms to protect themselves against the detrimental consequences of stress and aging. Many of the stress responsive mechanisms require altering the composition of their proteomes. This remodeling often includes enhancing the networks of stress responsive proteins and chaperones, which are targeted for specific subcellular compartments or organelles that are stressed.

290 It has been proposed that the alteration of mitochondrial import plays a role in the induction of 291 mitochondrial unfolded protein response ⁷. However, the regulation of mitochondrial import upon 292 stress has not been investigated in depth. In this work, we revealed the augmentation of 293 mitochondrial protein import as a downstream effect of the mitochondrial stress response. This upregulation of import is specifically associated with the induction of UPR^{mt}, instead of being a 294 295 generic secondary effect of delayed aging or prolonged lifespan. We did not observe upregulation 296 of the mitochondrial membrane potential that correlates with the import competency. In fact, we found that in spite of decreased membrane potential, the UPR^{mt} resulted in increased import 297 298 activity. Taken together, our findings indicate that the enhancement of import is mediated by 299 transcriptional regulation of the mitochondrial import machinery, and our *in vitro* biochemical assays reveal increased import competency of these mitochondria. Intriguingly, the efficiency of 300 301 mitochondrial import serves as an active mechanism of increased longevity upon the activation of 302 the UPR^{mt}.

303 It is of great importance to establish the UPR^{mt} activation paradigm in mammalian cells and thereby
304 analyze its impact on the mitochondrial import. Notably, loss of Tfam in T cells have reduced

mitochondrial respiration and lower ETC components, but the levels of TOM20 seem to be
elevated ³⁵. As lowered levels of ETC components would presumably induce UPR^{mt}, this
observation in mammalian cells is consistent with our finding that import machinery is upregulated
upon UPR^{mt} activation.

309 It was previously proposed that mitochondrial import deteriorates upon mitochondrial stress and 310 thereby excludes ATFS-1 from mitochondria, allowing it to enter the nucleus to induce the expression of downstream stress response genes ⁷. Surprisingly, we found that the UPR^{mt}-311 312 dependent upregulation of import is not only true for general import, but also the case for ATFS-313 1, the import-deficiency-dependent messenger of stress. Our findings raise the question of if and how the UPR^{mt} is maintained upon upregulation of the UPR^{mt}. It was recently revealed that 314 315 mitochondrial stress during larval development induces chromatin changes that are perpetuated 316 into adulthood and make up a critical part of the UPR^{mt 8,9}. Accordingly, UPR^{mt} induced by a 317 transient deficiency in import may be sufficient to self-sustain the downstream effects, including 318 the prolonged upregulation of import. In fact, it was found that the expression of ATFS-1 itself is upregulated upon induction of the UPR^{mt 7}, suggesting that once the UPR^{mt} is activated, nuclear 319 320 ATFS-1 might be kept at a higher level despite the recovery of mitochondrial import efficiency.

Notably, mitochondria exhibit a high level of heterogeneity within cells ³⁶. It is conceivable that among the large population of mitochondria within a cell, some might remain at a low-import status and constantly send stress signal to the nucleus, whereas the rescuing proteins, once made, are sent to relatively healthy sub-population of mitochondria, or are used in the genesis and assembly of a new cohort of mitochondria. Therefore, the higher level of import efficiency upon UPR^{mt} in the *in vitro* import assay may be due to a fraction of mitochondria being healthier and

- 327 more resilient due to UPR^{mt} activation and could be better preserved in the extraction process. In
- 328 the future, it will be imperative to monitor the mitochondrial import of individual mitochondria *in*
- 329 *vivo* during the aging process and under conditions of UPR^{mt} induction.

330 Figure Legends

331 Figure 1. *C. elegans* mitochondrial protein *in vitro* import assay

332 a, Schematic diagram of the C. elegans in vitro mitochondrial protein import assay. b and c, su9-333 DHFR was transcribed and translated in a single reaction with the Quick Coupled 334 Transcription/Translation System (TnT reaction). Mitochondria extraction was made from 335 synchronized N2 wild type worms at day 1 of adulthood and quantified with BCA analysis. 50ug 336 mitochondrial protein was used in each reaction. The substrate protein was incubated with 337 mitochondria extraction in import buffer containing an ATP regeneration system for 10, 20, or 30 minutes at 25°C. Mitochondria were subsequently treated with proteinase K to remove non-338 339 imported proteins. Upon being imported, the MTS of su9 is cleaved. 2ug/ml valinomycin was used 340 to disrupt the membrane potential ($\Delta\Psi$), thus inhibiting import. The precursor (p) and mature 341 protein(m) were detected with the DHFR antibody by western blot analysis. Right lane: 20% of 342 the su9-DHFR substrate used in the import assay representing the precursor (p). d and e, Germline-343 deficient, mutant glp-1(e2141ts) worms were bleach synchronized, grown at the restrictive 344 temperature, 25°C, and treated with RNAi against tomm-20 or timm-17 until the first day of 345 adulthood. Control worms were grown on bacteria containing empty vector alone. Mitochondria 346 were isolated and subjected to the import assay. c and e, The efficiency of mitochondrial import 347 was quantified by measuring the mature imported protein as detected by the DHFR antibody and 348 analyzed with unpaired student's t-test. All graphs are presented as mean \pm SD of two or more 349 biological repeats. *P<0.05. Arrowheads: mature (imported) DHFR with the MTS cleaved off.

Extended Data Figure 1. Germline-deficiency leads to the reduction of import competency; Somatic and germ cells differ in their import capacity.

352 a, A biological replicate of Fig. 1b. b, A biological replicate of Fig. 1d. c-e, N2 wild type worms 353 were synchronized and treated with RNAi against cco-1. Mitochondria were isolated at day 1 of 354 adulthood and subjected to the import assay (c and d are biological replicates). f-k, Temperature-355 sensitive germline-deficient glp-1(e2141ts) and glp-4(bn2ts) and spermatogenesis mutant strain 356 CF512, were grown at the restrictive, 25°C (f), or permissive temperature, 15°C (i). Mitochondria 357 were isolated at day 1 of adulthood and subjected to the import assay with 30 minutes incubation time (f and g are biological replicates, and i and j are biological replicates). I-n, *glp-1(e2141ts)* 358 359 mutant worms were synchronized and shifted at different developmental stages to the restrictive 360 temperature. Mitochondria were then isolated and subjected to the import assay (I and m are biological replicates). o-q, CF512 worms were synchronized and raised at 15°C or 25°C. 361 362 Mitochondria were then isolated and subjected to the import assay (o and p are biological 363 replicates). The efficiency of mitochondrial import was quantified by measuring the mature 364 imported protein as detected by the DHFR antibody, followed by analysis with unpaired student's 365 t-test (e, h, k, n, q). All graphs are presented as mean \pm SD of two to four biological repeats. *P<0.05, **P<0.01. Arrowheads: mature (imported) DHFR with the MTS cleaved off. 366

367

368 Figure 2. The UPR^{mt} promotes mitochondrial import.

a-e, To induce UPR^{mt} during development, *glp-1(e2141ts*) mutant animals were grown at 25°C on 369 370 bacteria expressing *cco-1* dsRNA (**a,b,e**) or *spg*-7 dsRNA (**c,d**) from the time of hatching until the 371 first day of adulthood (animals were treated with 1:1 mixture of bacteria containing the empty 372 RNAi vector alone (EV) to match with the double RNAi treatment). To suppress UPR^{mt}, animals were treated with double RNAi (1:1 mixture of bacteria replacing EV with bacteria expressing 373 374 *dve-1* dsRNA (**a**,**b**,**e**) or *atfs-1* dsRNA (**c**,**d**). Control worms were grown on bacteria containing 375 empty vector (EV) alone. Mitochondria were isolated from the animals at day 1 of adulthood and subjected to the import assay followed by western blot analysis (a, c). Import efficiency was 376 377 quantified by measuring the mature imported protein as detected by the DHFR antibody, followed 378 by analysis with unpaired student's t-test (b, d). e, RNA was isolated on day 1 of adulthood, and 379 qPCR analysis was performed. f and g, glp-1 animals were grown at 25°C on bacteria expressing 380 timm-23, cco-1, or daf-2 dsRNA (each was diluted to 1:1 ratio with bacteria containing the empty 381 RNAi vector alone) from hatching until the first day of adulthood. Control worms were grown on bacteria containing the empty vector alone. Quantification is shown in g with unpaired student's 382 t-test. The signal intensity of TIMM-23 was normalized to that of NDUFS3. h and i, hsp-6p::GFP 383 animals were grown at 20°C and treated with RNAi or empty RNAi vector control from hatching 384 385 until the L4 stage, then transferred to plates of the same RNAi treatment with the addition of TMRE 386 and grown overnight. Bacteria expressing *cco-1* dsRNA (right) or *mrps-5* dsRNA (middle) (both were diluted 20% with bacteria containing the empty RNAi vector alone) were used to induced 387 388 UPR^{mt}. Control worms were grown on bacteria containing the RNAi vector alone (left). Fluorescent intensity was quantified with Image J. All graphs are presented as mean \pm SD of three 389

- 390 or more biological repeats. *P<0.05, **P<0.01. Arrowheads: mature (imported) DHFR with the
- 391 MTS cleaved off.

392

393 Extended Data Figure 2. The UPR^{mt} promotes mitochondrial import.

394 **a**, A biological replicate of Fig. 2a. b, A biological replicate of Fig. 2c. c-e, glp-1(e2141ts) mutant animals were grown at 25°C on bacteria expressing mrps-5 dsRNA (20% diluted with bacteria 395 396 containing the empty RNAi vector alone) from hatching until the first day of adulthood. 397 Mitochondria were isolated on day 1 of adulthood and subjected to import assay followed by western blot analysis (c and d are biological replicates). f and g, To induce UPR^{mt} during 398 399 development, *hsp-6p*::GFP animals were grown at 25°C on bacteria expressing cco-1 dsRNA (f) 400 or spg-7 dsRNA (g) (diluted to 1:1 ratio with bacteria containing the empty RNAi vector alone) from the time of hatching until the first day of adulthood. To suppress UPR^{mt}, animals were treated 401 402 with double RNAi (1:1 mixture of bacteria replacing the empty RNAi vector with *dve-1* dsRNA 403 (f) or *atfs-1* dsRNA (g). Control worms were grown on bacteria containing empty vector alone. h 404 and i, glp-1(e2141ts) animals were grown at 25°C on bacteria expressing spg-7 dsRNA (h) or daf-405 2 dsRNA (i) (diluted to 1:1 ratio with bacteria containing the empty RNAi vector alone) from 406 hatching until the first of adulthood. RNA was isolated on day 1 of adulthood, and qPCR analysis 407 was performed. Expression was normalized against three housekeeping genes. j-l, *glp-1(e2141ts*) animals were grown at 25°C on bacteria expressing *daf-2* dsRNA (diluted to 1:1 ratio with bacteria 408 containing the empty RNAi vector alone) from hatching until the first day of adulthood. 409 410 Mitochondria were isolated on day 1 of adulthood and subjected to import assay followed by 411 western blot analysis (j and k are biological replicates). m-o, CF512 fer-15(b26) II; fem-1(hc17ts) I and CF596 *daf-2(mu150)* III; *fer-15(b26)*; *fem-1(hc17ts)* worms were grown at 20° C until larval 412 413 stage L2 and then transferred to 25°C. Mitochondria were isolated on day 1 of adulthood and 414 subjected to import assay followed by western blot analysis (**m** and **n** are biological replicates).

- 415 Import efficiency was quantified by measuring the mature imported protein as detected by the
- 416 DHFR antibody, followed by analysis with unpaired student's t-test (e, l, and o). All graphs are
- 417 presented as mean \pm SD of three or more biological repeats. *P<0.05, **P<0.01. Arrowheads:
- 418 mature (imported) DHFR with the MTS cleaved off.

419 Figure 3. The Mitochondrial targeting sequence of ATFS-1 is less import competent.

420 **a-c**, Comparison of import competency between su9-DHFR and ATFS-1-DHFR. The N-terminus 73 amino acid of ATFS-1 was used as MTS. glp-1(e2141ts) animals were grown at 25°C on 421 422 bacteria expressing mrps-5 dsRNA (20% diluted with bacteria containing the empty RNAi vector 423 alone) from hatching until the first day of adulthood. Mitochondria were isolated on day 1 of 424 adulthood and subjected to import assay with 30 minutes incubation time, followed by western 425 blot analysis. Import efficiency was quantified by measuring the mature imported protein as 426 detected by the DHFR antibody. Quantification of imported DHFR is shown in **b** and **c**. **b**, Import of ATFS1-DHFR was compared between mrps-5 RNAi and empty vector control with unpaired 427 student's t-test. The graph is presented as mean \pm SD of four biological repeats (*P<0.05, 428 **P<0.01). c. Import of DHFR with the MTS of su9 and ATFS-1 were compared with two-way 429 ANOVA. No interaction was found. The difference in MTS and the treatment with mrps-5 RNAi 430 431 both have significant effects on the import of DHFR. The graph is presented as mean \pm SD of two biological repeats. *P<0.05, **P<0.01. d, Mitochondrial targeting capacity abolished by point 432 mutations et15 or et18 in the MTS of ATFS-1. Mitochondria extraction was made from 433 synchronized N2 wild type worms at day 1 of adulthood and subjected to import assay with 434 different substrates. Import with *et15* or *et18* was below the detectable level. Solid arrowheads: 435 436 mature (imported) su9-DHFR with the su-9 MTS cleaved off. Open arrowheads: mature 437 (imported) ATFS1-DHFR with the MTS of ATFS-1 cleaved off.

438

439 Extended Data Figure 3

- 440 a, A biological replicate of Fig. 3a. b, A biological replicate of Fig. 3d. Incubation time: 30
- 441 minutes. Solid arrowheads: mature (imported) su9-DHFR with the su9 MTS cleaved off. Open
- 442 arrowheads: mature (imported) ATFS1-DHFR with the MTS of ATFS-1 cleaved off.

443

Figure 4. Import machinery is required for UPR^{mt}-dependent lifespan extension, but UPR^{mt} does not prevent the age-associated decline of import.

a, Subcellular fractionation and western blot of mitochondrial chaperone HSP-6. *glp-1* animals 446 447 were grown at 25°C on bacteria expressing cco-1, spg-7, or daf-2 dsRNA (each was diluted to 1:1 448 ratio with bacteria containing the empty RNAi vector alone) from hatching until the first day of 449 adulthood. Control worms were grown on bacteria containing the empty RNAi vector alone. 450 Different fractions were separated via differential centrifugation. b, Signal intensity was 451 normalized to alpha-tubulin (total and cytosolic fraction) or NDUFS3 (mitochondrial fraction). 452 HSP-6 level in each fraction was compared to that of the respective empty RNAi vector control 453 and analyzed with unpaired student's t-test. The graph is presented as mean \pm SD of four biological 454 repeats (*P<0.05, **P<0.01). c, Adult lifespans of CF512 fer-15(b26ts); fem-1(hc17) animals 455 grown on bacteria expressing dsRNA from the time of hatching. Blue lines, the lifespan of animals grown on control bacteria containing the RNAi vector alone; red lines, the lifespan of animals 456 457 grown on bacteria expressing the dsRNA of *cco-1*; purple lines, the lifespan of animals grown on 458 bacteria expressing the dsRNA of *cco-1* and the dsRNA of *tomm-22*; green lines, the lifespan of 459 animals grown on bacteria expressing the dsRNA of tomm-22. Log-rank (Mantel-Cox) method 460 was used to determine the significant differences (*P<0.05, ****P<0.0001). d, Median lifespans with four replications are plotted and analyzed with unpaired student's t-test. **P<0.01. e and f, 461 glp-1(e2141ts) worms were synchronized and grown at 25°C on bacteria expressing dsRNA from 462 463 the time of hatching. Mitochondria were isolated on day 1 of adulthood and subjected to import 464 assay followed by western blot analysis. Import efficiency was quantified by measuring the mature 465 imported protein as detected by DHFR antibody, followed by analysis with unpaired student's t-

466 test (f). g and h, glp-1(e2141ts) worms were synchronized in two batches and grown at 25°C. 467 When the two batches reached day 1 and 5 of adulthood, respectively, mitochondria were isolated in parallel and subjected to import assay with 30 minutes incubation time followed by western blot 468 469 analysis. Import efficiency was quantified by measuring the mature imported protein as detected 470 by the DHFR antibody, followed by analysis with unpaired student's t-test (h). All graphs are 471 presented as mean \pm SD of two to four biological repeats (*P<0.05, **P<0.01). Arrowheads: mature (imported) DHFR with the MTS cleaved off.

472

473 Extended Data Figure 4. Import machinery is required for UPR^{mt}-dependent lifespan 474 extension.

475 a, The enrichment of subcellular extracts was confirmed with western blot analysis using the 476 subcellular fraction-specific antibodies against NDUFS3 (mitochondria) and alpha-tubulin 477 (cytosol). b, A biological replicate of Fig. 4e. c, Adult lifespan of CF512 animals grown on bacteria expressing dsRNA from the time of hatching. Blue line, the lifespan of animals grown on 478 479 control bacteria containing the RNAi vector alone; red line, the lifespan of animals grown on 480 bacteria expressing the dsRNA of *cco-1*; purple line, the lifespan of animals grown on bacteria expressing the dsRNA of *cco-1* and the dsRNA of *tim-17*; green line, the lifespan of animals grown 481 482 on bacteria expressing the dsRNA of tim-17. Log-rank (Mantel-Cox) method was used to 483 determine the significant differences (****P<0.0001). d-f, CF512 worms were synchronized in 484 three batches and grown at 25°C. When the three batches reached day 1, 5, and 9 of adulthood, 485 respectively, mitochondria were isolated in parallel and subjected to import assay followed by 486 western blot analysis (c and d are biological replicates). Import efficiency was quantified by 487 measuring the mature imported protein as detected by DHFR antibody, followed by analysis with unpaired student's t-test (e). g, A biological replicate of Fig. 4g. h, glp-1(e2141ts) worms were 488 synchronized and grown at 25°C on bacteria expressing dsRNA from the time of hatching. RNA 489 490 was isolated on day 5 of adulthood, and qPCR analysis was performed. Expression was normalized 491 against three housekeeping genes. All graphs are presented as mean \pm SD of two to three biological 492 repeats (*P<0.05, **P<0.01). Arrowheads: mature (imported) DHFR with the MTS cleaved off.

493

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509 Methods

510 Strains

- 511 CB4037 glp-1(e2141) III, SS104 glp-4(bn2) I, CF512 fer-15(b26) II, fem-1(hc17ts) IV, CF596
- 512 *daf-2(mu150) III; fer-15(b26); fem-1(hc17ts)*, SJ4100 (*zcIs13[hsp-6p::gfp]*), N2 wild-type strains
- 513 were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN).

514 RNAi Feeding

515 Worms were grown from the hatch on HT115 *Escherichia coli* containing an empty vector control 516 or expressing double-stranded RNA. RNAi strains were from the Vidal library if present or the 517 Ahringer library if absent from the Vidal library.

518 Import assay

pGEM4-su9(1-69)-DHFR plasmid was a gift from Dr. Thomas Langer ³⁷. Fusion protein su9-519 DHFR was transcribed, translated, and biotinvlated in a single reaction with the TnT® SP6 Ouick 520 521 Coupled Transcription/Translation System (Promega, L2080) and Transcend[™] tRNA (Promega 522 L5016). Mitochondria extraction was made from synchronized worms at the designated age in 523 mitochondria extraction buffer (5 mM Tris-HCl pH 7.4, 210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA). Protease Inhibitor (Protease Inhibitor Cocktail Set III, EDTA-Free, Calbiochem 524 539134) was used at 1:1000). Worms were mechanically homogenized with Dura-Grind[™] 525 526 Stainless Steel Dounce Tissue Grinder (Wheaton 357572), and mitochondria were isolated via 527 differential centrifugation. Mitochondria pellets were resuspended in buffer C (20mM potassium

HEPES, 0.6M sorbitol). Protein concentration was measured using BCA Protein Assay Kit (Pierce
23225), and the same amount is used in each import reaction.

Mitochondrial import assay was performed as previously described ¹⁴ with some modification. The 530 531 biotinylated protein was incubated with 50ug fresh mitochondria extraction in import buffer 532 containing ATP regeneration system (Creatine kinase (Roche 10127566001), Creatine Phosphate 533 (Sigma-Aldrich 10621714001) for 10 to 45 minutes at 25°C with gentle shaking. Import assay 534 with a single time point was performed with a 30-minutes incubation time unless otherwise noted. 535 Membrane potential ($\Delta \Psi$) was disrupted with valinomycin in control. Mitochondria were subsequently treated with proteinase K to degrade preproteins that are attached to the surface of 536 537 the mitochondria. Mitochondria were spun down and resuspended in mitochondria extraction buffer. SDS (6×) loading buffer was added to each sample. Samples were heated at 95°C for 5 min 538 539 and resolved by NuPAGE Bis-Tris mini gels, followed by western blot with DHFR antibody and 540 streptavidin. Import efficiency was quantified by measuring the mature imported protein as 541 detected by the DHFR antibody in ImageStudio (LiCor). Signal intensity was normalized against 542 the signal intensity of control treatment with 30-minute incubation time unless otherwise noted. 543 Data were analyzed using unpaired t-test with Prism (GraphPad4).

544 Subcellular Fractionation

545 Synchronized worms were lysed, and mitochondria were isolated as previously described ³⁸.
546 Supernatant before and after the centrifugation for mitochondria are kept as total and cytosolic
547 portion, respectively.

qPCR 548

549	Total RNA was harvested from worms at the early adult Day 1 stage using TRIzol® LS Reag	gent
550	(Life Technologies). After freezing and thawing three times, RNA was purified on RNeasy r	nini
551	columns (QIAGEN), and cDNA was synthesized using the QuantiTect Reverse Transcription	kit
552	(QIAGEN). SybrGreen quantitative RT-PCR experiments were performed as described in	the
553	manual using QuantStudio [™] 6 Flex Real-Time PCR System. Internal controls utilized a geome	tric
554	mean of cdc-42, pmp-3, and Y45F10D.4. Experiments were repeated three times. Primers used	for
555	qPCR are listed below.	
556	hsp-6 forward 5'-CAAACTCCTGTGTCAGTATCATGGAAGG-3'	
557	hsp-6 reverse 5'-GCTGGCTTTGACAATCTTGTATGGAACG-3'	
558	tomm-20 forward 5'-CGGCTACTGCATTTACTTCGA-3'	
559	tomm-20 reverse 5'-TCATTGCCTGCTGCAGCTGGA-3'	
560	tomm-22 forward 5'-CGACTTCGTTCAGCAGTTCAT-3'	
561	tomm-22 reverse 5'-GCGATCAATGACGTTGTAGATA-3'	
562	tomm-40 forward 5'-AGCTCGTGATGTCTTCCCAAC-3'	
563	tomm-40 reverse 5'-TCCAAATCGGTATCCGGTGTT-3'	
564	timm-17B.1 forward 5'-GATTGTTGTCTTGTCGCCATCC-3'	33

- timm-17B.1 reverse 5'-ATCACCTTTGGTCCTGAACGG-3'
- timm-23 forward 5'-AGTGCCGGAATGAACTTCTC-3'
- timm-23 reverse 5'- GTTGATCCAAGGCGAGGAC-3'
- tin-44 forward 5'-GGGATACGATTAACTCGGACA-3'
- tin-44 reverse 5'-CTGCATTCGAGCTTTCAACTG-3'
- 570 mppa-1 forward 5'-CGATTTTGTGACTGTTGGCGT-3'
- 571 mppa-1 reverse 5'-GCTTGAGAACGATTCCGATGA-3'
- 572 mppb-1 forward 5'-GCACAAGTTCAGCCGAAATCA-3'
- 573 mppb-1 reverse 5'-TTCTCATTCTCGTAGCGACTG-3'
- 574 cdc-42 forward 5'- AGGAACGTCTTCCTTGTCTCC -3'
- 575 cdc-42 reverse 5'- GGACATAGAAAGAAAAACACAGTCAC -3'
- 576 pmp-3 forward 5'- CGGTGTTAAAACTCACTGGAGA -3'
- 577 pmp-3 reverse 5'- TCGTGAAGTTCCATAACACGA -3'
- 578 Y45F10D.4 forward 5'- AAGCGTCGGAACAGGAATC -3'

579 Y45F10D.4 reverse 5'- TTTTTCCGTTATCGTCGACTC -3'

580 Antibodies

A polyclonal rabbit antibody to TIMM-23 was generated against the synthesized polypeptide of amino acid 95-230, and affinity-purified (ABClonal Science, Inc.). Other antibodies and probes used for western blot were as follows: anti-DHFR antibody (Sigma-Aldrich D1067), anti-HSP-6 antibody (Enzo Life Science ADI-SPS-825); and anti-NDUF3 [17D95] antibody (Abcam ab14711); IRDye® 680CW Donkey anti-Mouse IgG (H + L) (LI-COR 926-68072); IRDye 680LT Donkey anti-Rabbit IgG (H + L), (LI-COR 926-68023).

587 TMRE Staining

TMRE staining was performed according to the previous study ³⁹. TMRE was dissolved in DMSO at a concentration of 50μ M and added into fresh bacteria culture at a final concentration of 0.1μ M before seeding the plates. Worms were synchronized by egg bleach and grown on *E. coli* HT115 for RNAi from the hatch and transferred to RNAi plates containing TMRE at the L3/L4 stage. Worms were imaged after growing overnight on TMRE plates. TMRE staining was quantified with ImageJ.

594 CCCP is dissolved in DMSO at a concentration of 10mM and added into bacteria culture at a final595 concentration of 50µM before seeding the plates.

596 Lifespan Analysis

- 597 Lifespan experiments were performed with CF512 worms at 25°C as previously described ¹⁸.
- 598 Worms were synchronized by egg bleach and grown on *E. coli* HT115 for RNAi from the hatch.
- 599 Worms were scored every second day. Prism 6 software was used for statistical analysis. Log-rank
- 600 (Mantel-Cox) method was used to determine the significant difference.

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Figure 1



Extended Data Figure 1







Figure 2



Extended Data Figure 2

anti-DHFR







30'

Figure 3



Extended Data Figure 3



Figure 4



Extended Data Figure 4

