

# The UPR<sup>mt</sup> Preserves Mitochondrial Import to Extend Lifespan

Nan Xin<sup>1, 4</sup>, Jenni Durieux<sup>1</sup>, Chunxia Yang<sup>4</sup>, Suzanne Wolff<sup>1</sup>, Hyun-Eui Kim<sup>4, \*</sup>, Andrew Dillin<sup>1, 2, 3, 5, \*</sup>

<sup>1</sup> Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, 94720, USA

<sup>2</sup> Howard Hughes Medical Institute

<sup>3</sup> The Paul F. Glenn Center for Aging Research, University of California, Berkeley, Berkeley, CA 94720, USA

<sup>4</sup> Department of Integrated Biology and Pharmacology, University of Texas, Health Science Center, Houston, TX 77030, USA

<sup>5</sup> Lead contact

\* Correspondence: [hyun-eui.kim@uth.tmc.edu](mailto:hyun-eui.kim@uth.tmc.edu) (H-E.K.), [dillin@berkeley.edu](mailto:dillin@berkeley.edu) (A.D.)

## Author Contribution

N.X. and A.D. conceived the study. J.D. and H-E.K. planned 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<sup>mt</sup>) is dedicated to promote mitochondrial  
3 proteostasis and is linked to extreme longevity in worms, flies, and mice. The key regulator of this  
4 process is the transcription factor, ATFS-1. In the absence of mitochondrial stress, ATFS-1 is  
5 transported to the mitochondria and degraded. During conditions of mitochondrial stress, ATFS-1  
6 is excluded from the mitochondria and enters the nucleus to regulate the expression of UPR<sup>mt</sup> genes.  
7 However, there exists a dichotomy in regards to induction of the UPR<sup>mt</sup> and mitochondrial import.  
8 The repair proteins synthesized as a direct result of UPR<sup>mt</sup> activation must be transported into  
9 damaged mitochondria that had previously excluded ATFS-1 due to reduced import efficiency. To  
10 address this conundrum, we analyzed the role of the import machinery under conditions where the  
11 UPR<sup>mt</sup> 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<sup>mt</sup> 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<sup>mt</sup>-mediated increase and lifespan extension. With  
16 age, import capacity decreases, and activation of the UPR<sup>mt</sup> delays this decline and increases  
17 longevity. Finally, we find that ATFS-1 has a significantly weaker mitochondrial targeting  
18 sequence (MTS), allowing for dynamic subcellular localization during the initial stages of UPR<sup>mt</sup>  
19 activation.

## 20 **Introduction**

21 During the course of eukaryotic evolution and the development of sequestered organelles,  
22 communication events co-evolved to allow proper homeostasis within the cell that encompassed  
23 master coordination by the nucleus. Such events have been discovered to include the unfolded  
24 protein response of the endoplasmic reticulum (UPR<sup>ER</sup>), the mitochondria (UPR<sup>mt</sup>), and the  
25 cytoplasmic heat shock response (HSR) <sup>1-3</sup>. The primary mode of action of each of these stress  
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  
28 organelle. Genes encoding compartment-specific chaperones are the sentinel transcriptional  
29 targets of such responses.

30 Mitochondria pose the most challenging organelle to coordinate stress responsiveness with the  
31 nucleus for several reasons. One, the mitochondrion is encapsulated within a double membrane  
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  
35 comprise some of the largest complexes found in eukaryotic cells <sup>4,5</sup>. Three, the by-products of  
36 oxidative respiration, superoxide radicals, pose a constant challenge to mitochondrial integrity <sup>6</sup>.  
37 Four, import of proteins into the mitochondrion requires energy in the form of membrane potential  
38 and ATP created by the electron transport chain <sup>4</sup>, and each cell can contain hundreds of  
39 mitochondria that fuse and divide at any given time to change the cellular mitochondrial landscape.  
40 Taken together, monitoring the integrity of the mitochondrion and all of its various protein

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

43 Mitochondria can orchestrate and coordinate a wide number of different stress-response  
44 mechanisms under various cellular and subcellular perturbations. Such responses include the  
45 UPR<sup>mt</sup>, mitophagy, and programmed cell death. In response to mild mitochondrial stress, the  
46 UPR<sup>mt</sup>, a specific transcriptional stress response system that is mediated by ATFS-1, DVE-1, UBL-  
47 5, LIN-65, MET-2, and PHF-8, is activated to increase the production of mitochondrial localized  
48 chaperones and proteases to help relieve the stress <sup>7-11</sup>. One of the major contributors to this  
49 response is ATFS-1. During mitochondrial stress, mitochondrial import efficiency is compromised,  
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 <sup>7-11</sup>. Inherent within this model is the balance that  
56 must be maintained between the membrane potential, import machinery, and the ability to induce  
57 the UPR<sup>mt</sup>. However, the link between membrane potential, mitochondrial import, and the UPR<sup>mt</sup>  
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<sup>mt</sup> and mitochondrial import. The repair proteins synthesized as a  
61 direct result of UPR<sup>mt</sup> 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  
64 mitochondria during stress, how then are other proteins allowed entry, especially mitochondria  
65 with reduced membrane potential? Could there be coordination to increase mitochondrial import  
66 efficiency once the UPR<sup>mt</sup> is induced? Is the mitochondrial import machinery a distinct branch of  
67 the UPR<sup>mt</sup> to overcome the lack of integrity of damaged mitochondria? To address these questions,  
68 we analyzed the role of the import machinery under conditions where the UPR<sup>mt</sup> was induced.  
69 Using *in vitro* biochemical assays of mitochondrial import and *in vivo* analysis of mitochondrially  
70 localized proteins, we find the efficiency of mitochondrial import increases when the UPR<sup>mt</sup> is  
71 activated. More surprisingly, the increased import due to UPR<sup>mt</sup> induction occurs when the  
72 mitochondrial membrane potential is decreased. Finally, we find that the induction of the import  
73 machinery is essential for UPR<sup>mt</sup>-mediated lifespan extension, and ectopic induction of the UPR<sup>mt</sup>  
74 preserves import into late life.

## 75 **Results**

### 76 **Assessing mitochondrial import capacity from *C. elegans* mitochondria**

77 All but a few mitochondrial proteins are transcribed from the nuclear genome and imported post-  
78 translationally through the translocase of the outer/inner membrane (TOM/TIM) complex <sup>4,12</sup>.  
79 Mitochondrial protein import depends on the mitochondrial membrane potential ( $\Delta\Psi$ ), ATP, and  
80 is under the direction of mitochondrial targeting sequences (MTS), which is cleaved after import  
81 <sup>13</sup>. To measure the efficiency of mitochondrial import, we adapted and validated a method in which  
82 substrate proteins are synthesized in an *in vitro* transcription/translation reaction, and subsequently  
83 imported into isolated mitochondria <sup>14,15</sup> (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  
85 fragment of the cytosolic protein dihydrofolate reductase (DHFR) from mice<sup>16</sup>. An ATP  
86 regeneration system<sup>17</sup> was applied to increase the efficiency of import. Importantly, a DHFR  
87 antibody can readily detect the fusion protein being imported (Fig. 1b, c, Extended Data Fig. 1a),  
88 as indicated by: 1) the change in size from a precursor protein to a mature DHFR; 2) the absence  
89 of mature DHFR upon disruption of membrane potential ( $\Delta\Psi$ ); 3) the absence of precursor protein  
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*.**

96 To determine whether induction of the UPR<sup>mt</sup> had any impact upon mitochondrial import  
97 efficiency, we first induced the UPR<sup>mt</sup> in wild-type N2 worms with RNAi against cytochrome c  
98 oxidase-1 subunit (*cco-1/cox-5B*), a component of the electron transport chain complex IV, and  
99 found that import efficiency was reduced in mitochondrial preparations isolated from whole  
100 animals (Extended Data Fig. 1c-e). This finding appears to be consistent with the current model of  
101 UPR<sup>mt</sup> 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<sup>18</sup>. 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  
105 germline accounts for the vast majority of mtDNA amplification in *C. elegans*<sup>19</sup>. As the germline

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

110 We, therefore, tested whether germline cells differ in import competency relative to post-mitotic  
111 cells. To this end, we used three temperature-sensitive sterile strains that differentially affect the  
112 development of the germline at the restrictive temperature. In particular, CB4037 *glp-1(e2141ts)*  
113 <sup>20</sup> and SS104 *glp-4(bn2ts)* <sup>21</sup> are sterile strains that lack the majority of the germline, whereas  
114 CF512 *fer-15(b26ts); fem-1(hc17)* <sup>22</sup> is sterile due to the conversion of sperm into oocytes. Of note,  
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  
121 observed no significant difference between these strains when raised at the permissive temperature,  
122 15°C (Extended Data Fig. 1i-k), which allows normal germline development. Additionally,  
123 temporal shifting of *glp-1(e1241ts)* mutant animals from 15°C to 25°C during development allows  
124 limited germline development, with earlier shifts resulting in fewer germ cells, and later shifts  
125 having a near-complete complement of germ cells. By shifting at a series of time points, we found  
126 that import capacity was positively correlated with the number of germline cells present in the  
127 animals (Extended Data Fig. 1l-n). In contrast, the CF512 strain, which has a normal female

128 germline at both permissive and restrictive temperatures, shows higher import competency at 25°C  
129 (Extended Data Fig. 1o-q). These results suggest that germline mitochondria are highly import-  
130 competent in comparison to post-mitotic, somatic cells in *C. elegans*.

### 131 **Mitochondrial import is enhanced upon UPR<sup>mt</sup> induction**

132 To understand if and how mitochondrial import is regulated upon mitochondrial stress in somatic  
133 cells, we induced UPR<sup>mt</sup> in *C. elegans* and examined the import capacity of isolated mitochondria  
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-*  
136 *1(e2141ts)* mutant animals<sup>18</sup>, to only assay mitochondria from somatic tissues where the UPR<sup>mt</sup>  
137 impacts longevity and health<sup>23,24</sup>. 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  
144 mitochondrial inner membrane<sup>25</sup>. MRPS-5 is a mitochondrial ribosome protein<sup>23</sup>. Knockdown of  
145 either *spg-7* or *mrps-5*, via RNAi, induces the UPR<sup>mt</sup> and leads to lifespan extension of worms  
146 composed of post-mitotic, somatic cells<sup>23,26</sup>. We found that mitochondrial import was also  
147 significantly enhanced upon either *spg-7* RNAi (Fig. 2c, d, Extended Data Fig. 2b) or *mrps-5* RNAi  
148 (Extended Data Fig. 2c-e). Taken together, both results suggest that the activation of the UPR<sup>mt</sup> is  
149 widely associated with enhanced mitochondrial import.

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

## 162 **Mitochondrial import machinery is upregulated upon UPR<sup>mt</sup> induction.**

163 The UPR<sup>mt</sup> promotes mitochondrial protein homeostasis through signaling to the nucleus to induce  
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  
166 proteins that constitute the import machinery, TIM/TOM complexes, are exclusively encoded by  
167 nuclear genes. Therefore, it is conceivable that the UPR<sup>mt</sup> may enhance import through  
168 upregulating the expression of the TIM/TOM complex components. Indeed, the import machinery  
169 components *timm-17* and *timm-23* were upregulated upon UPR<sup>mt</sup> induction by *spg-7* RNAi <sup>7</sup>.  
170 Similarly, *timm-23* was also found to be moderately upregulated upon *cco-1* RNAi treatment <sup>8</sup>.  
171 However, when we examined the RNA-sequencing data of animals treated with *cco-1* RNAi, we

172 found no significant change in the transcription of most other import machinery components<sup>8,9</sup>.  
173 As N2 worms were used in this RNA-seq analysis, the loss of germline caused by *cco-1*  
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  
176 tested germline-deficient *glp-1(e2141ts)* mutant animals for the transcription of a series of genes  
177 encoding the mitochondrial import machinery. Comparing the synchronized and age-matched  
178 worms, we found that transcription of the TIM/TOM genes upon *cco-1* RNAi treatment was  
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<sup>mt</sup> activation. This is consistent with previous findings, which suggests that  
185 TIM23 protein might be the rate-limiting factor in mitochondrial import<sup>28</sup>. 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  
189 the mitochondrial targeting sequences (MTS)<sup>29</sup>. We found that *hsp-6*, *tin-44*, as well as *mppa-1*  
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<sup>mt</sup> in somatic cells.  
193 Similarly, the TIM/TOM import machinery was also upregulated upon *spg-7* RNAi (Extended

194 Data Fig. 2h). Additionally, the *cco-1* induced upregulation of import machinery genes was  
195 suppressed by RNAi against *dve-1* (Fig. 2e).

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

200 Given that mitochondrial protein import requires membrane potential ( $\Delta\Psi$ ), we examined if  $\Delta\Psi$  is  
201 enhanced upon UPR<sup>mt</sup> activation. Surprisingly, in early Day 1 adult, the same stage when enhanced  
202 mitochondrial import was detected, we did not observe stronger TMRE staining, a marker of  $\Delta\Psi$ ,  
203 in worms with activated UPR<sup>mt</sup> (Fig. 2h, i). On the contrary, membrane potential was found to be  
204 reversely correlated with the activation of the UPR<sup>mt</sup>. In particular, knockdown of *cco-1* induced  
205 the UPR<sup>mt</sup> more robustly than knockdown of *mrps-5*, as indicated by the level of *hsp-6p::gfp*  
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  
214 into the mitochondria to restore their proper function. However, previous findings suggest that  
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 genes <sup>2</sup>. Is the import of ATFS-  
217 1 differentially regulated? To interrogate this possibility, we made a chimeric protein with the  
218 predicted mitochondrial targeting sequence of ATFS-1 (N terminal 73 amino acids) <sup>7</sup> fused with  
219 DHFR (ATFS1-DHFR). We found that upon the induction of the UPR<sup>mt</sup>, 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  
226 depolarization to send a stress signal to the nucleus by translocation <sup>30</sup>. Point mutations in the MTS  
227 of *atfs-1*, such as *et15* or *et18*, display constitutively active UPR<sup>mt</sup> <sup>33</sup>, 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<sup>mt</sup> induction requires intact TIM/TOM complexes.**

233 Our findings suggest that the mitochondrial import machinery is upregulated at the transcriptional  
234 level upon UPR<sup>mt</sup> induction, thereby elevating import capacity and allowing stress-responsive



235 proteins to translocate into the mitochondrial matrix and restore proteostasis. One such stress-  
236 responsive proteins is the mitochondrial chaperone mtHSP70(HSP-6), which is transcriptionally  
237 upregulated upon the activation of UPR<sup>mt</sup>. Analyzing the subcellular fractionation of mitochondria  
238 from UPR<sup>mt</sup> induced animals, we find increased levels of HSP-6 in the mitochondrial fraction (Fig.  
239 4a, b, extended data Fig. 4a), suggesting that import *in vivo* is maintained at a level that allows  
240 efficient mitochondrial translocation of the elevated level of stress-responsive proteins, despite the  
241 reduction of membrane potential (Fig. 2h, i).

242

243 The next question we asked was whether the improvement of import is a necessary element for  
244 UPR<sup>mt</sup>-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  
248 effect on lifespan in wild-type animals (Fig. 4c, d). Similarly, treating worms with *timm-17* RNAi  
249 also partially suppressed the lifespan extension (Extended Data Fig. 4c). Taken together, these  
250 results indicate that intact import machinery is essential for UPR<sup>mt</sup>-induced lifespan extension.

## 251 **Induction of the UPR<sup>mt</sup> maintains mitochondrial import during aging**

252 Intrigued by the finding that enhanced import efficiency was required for UPR<sup>mt</sup>-mediated  
253 longevity, we asked how import efficiency might play a role in normal aging. As organisms age  
254 mitochondria gradually depolarize and the membrane potential, the major driving force of import,  
255 declines<sup>22</sup>. We tested import efficiency across mitochondria isolated from aging cohorts of *C.*  
256 *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  
258 the process of aging. We observed a more than one-half reduction in the import efficiency of su9-  
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  
261 import was observed in both *glp-1(e2141ts)* worms, which lack a germline (Fig. 4g, h, Extended  
262 Data Fig. g), and CF512 *fer-15(b26ts); fem-1(hc17)* worms, which have intact female germline  
263 (Extended Data Fig. 4d-f), suggesting that import in somatic tissue and germline are both affected  
264 by aging.

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

270 As lifespan extension is a common effect of UPR<sup>mt</sup> activation, it is intriguing to know whether the  
271 enhancement in mitochondrial import capacity we observed is a secondary effect of delayed aging  
272 or more specific to UPR<sup>mt</sup> 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-*  
278 *2(e1370)* mutation (Extended Data Fig. 2j-l and m-o), despite the dramatic increase in lifespan in

279 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  
282 together, these findings indicate that the enhancement of import in somatic cells is unique to UPR<sup>mt</sup>  
283 activation, and the aging process itself reduces import that can be combated by UPR<sup>mt</sup> activation.

## 284 **Discussion**

285 Metazoans have evolved various defense mechanisms to protect themselves against the detrimental  
286 consequences of stress and aging. Many of the stress responsive mechanisms require altering the  
287 composition of their proteomes. This remodeling often includes enhancing the networks of stress  
288 responsive proteins and chaperones, which are targeted for specific subcellular compartments or  
289 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<sup>7</sup>. 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  
294 upregulation of import is specifically associated with the induction of UPR<sup>mt</sup>, instead of being a  
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  
297 found that in spite of decreased membrane potential, the UPR<sup>mt</sup> resulted in increased import  
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  
300 assays reveal increased import competency of these mitochondria. Intriguingly, the efficiency of  
301 mitochondrial import serves as an active mechanism of increased longevity upon the activation of  
302 the UPR<sup>mt</sup>.

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

305 mitochondrial respiration and lower ETC components, but the levels of TOM20 seem to be  
306 elevated <sup>35</sup>. As lowered levels of ETC components would presumably induce UPR<sup>mt</sup>, this  
307 observation in mammalian cells is consistent with our finding that import machinery is upregulated  
308 upon UPR<sup>mt</sup> 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  
311 expression of downstream stress response genes <sup>7</sup>. Surprisingly, we found that the UPR<sup>mt</sup>-  
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  
314 how the UPR<sup>mt</sup> is maintained upon upregulation of the UPR<sup>mt</sup>. It was recently revealed that  
315 mitochondrial stress during larval development induces chromatin changes that are perpetuated  
316 into adulthood and make up a critical part of the UPR<sup>mt</sup> <sup>8,9</sup>. Accordingly, UPR<sup>mt</sup> 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  
319 upregulated upon induction of the UPR<sup>mt</sup> <sup>7</sup>, suggesting that once the UPR<sup>mt</sup> is activated, nuclear  
320 ATFS-1 might be kept at a higher level despite the recovery of mitochondrial import efficiency.

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

327 more resilient due to UPR<sup>mt</sup> 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<sup>mt</sup> 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  
338 minutes at 25°C. Mitochondria were subsequently treated with proteinase K to remove non-  
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.

350 **Extended Data Figure 1. Germline-deficiency leads to the reduction of import competency;**  
351 **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  
358 time (**f** and **g** are biological replicates, and **i** and **j** are biological replicates). **l-n**, *glp-1(e2141ts)*  
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 (**l** and **m** are  
361 biological replicates). **o-q**, CF512 worms were synchronized and raised at 15°C or 25°C.  
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.  
366 \*P<0.05, \*\*P<0.01. Arrowheads: mature (imported) DHFR with the MTS cleaved off.

367



368 **Figure 2. The UPR<sup>mt</sup> promotes mitochondrial import.**

369 **a-e**, To induce UPR<sup>mt</sup> during development, *glp-1(e2141ts)* mutant animals were grown at 25°C on  
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<sup>mt</sup>, animals  
373 were treated with double RNAi (1:1 mixture of bacteria replacing EV with bacteria expressing  
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  
376 subjected to the import assay followed by western blot analysis (**a, c**). Import efficiency was  
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  
382 bacteria containing the empty vector alone. Quantification is shown in **g** with unpaired student's  
383 t-test. The signal intensity of TIMM-23 was normalized to that of NDUFS3. **h** and **i**, *hsp-6p::GFP*  
384 animals were grown at 20°C and treated with RNAi or empty RNAi vector control from hatching  
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  
387 were diluted 20% with bacteria containing the empty RNAi vector alone) were used to induced  
388 UPR<sup>mt</sup>. Control worms were grown on bacteria containing the RNAi vector alone (left).  
389 Fluorescent intensity was quantified with Image J. All graphs are presented as mean ± SD of three

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<sup>mt</sup> promotes mitochondrial import.**

394 **a**, A biological replicate of Fig. 2**a**. **b**, A biological replicate of Fig. 2**c**. **c-e**, *glp-1(e2141ts)* mutant  
395 animals were grown at 25°C on bacteria expressing *mrps-5* dsRNA (20% diluted with bacteria  
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  
398 western blot analysis (**c** and **d** are biological replicates). **f** and **g**, To induce UPR<sup>mt</sup> during  
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)  
401 from the time of hatching until the first day of adulthood. To suppress UPR<sup>mt</sup>, animals were treated  
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)*  
408 animals were grown at 25°C on bacteria expressing *daf-2* dsRNA (diluted to 1:1 ratio with bacteria  
409 containing the empty RNAi vector alone) from hatching until the first day of adulthood.  
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)*  
412 *I* and CF596 *daf-2(mu150) III; fer-15(b26); fem-1(hc17ts)* worms were grown at 20° C until larval  
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  
421 73 amino acid of ATFS-1 was used as MTS. *glp-1(e2141ts)* animals were grown at 25°C on  
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  
427 of ATFS1-DHFR was compared between *mrps-5* RNAi and empty vector control with unpaired  
428 student's t-test. The graph is presented as mean  $\pm$  SD of four biological repeats (\*P<0.05,  
429 \*\*P<0.01). **c**, Import of DHFR with the MTS of su9 and ATFS-1 were compared with two-way  
430 ANOVA. No interaction was found. The difference in MTS and the treatment with *mrps-5* RNAi  
431 both have significant effects on the import of DHFR. The graph is presented as mean  $\pm$  SD of two  
432 biological repeats. \*P<0.05, \*\*P<0.01. **d**, Mitochondrial targeting capacity abolished by point  
433 mutations *et15* or *et18* in the MTS of ATFS-1. Mitochondria extraction was made from  
434 synchronized N2 wild type worms at day 1 of adulthood and subjected to import assay with  
435 different substrates. Import with *et15* or *et18* was below the detectable level. Solid arrowheads:  
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

444 **Figure 4. Import machinery is required for UPR<sup>mt</sup>-dependent lifespan extension, but UPR<sup>mt</sup>**  
445 **does not prevent the age-associated decline of import.**

446 **a**, Subcellular fractionation and western blot of mitochondrial chaperone HSP-6. *glp-1* animals  
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 ± 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  
456 grown on control bacteria containing the RNAi vector alone; red lines, the lifespan of animals  
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  
461 with four replications are plotted and analyzed with unpaired student's t-test. \*\*P<0.01. **e** and **f**,  
462 *glp-1(e2141ts)* worms were synchronized and grown at 25°C on bacteria expressing dsRNA from  
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  
468 in parallel and subjected to import assay with 30 minutes incubation time followed by western blot  
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:  
472 mature (imported) DHFR with the MTS cleaved off.



473 **Extended Data Figure 4. Import machinery is required for UPR<sup>mt</sup>-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  
478 bacteria expressing dsRNA from the time of hatching. Blue line, the lifespan of animals grown on  
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  
481 expressing the dsRNA of *cco-1* and the dsRNA of *tim-17*; green line, the lifespan of animals grown  
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  
488 unpaired student's t-test (**e**). **g**, A biological replicate of Fig. **4g**. **h**, *glp-1(e2141ts)* worms were  
489 synchronized and grown at 25°C on bacteria expressing dsRNA from the time of hatching. RNA  
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 ± SD of two to three biological  
492 repeats (\*P<0.05, \*\*P<0.01). Arrowheads: mature (imported) DHFR with the MTS cleaved off.

493

494 **Acknowledgment**

495 We thank the Caenorhabditis Genetic Center and Shohei Mitani of the National BioResource  
496 Project for providing strains.

497 This research was supported by NIH F32 (5F32AG051353-03), NIH R01 (R37AG024365 and  
498 R01ES021667), University of Texas Health Science Center at Houston (37516-12002) and the  
499 Rising STARS program of UT systems (26532). This work used the Vincent J. Coates Genomics  
500 Sequencing Laboratory at UC Berkeley, supported by NIH S10 Instrumentation Grants  
501 S10RR029668 and S10RR027303.

502 We thank Dr. Stephan Rolland from Ludwig Maximilian University of Munich for sharing a  
503 sequence-verified *tim-23* RNAi strain.

504 We thank Dr. Ryo Higuchi-Sanabria, Dr. Hope Henderson, and the Dillin Lab for comments that  
505 greatly improved the manuscript.

506 We would also like to show our gratitude to Dr. Ye Tian (Dillin Lab alumni, Institute of Genetics  
507 and Developmental Biology, CAS), for sharing her insight with us during the course of this  
508 research.

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 (*zcls13[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**

519 pGEM4-su9(1-69)-DHFR plasmid was a gift from Dr. Thomas Langer<sup>37</sup>. Fusion protein su9-  
520 DHFR was transcribed, translated, and biotinylated in a single reaction with the TnT® SP6 Quick  
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  
524 mM EDTA). Protease Inhibitor (Protease Inhibitor Cocktail Set III, EDTA-Free, Calbiochem  
525 539134) was used at 1:1000). Worms were mechanically homogenized with Dura-Grind™  
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

528 HEPES, 0.6M sorbitol). Protein concentration was measured using BCA Protein Assay Kit (Pierce  
529 23225), and the same amount is used in each import reaction.

530 Mitochondrial import assay was performed as previously described<sup>14</sup> with some modification. The  
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  
536 subsequently treated with proteinase K to degrade preproteins that are attached to the surface of  
537 the mitochondria. Mitochondria were spun down and resuspended in mitochondria extraction  
538 buffer. SDS (6×) loading buffer was added to each sample. Samples were heated at 95°C for 5 min  
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<sup>38</sup>.  
546 Supernatant before and after the centrifugation for mitochondria are kept as total and cytosolic  
547 portion, respectively.

548 **qPCR**

549 Total RNA was harvested from worms at the early adult Day 1 stage using TRIzol® LS Reagent  
550 (Life Technologies). After freezing and thawing three times, RNA was purified on RNeasy mini  
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 geometric  
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'-CAAACCTCCTGTGTCAGTATCATGGAAGG-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'-CGACTTCGTTTCAGCAGTTCAT-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'-GATTGTTGTCTTGTGCGCCATCC-3'

- 565 timm-17B.1 reverse 5'-ATCACCTTTGGTCCTGAACGG-3'
- 566 timm-23 forward 5'-AGTGCCGGAATGAACTTCTC-3'
- 567 timm-23 reverse 5'-GTTGATCCAAGGCGAGGAC-3'
- 568 tin-44 forward 5'-GGGATACGATTA ACTCGGACA-3'
- 569 tin-44 reverse 5'-CTGCATTGAGCTTTCAACTG-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'-CGGTGTTAAA ACTCACTGGAGA-3'
- 577 pmp-3 reverse 5'-TCGTGAAGTTCCATAACACGA-3'
- 578 Y45F10D.4 forward 5'-AAGCGTCGGAACAGGAATC-3'

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

## 580 **Antibodies**

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

## 587 **TMRE Staining**

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

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

## 596 **Lifespan Analysis**

597 Lifespan experiments were performed with CF512 worms at 25°C as previously described <sup>18</sup>.  
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.



## Reference:

1. Liu, Y. & Chang, A. Heat shock response relieves ER stress. *EMBO J.* **27**, 1049–1059 (2008).
2. Haynes, C. M. & Ron, D. The mitochondrial UPR - protecting organelle protein homeostasis. *J. Cell Sci.* **123**, 3849–3855 (2010).
3. Kirstein-Miles, J. & Morimoto, R. I. *Caenorhabditis elegans* as a model system to study intercompartmental proteostasis: Interrelation of mitochondrial function, longevity, and neurodegenerative diseases. *Dev. Dyn.* **239**, 1529–1538 (2010).
4. Wiedemann, N. & Pfanner, N. Mitochondrial Machineries for Protein Import and Assembly. *Annu. Rev. Biochem.* **86**, 685–714 (2017).
5. Hirst, J. Mitochondrial complex I. *Annu. Rev. Biochem.* **82**, 551–575 (2013).
6. Murphy, M. P. How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1–13 (2009).
7. Nargund, A. M., Pellegrino, M. W., Fiorese, C. J., Baker, B. M. & Haynes, C. M. Mitochondrial Import Efficiency of ATFS-1 Regulates Mitochondrial UPR Activation. *Science* **337**, 587–590 (2012).
8. Tian, Y. *et al.* Mitochondrial Stress Induces Chromatin Reorganization to Promote Longevity and UPR(mt). *Cell* **165**, 1197–1208 (2016).

9. Merkwirth, C. *et al.* Two Conserved Histone Demethylases Regulate Mitochondrial Stress-Induced Longevity. *Cell* **165**, 1209–1223 (2016).
10. Haynes, C. M., Petrova, K., Benedetti, C., Yang, Y. & Ron, D. ClpP Mediates Activation of a Mitochondrial Unfolded Protein Response in *C. elegans*. *Dev. Cell* **13**, 467–480 (2007).
11. Benedetti, C., Haynes, C. M., Yang, Y., Harding, H. P. & Ron, D. Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. *Genetics* **174**, 229–239 (2006).
12. Wiedemann, N., Frazier, A. E. & Pfanner, N. The Protein Import Machinery of Mitochondria. *J. Biol. Chem.* **279**, 14473–14476 (2004).
13. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing Mitochondrial Proteins: Machineries and Mechanisms. *Cell* **138**, 628–644 (2009).
14. Ryan, M. T., Voos, W. & Pfanner, N. Chapter 11 Assaying protein import into mitochondria. in *Methods in Cell Biology* vol. 65 189–215 (Academic Press, 2001).
15. Stuart, R. A. & Koehler, C. M. In vitro analysis of yeast mitochondrial protein import. *Curr. Protoc. Cell Biol.* **Chapter 11**, 11.19 (2007).

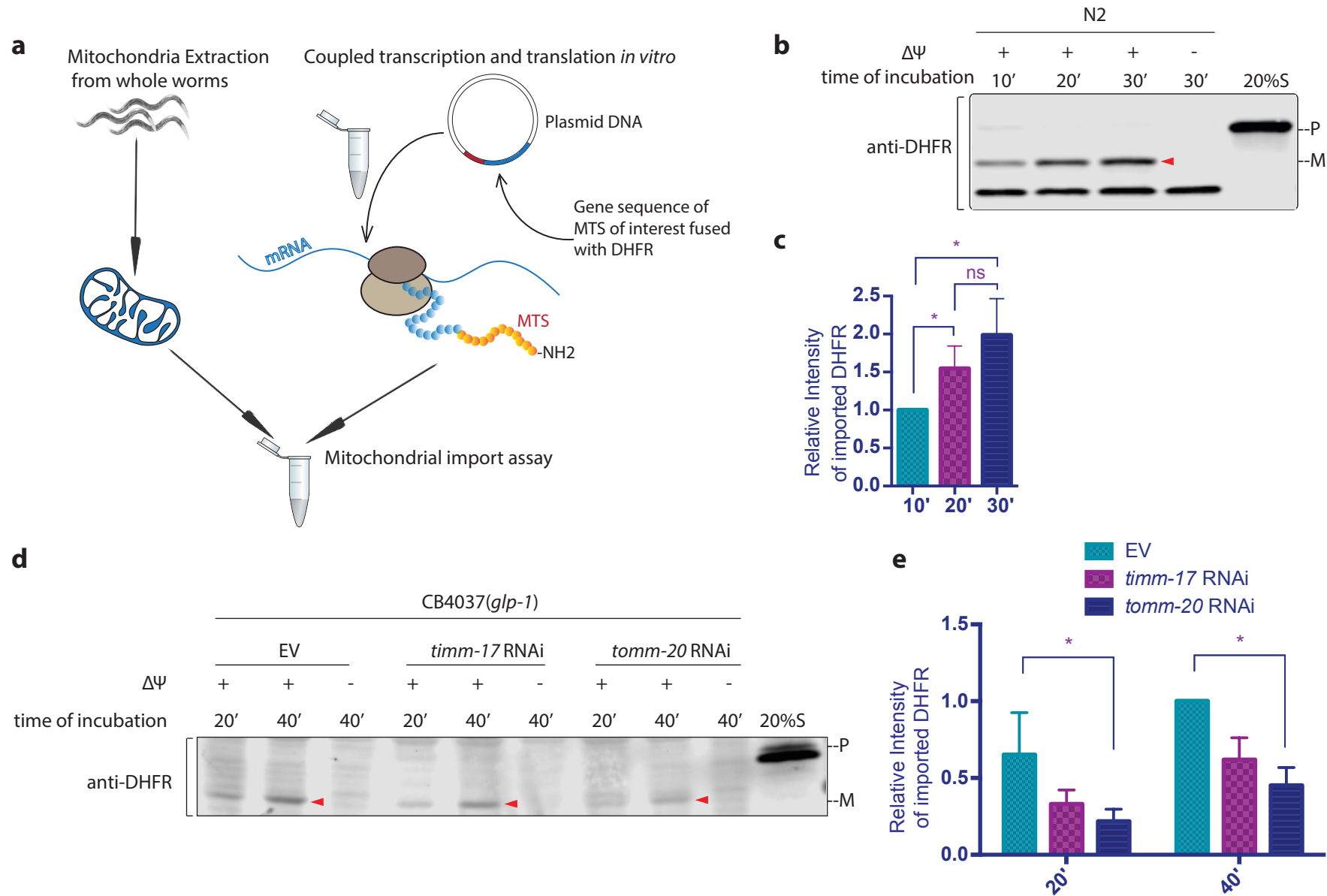
16. Pfanner, N., Müller, H. K., Harmey, M. A. & Neupert, W. Mitochondrial protein import: involvement of the mature part of a cleavable precursor protein in the binding to receptor sites. *EMBO J.* **6**, 3449–3454 (1987).
17. Khalimonchuk, O. *et al.* Sequential Processing of a Mitochondrial Tandem Protein: Insights into Protein Import in *Schizosaccharomyces pombe*. *Eukaryot. Cell* **5**, 997–1006 (2006).
18. Dillin, A. *et al.* Rates of Behavior and Aging Specified by Mitochondrial Function During Development. *Science* **298**, 2398–2401 (2002).
19. Tsang, W. Y. & Lemire, B. D. Mitochondrial Genome Content Is Regulated during Nematode Development. *Biochem. Biophys. Res. Commun.* **291**, 8–16 (2002).
20. Kodoyianni, V., Maine, E. M. & Kimble, J. Molecular basis of loss-of-function mutations in the *glp-1* gene of *Caenorhabditis elegans*. *Mol. Biol. Cell* **3**, 1199–1213 (1992).
21. Arantes-Oliveira, N., Apfeld, J., Dillin, A. & Kenyon, C. Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*. *Science* **295**, 502–505 (2002).
22. Garigan, D. *et al.* Genetic Analysis of Tissue Aging in *Caenorhabditis elegans*: A Role for Heat-Shock Factor and Bacterial Proliferation. *Genetics* **161**, 1101–1112 (2002).
23. Houtkooper, R. H. *et al.* Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature* **497**, 451–457 (2013).

24. Durieux, J., Wolff, S. & Dillin, A. The Cell Non-Autonomous Nature of Electron Transport Chain-Mediated Longevity. *Cell* **144**, 79–91 (2011).
25. Nolden, M. *et al.* The m-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria. *Cell* **123**, 277–289 (2005).
26. Yoneda, T. *et al.* Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. *J. Cell Sci.* **117**, 4055–4066 (2004).
27. Baker, B. M., Nargund, A. M., Sun, T. & Haynes, C. M. Protective coupling of mitochondrial function and protein synthesis via the eIF2 $\alpha$  kinase GCN-2. *PLoS Genet.* **8**, e1002760 (2012).
28. Donzeau, M. *et al.* Tim23 Links the Inner and Outer Mitochondrial Membranes. *Cell* **101**, 401–412 (2000).
29. Gakh, O., Cavadini, P. & Isaya, G. Mitochondrial processing peptidases. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1592**, 63–77 (2002).
30. Rolland, S. G. *et al.* Compromised Mitochondrial Protein Import Acts as a Signal for UPRmt. *Cell Rep.* **28**, 1659-1669.e5 (2019).
31. Melber, A. & Haynes, C. M. UPRmt regulation and output: a stress response mediated by mitochondrial-nuclear communication. *Cell Res.* **28**, 281–295 (2018).

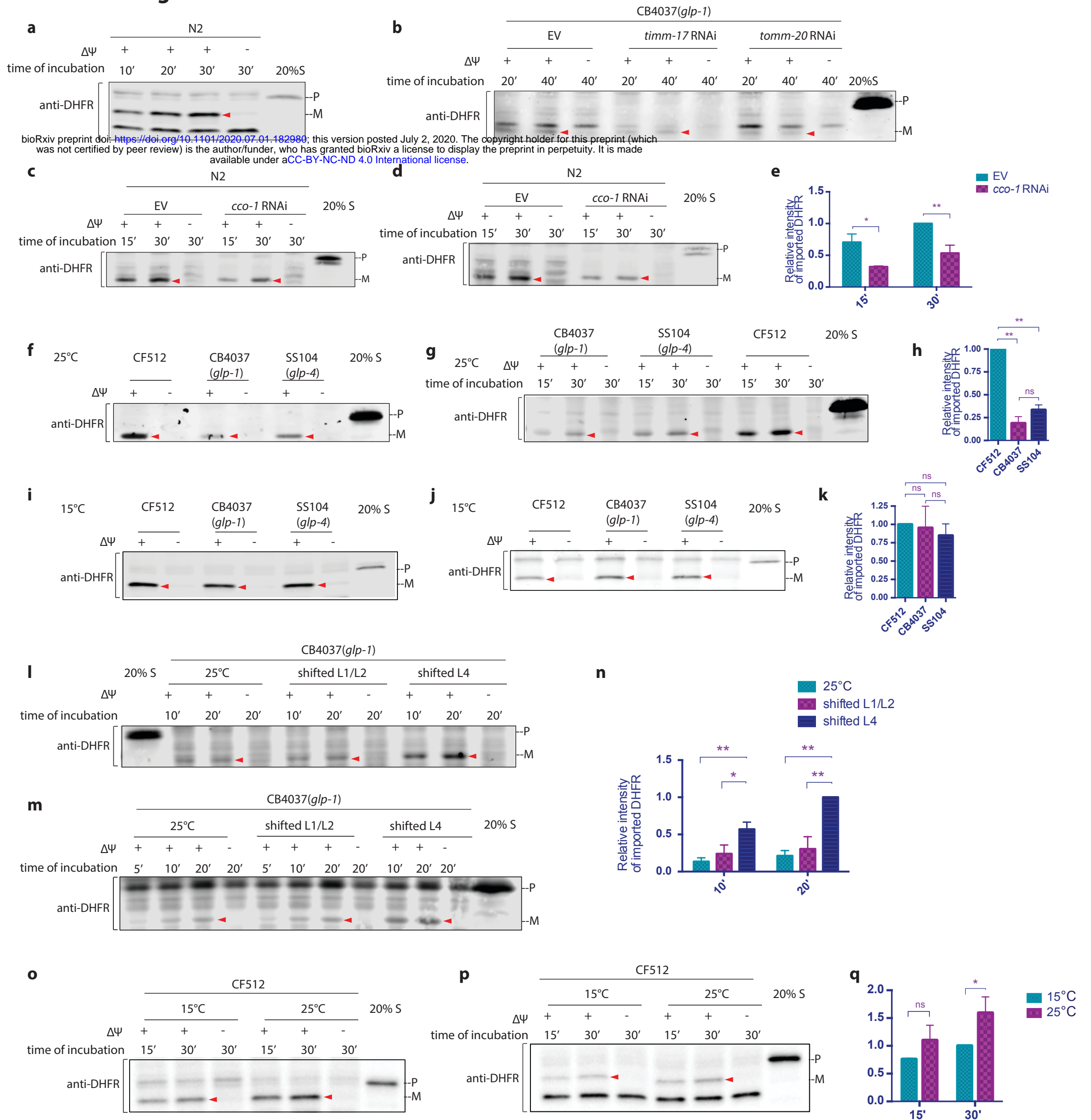
32. The mitochondrial unfolded protein response activator ATFS-1 protects cells from inhibition of the mevalonate pathway. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3625262/>.
33. Rauthan, M., Ranji, P., Abukar, R. & Pilon, M. A Mutation in *Caenorhabditis elegans* NDUF-7 Activates the Mitochondrial Stress Response and Prolongs Lifespan via ROS and CED-4. *G3 Genes Genomes Genet.* **5**, 1639–1648 (2015).
34. Parihar, M. S. & Brewer, G. J. Simultaneous age-related depolarization of mitochondrial membrane potential and increased mitochondrial reactive oxygen species production correlate with age-related glutamate excitotoxicity in rat hippocampal neurons. *J. Neurosci. Res.* **85**, 1018–1032 (2007).
35. Baixauli, F. *et al.* Mitochondrial Respiration Controls Lysosomal Function during Inflammatory T Cell Responses. *Cell Metab.* **22**, 485–498 (2015).
36. Kuznetsov, A. V. & Margreiter, R. Heterogeneity of Mitochondria and Mitochondrial Function within Cells as Another Level of Mitochondrial Complexity. *Int. J. Mol. Sci.* **10**, 1911–1929 (2009).
37. Teichmann, U. *et al.* Substitution of PIM1 Protease in Mitochondria by *Escherichia coli* Lon Protease. *J. Biol. Chem.* **271**, 10137–10142 (1996).

38. Daniele, J. R., Heydari, K., Arriaga, E. A. & Dillin, A. Identification and Characterization of Mitochondrial Subtypes in *Caenorhabditis elegans* via Analysis of Individual Mitochondria by Flow Cytometry. *Anal. Chem.* **88**, 6309–6316 (2016).
  
39. Cho, I., Song, H.-O. & Cho, J. H. Mitochondrial Uncoupling Attenuates Age-Dependent Neurodegeneration in *C. elegans*. *Mol. Cells* **40**, 864–870 (2017).

# Figure 1

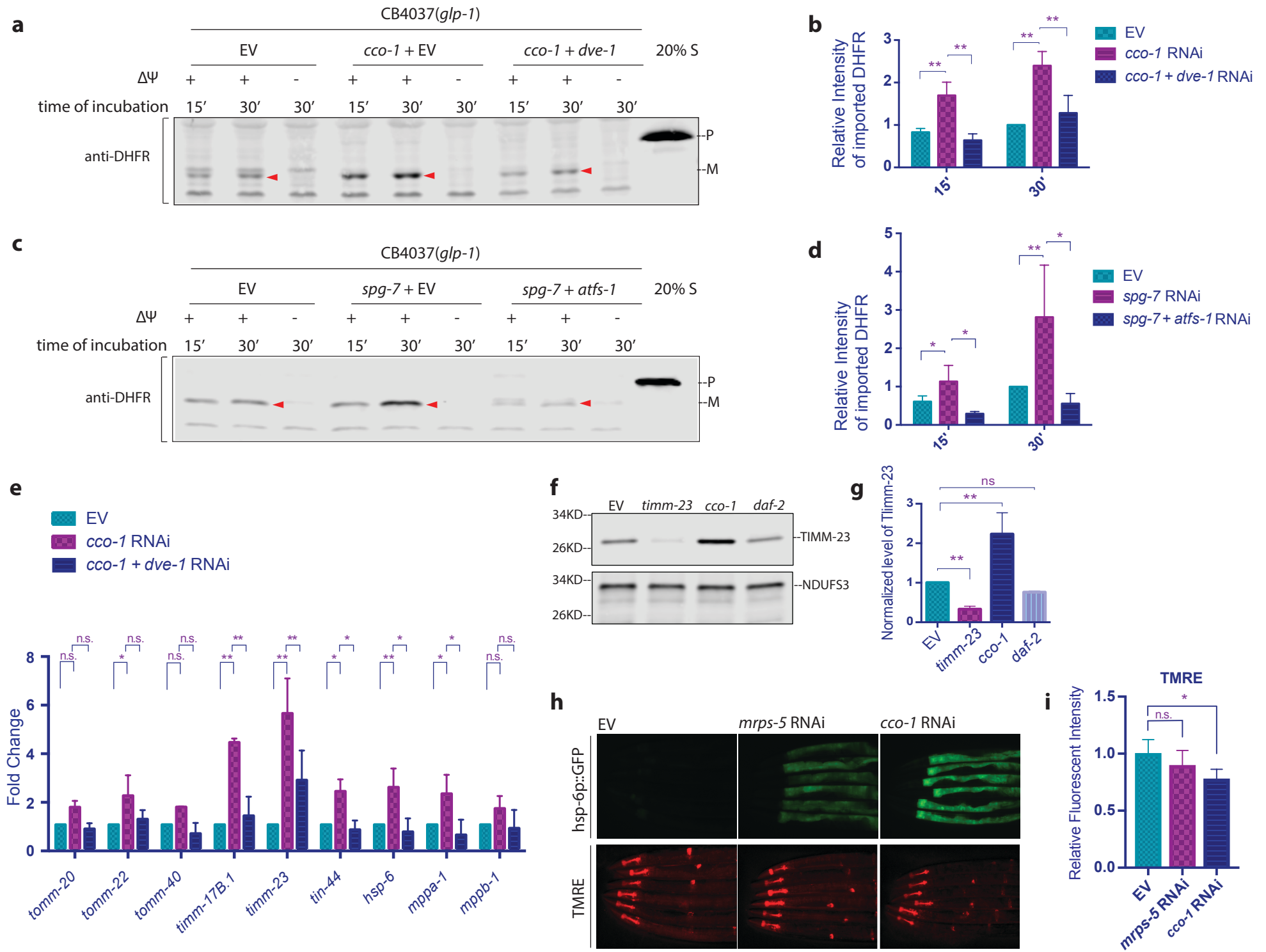


# Extended Data Figure 1



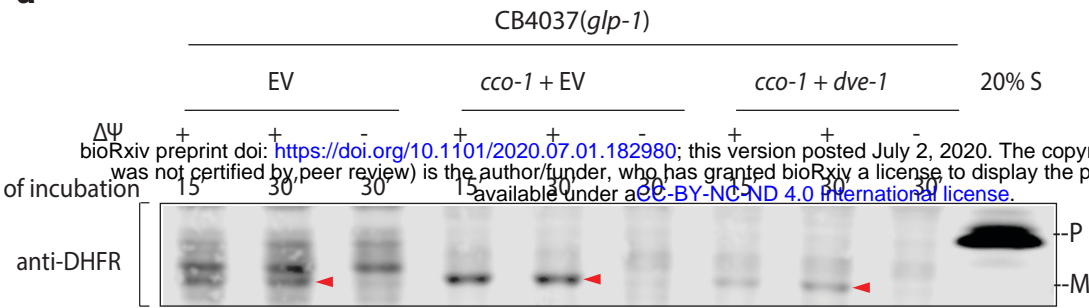


# Figure 2

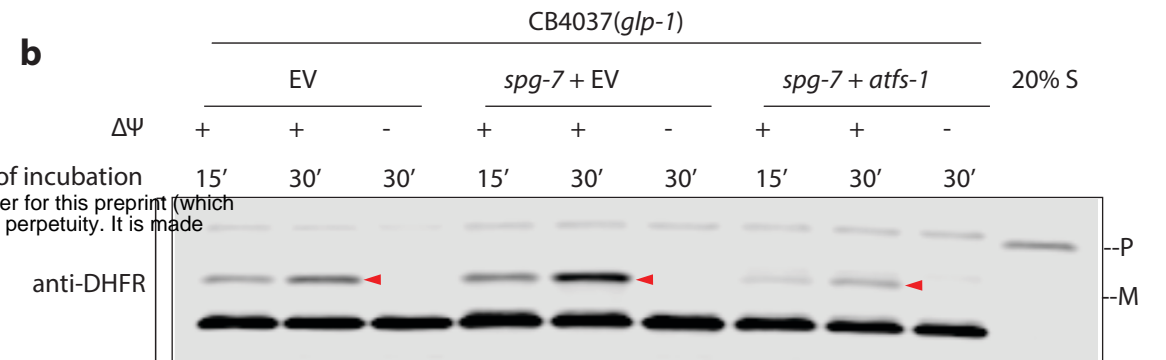


# Extended Data Figure 2

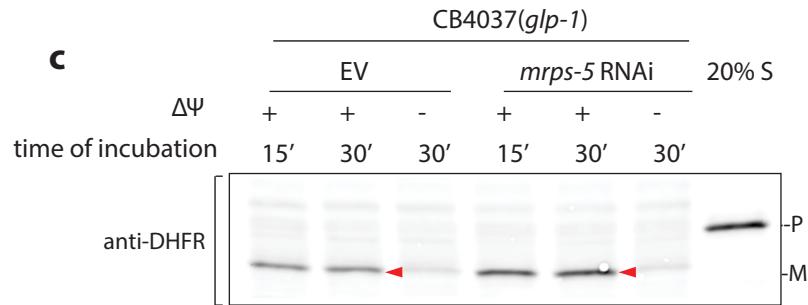
**a**



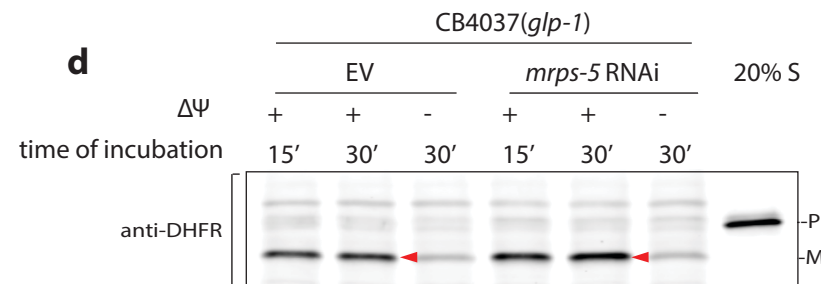
**b**



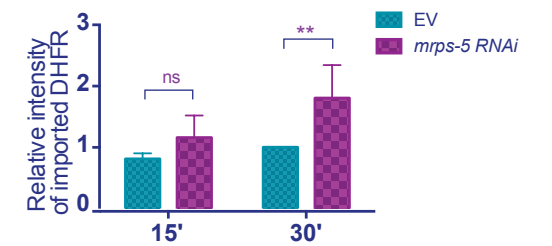
**c**



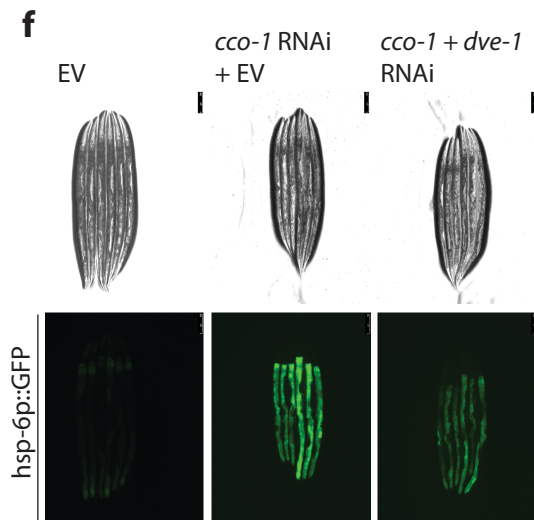
**d**



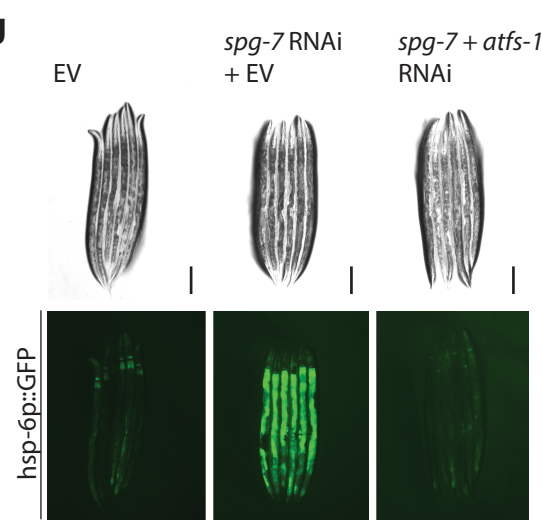
**e**



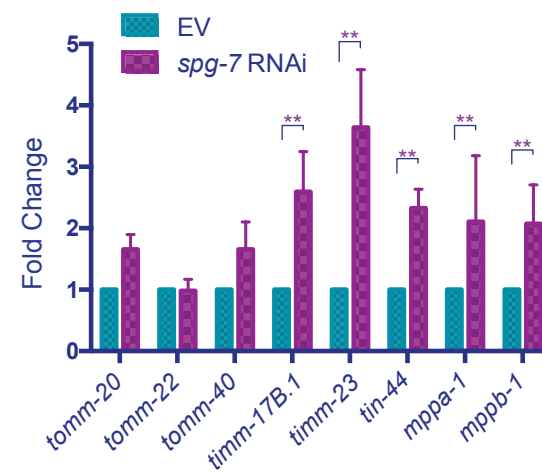
**f**



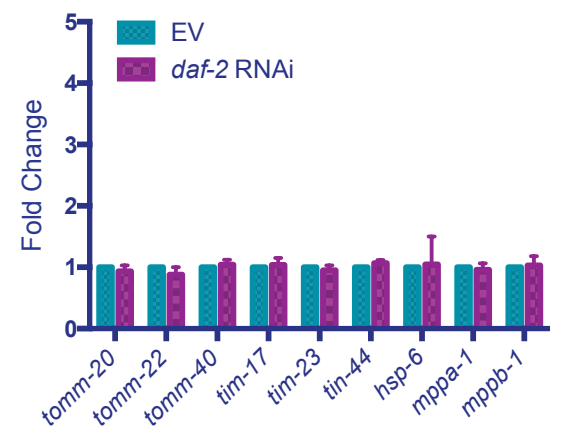
**g**



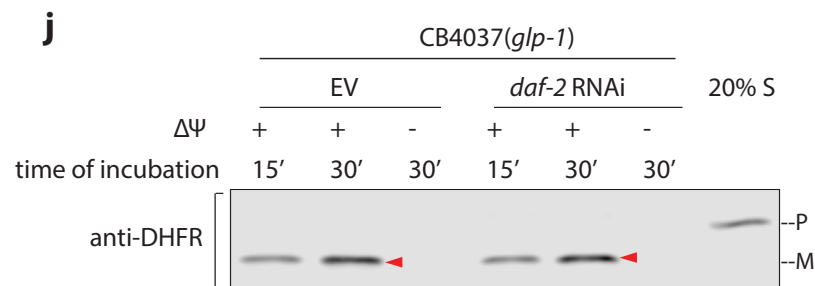
**h**



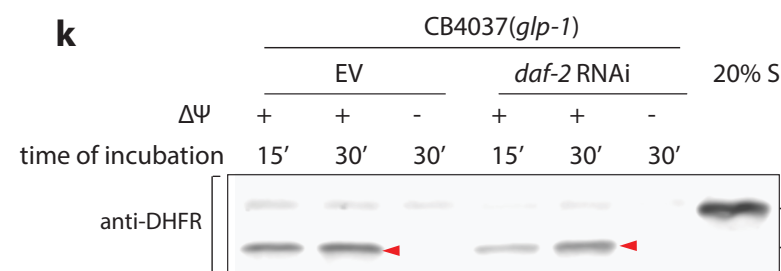
**i**



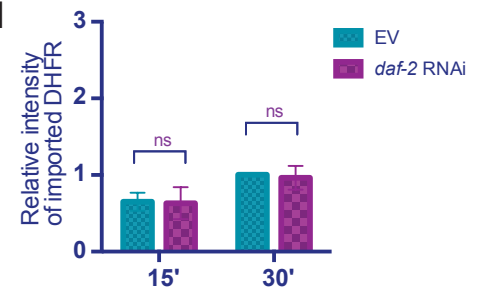
**j**



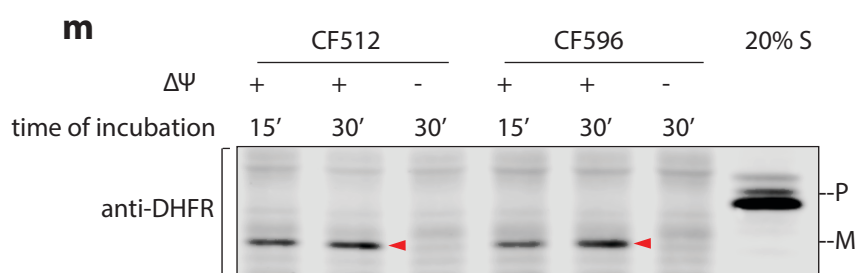
**k**



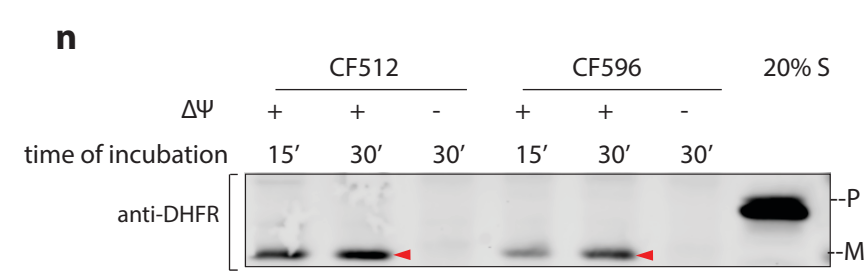
**l**



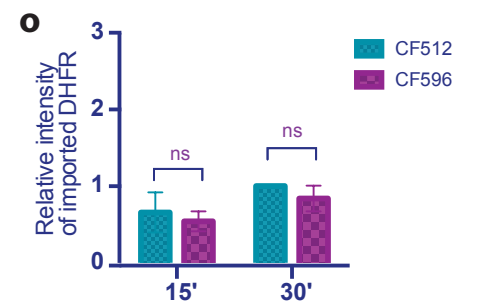
**m**



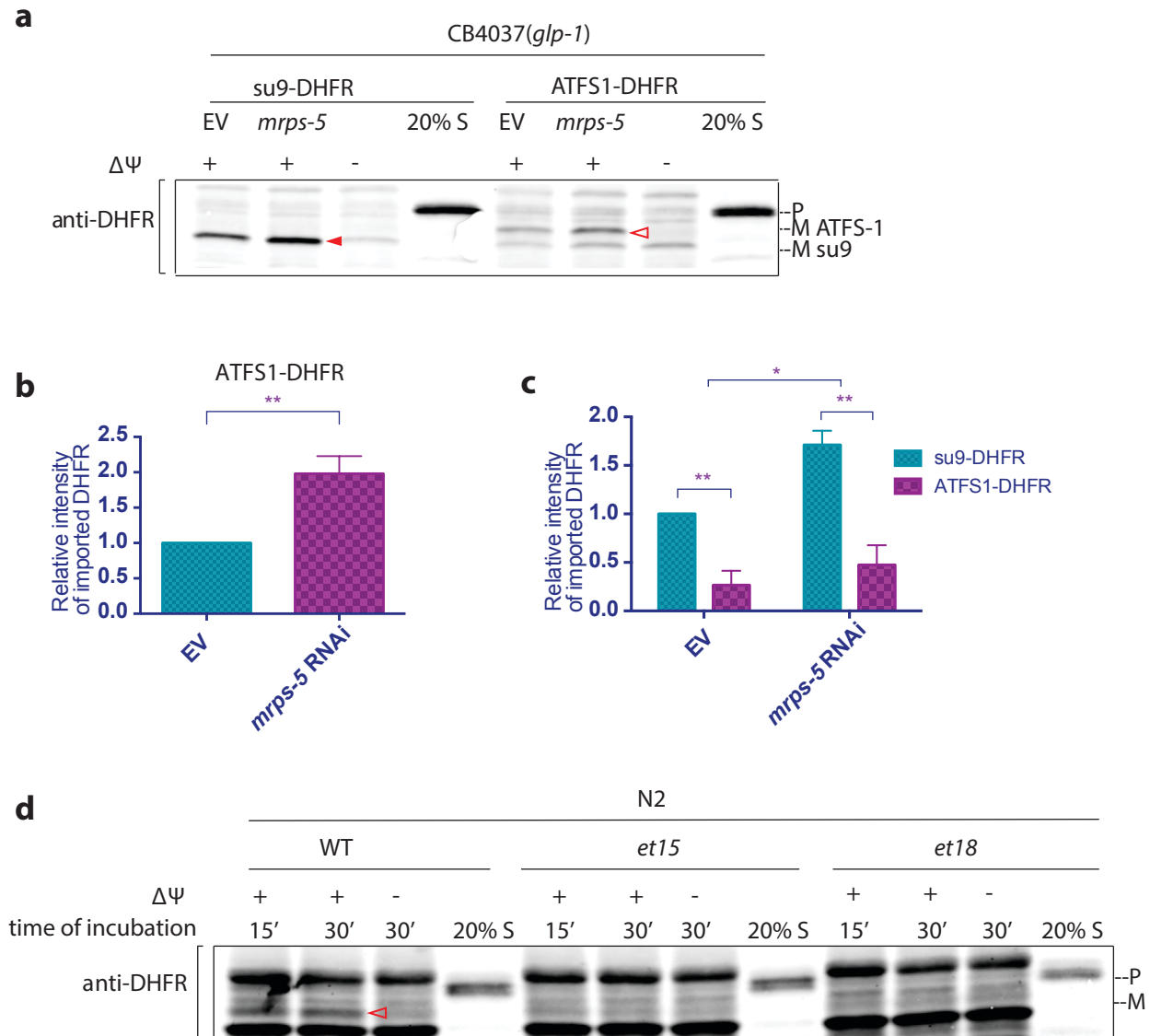
**n**



**o**

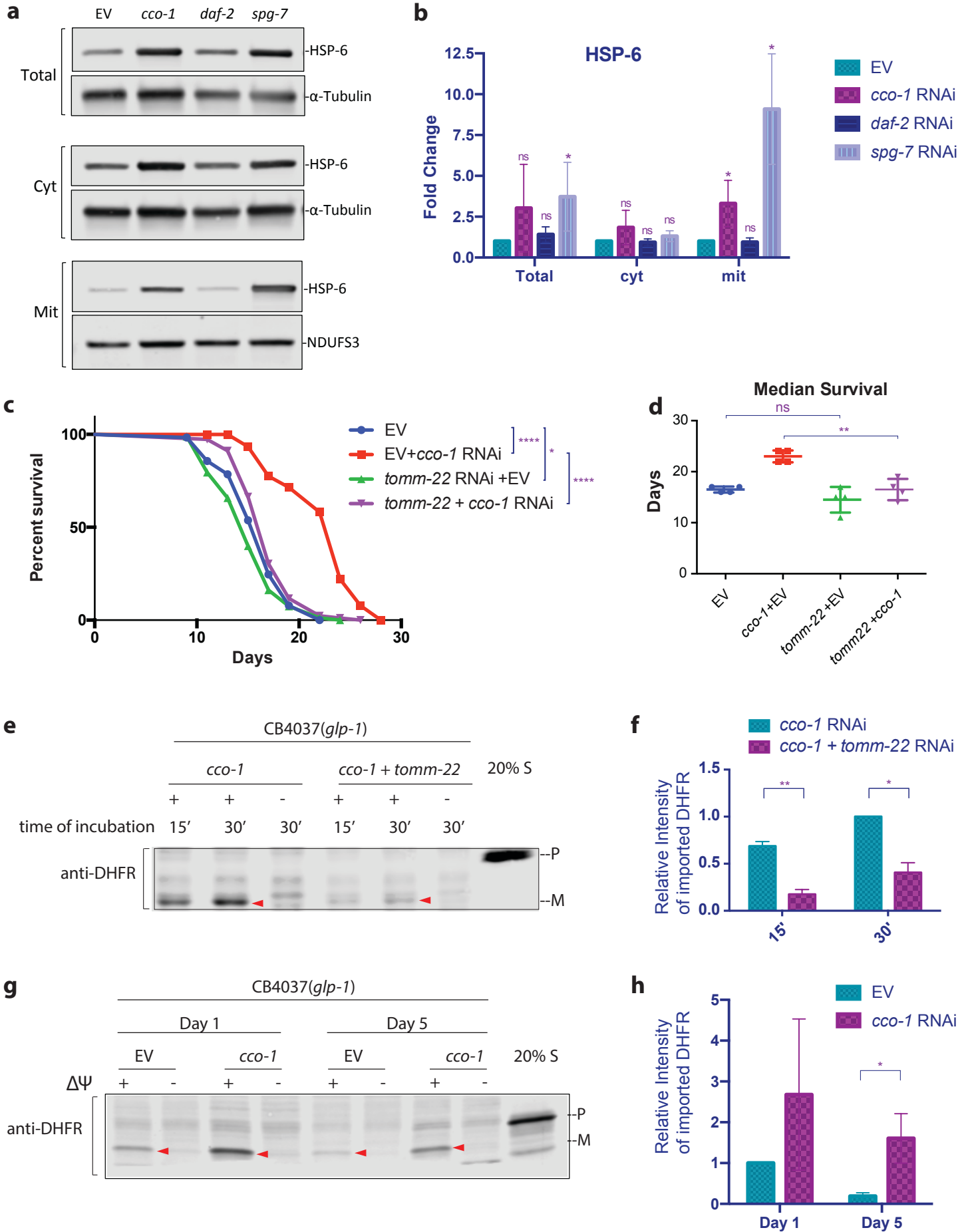


## Figure 3





## Figure 4



## Extended Data Figure 4

