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Mitochondrial unfolded protein response transcription factor ATFS-1 increases resistance to exogenous stressors through upregulation of multiple stress response pathways

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Short title: ATFS-1 promotes stress resistance

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1 **Abstract**

2

3 The mitochondrial unfolded protein response (mitoUPR) is an evolutionarily conserved pathway
4 that responds to various insults to the mitochondria through transcriptional changes that
5 restore mitochondrial homeostasis in order to facilitate cell survival. Gene expression changes
6 resulting from the activation of the mitoUPR are mediated by the transcription factor ATFS-
7 1/ATF-5. To further define the mechanisms through which the mitoUPR protects the cell during
8 mitochondrial dysfunction, we characterized the role of ATFS-1 in responding to organismal
9 stress. We found that activation of ATFS-1 is sufficient to cause upregulation of genes involved
10 in multiple stress response pathways, including the DAF-16-mediated stress response pathway,
11 the SKN-1-mediated oxidative stress response pathway, the HIF-mediated hypoxia response
12 pathway, the p38-mediated innate immune response pathway, and antioxidant genes.
13 Moreover, ATFS-1 is required for the upregulation of stress response genes after exposure to
14 exogenous stressors, especially oxidative stress and bacterial pathogens. Constitutive activation
15 of ATFS-1 increases resistance to multiple acute exogenous stressors, while disruption of *atfs-1*
16 decreases stress resistance. Although ATFS-1-dependent genes are upregulated in multiple
17 long-lived mutants, constitutive activation of ATFS-1 in wild-type animals results in decreased
18 lifespan. Overall, our work demonstrates that ATFS-1 serves a vital role in organismal survival of
19 acute stresses through its ability to activate multiple stress response pathways, but that chronic
20 ATFS-1 activation is detrimental for longevity.

21

22

23 **Keywords:** Aging; Mitochondria; Mitochondrial unfolded protein response; ATFS-1; Stress
24 resistance; *C. elegans*; Genetics; Lifespan; RNA sequencing

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25 **Introduction**

26

27 The mitochondrial unfolded protein response (mitoUPR) is a stress response pathway
28 that acts to reestablish mitochondrial homeostasis through inducing transcriptional changes of
29 genes involved in metabolism and restoration of mitochondrial protein folding [1]. Various
30 perturbations to the mitochondria can activate mitoUPR, including excess reactive oxygen
31 species (ROS) and defects in mitochondrial import machinery [2]. The mitoUPR is mediated by
32 the transcription factor ATFS-1 (activating transcription factor associated with stress-1) in *C.*
33 *elegans* [3], or ATF5 in mammals [4].

34

35 ATFS-1/ATF5 regulates mitoUPR through its dual targeting domains, a mitochondrial
36 targeting sequence (MTS) and a nuclear localization signal (NLS). Under normal unstressed
37 conditions, the MTS causes ATFS-1 to enter the mitochondria through the HAF-1 import
38 channel. Inside the mitochondria, ATFS-1 is degraded by the Lon protease CLPP-1/CLP1 [3].
39 However, mitochondrial stress disrupts ATFS-1 import into the mitochondria, resulting in
40 cytoplasmic accumulation of ATFS-1. The NLS of the cytoplasmic ATFS-1 then targets it to the
41 nucleus, where ATFS-1 acts with the transcription factor DVE-1 and transcriptional regulator
42 UBL-5 to upregulate expression of chaperones, proteases, and other proteins [5].

43

44 In order to study the role of the mitoUPR in longevity, we previously disrupted *atfs-1* in
45 long-lived *nuo-6* mutants, which contain a point mutation that affects Complex I of the electron
46 transport chain [6]. *nuo-6* mutants have a mild impairment of mitochondrial function that leads
47 to increased lifespan and enhanced resistance to multiple stressors. We found that loss of *atfs-*
48 *1* not only decreased the lifespan of *nuo-6* worms, but also abolished the increased stress
49 resistance of these worms, thereby suggesting that ATFS-1 contributes to both longevity and
50 stress resistance in these worms [7].

51

52 While a role for the mitoUPR in longevity has been reported [8-11], and debated [12,
53 13], little is known about the role of ATFS-1 in response to exogenous stressors. Pellegrino *et al.*

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54 found that activation of ATFS-1 can increase organismal resistance to the pathogenic bacteria *P.*
55 *aeruginosa* [14], while Pena *et al.* showed that ATFS-1 activation can protect against anoxia-
56 reperfusion-induced death [15].
57
58 In this study, we use *C. elegans* to define the relationship between ATFS-1 and
59 organismal stress resistance, and explore the underlying mechanisms. We find that activation of
60 ATFS-1 is sufficient to upregulate genes from multiple stress response pathways and is
61 important for transcriptional changes induced by oxidative stress and bacterial pathogen
62 exposure. Constitutive activation of ATFS-1 is also sufficient to increase resistance to multiple
63 stressors. While ATFS-1-dependent genes are upregulated in several long-lived mutants
64 representative of multiple pathways of lifespan extension, chronic activation of ATFS-1 does not
65 extend longevity. Overall, our results demonstrate a crucial role for ATFS-1 in organismal stress
66 response through activation of multiple stress response pathways.

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67 **Results**

68

69 **ATFS-1 activates genes from multiple stress response pathways**

70 Mild impairment of mitochondrial function through a mutation in *nuo-6* results in the activation
71 of the mitoUPR. We previously performed a bioinformatic analysis of genes that are
72 upregulated in *nuo-6* mutants in an ATFS-1-dependent manner, and discovered an enrichment
73 for genes associated with the GO term "response to stress" [7]. Based on this observation, we
74 hypothesized that ATFS-1 may be able to activate other stress response pathways. To test this
75 hypothesis, we quantified the expression of established target genes from eight different stress
76 response pathways under conditions where ATFS-1 is either activated, or where ATFS-1 is
77 disrupted.

78

79 To activate ATFS-1, we used the *nuo-6* mutation. We also examined gene expression in
80 two different gain-of-function (GOF) mutants with constitutively active ATFS-1: *atfs-1(et15)* and
81 *atfs-1(et17)*. Both of these constitutively active ATFS-1 mutants have mutations in the MTS
82 causing increased nuclear localization of ATFS-1 [16]. We used a loss-of-function (LOF) *atfs-1*
83 deletion mutation (*gk3094*) to disrupt ATFS-1 function in wild-type and *nuo-6* mutants.

84

85 Expression of specific stress response target genes were examined: *hsp-6* in the
86 mitochondrial unfolded protein response (mitoUPR) pathway; *hsp-4* in the endoplasmic
87 reticulum unfolded protein response (ER-UPR) pathway; *hsp-16.2* in the cytoplasmic unfolded
88 protein response pathway (cytoUPR); *sod-3* in the DAF-16-mediated stress response pathway;
89 *gst-4* in the SKN-1-mediated stress response pathway; *nhr-57* in the HIF-1-mediated hypoxia
90 response pathway; *Y9C9A.8* in the p38-mediated innate immunity pathway; and *trx-2*, an
91 antioxidant gene (**Table S1**).

92

93 We found that compared to wild-type worms, *atfs-1(gk3094)* deletion mutants did not
94 have decreased expression levels for the target genes of any of the stress response pathways

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95 (**Fig. 1**). This indicates that ATFS-1 is not required for the basal expression levels of these stress
96 response genes.
97
98 Activation of the mitoUPR through mutation of *nuo-6* resulted in significant
99 upregulation of target genes from the mitoUPR (*hsp-6*; **Fig. 1A**), the DAF-16-mediated stress
100 response (*sod-3*; **Fig. 1D**), the SKN-1-mediated oxidative stress response (*gst-4*; **Fig. 1E**), the HIF-
101 1-mediated hypoxia response (*nhr-57*; **Fig. 1F**), the p38-mediated innate immunity pathway
102 (*Y9C9A.8*; **Fig. 1G**), and antioxidant defense (*trx-2*; **Fig. 1H**). Importantly, for all of these genes,
103 inhibiting the mitoUPR through deletion of *atfs-1* prevented the upregulation of the stress
104 response in *nuo-6;atfs-1(gk3094)* worms (**Fig. 1A, D-H**), indicating that ATFS-1 is required for
105 the activation of these stress pathway genes during mitochondrial stress.
106
107 Constitutive activation of ATFS-1 in *atfs-1(et 15)* and *atfs-1(et17)* mutants resulted in
108 upregulation of most of the same genes that are upregulated in *nuo-6* mutants, except for *gst-4*
109 in the SKN-1 pathway (**Fig. 1A, D-H**). This indicates that ATFS-1 activation is sufficient to induce
110 upregulation of specific stress response genes independently of mitochondrial stress. Activating
111 the mitoUPR through the *nuo-6* mutation, or through the constitutively-active ATFS-1 mutants
112 did not significantly increase the expression of target genes from the ER-UPR (*hsp-4*; **Fig. 1B**) or
113 the cyto-UPR (*hsp-16.2;* **Fig. 1C**).
114
115 To gain a more comprehensive view of the extent to which mitoUPR activation causes
116 upregulation of genes in other stress response pathways, we compared genes upregulated in
117 the constitutively active *atfs-1* mutant, *atfs-1(et15),* to genes upregulated by activation of
118 different stress response pathways. As a proof-of-principle, we first examined the overlap
119 between upregulated genes in *atfs-1(et15)* mutants and genes upregulated by activation of the
120 mitoUPR with *spg-7* RNAi in an ATFS-1-dependent manner [3].
121
122 We identified genes upregulated by the activation of other stress response pathways
123 from published gene expression studies, and the genes and relevant pathways are listed in

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124 **Table S3**. Target genes from the ER-UPR pathway were defined as genes upregulated by
125 tunicamycin exposure and dependent on either *ire-1*, *xbp-1*, *pek-1* or *atf-6* [17]. Cyto-UPR
126 pathway genes are genes upregulated by overexpression of heat shock factor 1 (HSF-1) and
127 genes bound by HSF-1 after a thirty-minute heat shock at 34°C [18, 19]. DAF-16 pathway genes
128 were identified by Tepper et al. by performing a meta-analysis of 46 previous gene expression
129 studies, comparing conditions in which DAF-16 is activated (e.g. *daf-2* mutants) and conditions
130 in which the activation is inhibited by disruption of *daf-16* (e.g. *daf-2;daf-16* mutants) [20]. SKN-
131 1 pathway genes were identified as genes that exhibit decreased expression after *skn-1* RNAi in
132 wild-type worms, genes that are upregulated in *glp-1* mutants in a SKN-1-dependent manner,
133 genes that are upregulated by germline stem cell removal in a SKN-1-dependent manner [21],
134 and genes upregulated in *daf-2* mutants in a SKN-1-dependent manner [22]. HIF-1-mediated
135 hypoxia genes are genes induced by hypoxia in a HIF-1-dependent manner [23]. Innate
136 immunity genes are defined as genes upregulated by exposure to *Pseudomonas aeruginosa*
137 strain PA14 in a PMK-1- and ATF-7-dependent manner [24], where PMK-1 and ATF-7 are part of
138 the p38-mediated innate immune signaling pathway. Finally, antioxidant genes is a
139 comprehensive list of genes involved in antioxidant defense such as superoxide dismutases
140 (*sod*), catalases (*ctl*), peroxiredoxins (*prdx*), or thioredoxins (*trx*).
141

142 In comparing genes upregulated in the constitutively active *atfs-1* mutant *et15* to these
143 previously published gene lists, we found that 51% of genes upregulated by *spg-7* RNAi in an
144 ATFS-1-dependent manner are also upregulated by constitutive activation of ATFS-1 (**Fig. 2A**).
145 Similarly, we found a highly significant overlap of upregulated genes between *atfs-1(et15)*
146 mutants and each of the examined stress response pathways. We found that *atfs-1(et15)* had a
147 25% overlap with genes of ER-UPR pathway (**Fig. 2B**); 22% overlap with genes of the Cyto-UPR
148 pathway (**Fig. 2C**); 26% overlap with genes of the DAF-16-mediated stress pathway (**Fig. 2D**);
149 30% overlap with genes of the SKN-1-mediated stress pathway (**Fig. 2E**); 23% overlap with
150 genes of the HIF-1-mediated hypoxia pathway (**Fig. 2F**); 22% overlap with genes of the p38-
151 mediated innate immunity pathway (**Fig. 2G**); and 33% overlap with antioxidant genes (**Fig. 2H**).

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152 Combined, this indicates that activation of ATFS-1 is sufficient to upregulate genes in multiple
153 stress response pathways.
154

155 **ATFS-1 is required for transcriptional responses to exogenous stressors**

156 Having shown that constitutive activation of ATFS-1 can induce upregulation of genes involved
157 in various stress response pathways, we next sought to determine the role of ATFS-1 in the
158 genetic response to different stressors. To do this, we exposed wild-type animals and *atfs-1*
159 loss-of-function mutants (*atfs-1(gk3094)*) to six different types of stress and quantified the
160 resulting upregulation of stress response genes using quantitative RT-PCR (qPCR). We found
161 that exposure to either oxidative stress (4 mM paraquat, 48 hours) or the bacterial pathogen
162 *Pseudomonas aeruginosa* strain PA14 induced a significant upregulation of stress response
163 genes in wild-type worms, but that this upregulation was suppressed by disruption of *atfs-1*
164 (**Fig. 3A,B**). In contrast, exposure to heat stress (35°C, 2 hours; **Fig. 3C**), osmotic stress (300 mM
165 NaCl, 24 hours; **Fig. 3D**), anoxic stress (24 hours; **Fig. 3E**), or ER stress (tunicamycin for 24 hours;
166 **Fig. 3F**) caused upregulation of stress response genes in both wild-type and *atfs-1(gk3094)*
167 worms to a similar extent. Combined, these results indicate that ATFS-1 is required for
168 upregulating stress response genes in response to exposure to oxidative stress or bacterial
169 pathogens.
170

171 **Modulation of ATFS-1 levels affects resistance to multiple stressors**

172 Due to the crucial role of ATFS-1 in upregulating genes in multiple stress response pathways, we
173 next sought to determine the extent to which activating ATFS-1 protects against exogenous
174 stressors. To do this, we quantified resistance to stress in two constitutively active *atfs-1* gain-
175 of-function mutants (*atfs-1(et15), atfs-1(et17)*) compared to wild-type worms. For comparison,
176 we also included an *atfs-1* loss-of-function deletion mutant (*atfs-1(gk3094)*), which we have
177 previously shown to have decreased resistance to oxidative, heat, osmotic and anoxic stress [7].
178

179 We measured resistance to acute oxidative stress by exposing worms to 300 µM
180 juglone. We found that both the gain-of-function mutants, *atfs-1(et15)* and *atfs-1(et17),* have

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181 increased resistance to acute oxidative stress compared to wild-type worms, while *atfs-*
182 *1(gk3094)* deletion mutants were less resistant compared to wild-type worms (**Fig. 4A**). To test
183 resistance to chronic oxidative stress, worms were transferred to plates containing 4 mM
184 paraquat beginning at day 1 of adulthood. Similar to the acute assay, *atfs-1(et17)* mutants were
185 more resistant to chronic oxidative stress, while *atfs-1(gk3094)* mutants were less resistant to
186 chronic oxidative stress compared to wild-type worms (**Fig. 4B**). Oddly, *atfs-1(et15)* gain-of-
187 function mutants exhibited decreased resistance to chronic oxidative stress.
188
189 Resistance to heat stress was measured at 37°C. None of the mutants showed increased
190 survival during heat stress, with *atfs-1(et15)* and *atfs-1(gk3094)* mutants both showing a
191 significant decrease in survival compared to wild-type worms (**Fig. 4C**). Resistance to osmotic
192 stress was quantified on plates containing 450 mM or 500 mM NaCl after 48 hours. At both
193 concentrations, the constitutively active *atfs-1* mutants had increased survival compared to
194 wild-type worms, while *atfs-1(gk3094)* deletion mutants had decreased survival, although the
195 difference was only significant at 500 mM (**Fig. 4D, E**). Resistance to anoxic stress was measured
196 by placing worms in an oxygen-free environment for 75 hours, followed by a 24-hour recovery.
197 We observed increased survival in *atfs-1(et15)* and *atfs-1(et17)* mutants and decreased survival
198 in *atfs-1(gk3094)* mutant compared to wild-type worms (**Fig. 4F**).
199
200 Lastly, to test resistance to bacterial pathogens, worms were exposed to *Pseudomonas*
201 *aeruginosa* strain PA14 in either a fast kill assay in which worms die from a toxin produced by
202 the bacteria, or a slow kill assay in which worms die due to the intestinal colonization of the
203 pathogenic bacteria [25]. In the fast kill assay, we found that constitutive activation of ATFS-1
204 increases survival in *atfs-1(et15)* and *atfs-1(et17)* mutants (**Fig. 4G**). We also observed increased
205 survival in *atfs-1(gk3094)* deletion mutants. For the slow kill assay, we used two established
206 protocols: one in which the assay is initiated at the L4 larval stage and performed at 25°C [14,
207 25, 26] and one in which the assay is initiated at day three of adulthood and performed at 20°C
208 [27]. Surprisingly, at 25°C, we found that *atfs-1(et17)* mutant had a small decrease in resistance
209 to PA14, while *atfs-1(gk3094)* mutants exhibited a small increase in resistance to PA14

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210 compared to wild-type worms (**Fig. 4H**). At 20°C, both *atfs-1(gk3094)* and *atfs-1(et17*) mutants
211 had a small increase in resistance to PA14 compared to wild-type worms (**Fig. I**).
212
213 All together, these data indicate that activation of ATFS-1 is sufficient to protect against
214 oxidative stress, osmotic stress, anoxia, and bacterial pathogens but not heat stress. They also
215 show that ATFS-1 is required for wild-type worms to survive oxidative stress, heat stress,
216 osmotic stress, and anoxia.
217

218 **Long-lived genetic mutants have upregulation of ATFS-1 target genes**

219 We previously showed that ATFS-1 target genes are upregulated in three long-lived
220 mitochondrial mutants: *clk-1, isp-1* and *nuo-6* [6, 7, 28, 29]. To determine if ATFS-1 target genes
221 are specifically upregulated in long-lived mitochondrial mutants, or if they are also upregulated
222 in other mutants with extended longevity, we compared gene expression in six additional long-
223 lived mutants, which act through other longevity-promoting pathways, to genes that are
224 upregulated by ATFS-1 activation. These long-lived mutants included *sod-2* mutants, which act
225 through increasing mitochondrial reactive oxygen species (ROS) [30]; *daf-2* mutants, which
226 have reduced insulin-IGF1 signaling [31]; *glp-1* mutants, which have germline ablation [32]; *ife-2*
227 mutants, which have reduced translation [33]; *osm-5* with reduced chemosensation [34]; and
228 *eat-2* with dietary restriction [35].
229
230 After identifying genes that are differentially expressed in each of these long-lived
231 mutants, we compared the differentially expressed genes to genes that are upregulated by
232 ATFS-1 activation. We defined ATFS-1-upregulated genes in two ways: (1) genes that are
233 upregulated by *spg-7* RNAi in an ATFS-1-dependent manner [3]; and (2) genes that are
234 upregulated in a constitutively active *atfs-1* mutant (*et15;* [7]).
235
236 We found that the majority of the long-lived mutants examined had a significant
237 enrichment of ATFS-1 target genes. In comparing the number of overlapping genes between
238 genes upregulated in each long-lived mutant and genes upregulated by *spg-7* RNAi in an ATFS-

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239 1-dependent manner, the degree of overlap was significantly greater than would be expected
240 by chance for *clk-1* (6.7 fold enrichment), *isp-1* (6.0 fold enrichment), *sod-2* (5.5 fold
241 enrichment), *nuo-6* (4.1 fold enrichment), *daf-2* (2.6 fold enrichment), *glp-1* (2.0 fold
242 enrichment), and *ife-2* mutants (1.5 fold enrichment)(**Fig. 5**). We did not find a significant
243 enrichment of ATFS-1 targets in *osm-5* and *eat-2* worms (**Fig. 5**).
244
245 In comparing the number of overlapping genes between genes upregulated in each
246 long-lived mutant and genes upregulated in the constitutively active *atfs-1(et15)* mutant, we
247 found that the degree of overlap was significantly greater than would be expected by chance
248 for *isp-1* (3.5 fold enrichment), *sod-2* (3.4 fold enrichment), *clk-1* (3.3 fold enrichment), *nuo-6*
249 (2.5 fold enrichment), *daf-2* (2.4 fold enrichment), *glp-1* (1.8 fold enrichment), *ife-2* (1.8 fold
250 enrichment), and *eat-2* mutants (1.5 fold enrichment) (**Fig. S1**). We did not observe a significant
251 enrichment of ATFS-1 targets in *osm-5* mutants (**Fig. S1**).
252
253 Overall, these results indicate that ATFS-1 target genes are upregulated in multiple long-
254 lived mutants, including mutants in which mitochondrial function is not directly disrupted.
255
256 **Constitutively active *atfs-1* mutants have decreased lifespan despite enhanced resistance to**
257 **stress**
258 Having shown that ATFS-1 target genes are activated in multiple long-lived mutants, we sought
259 to determine if ATFS-1 activation is sufficient to increase lifespan, and whether the presence of
260 ATFS-1 is required for normal longevity in wild-type worms. Despite having increased resistance
261 to multiple stresses, both constitutively active *atfs-1* mutants (*et15* and *et17*) have decreased
262 lifespan compared to wild-type worms (**Fig. 6A,B**), which is consistent with a previous study
263 finding shortened lifespan in *atfs-1(et17)* and *atfs-1(et18)* worms [13]. Despite having
264 decreased resistance to multiple stresses, *atfs-1* deletion mutants (*gk3094*), had a lifespan
265 comparable to wild-type worms (**Fig. 6C**), as we have previously observed [7]. Combined, this
266 indicates that ATFS-1 does not play a major role in lifespan determination in a wild-type
267 background despite having an important role in stress resistance.

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268 **Discussion**

269

270 Mitochondria are vital for organismal health as they perform multiple crucial functions within
271 the cell including energy generation, metabolic reactions and intracellular signaling.
272 Accordingly, it is important for cell and organismal survival maintain mitochondrial function
273 during times of acute stress, and throughout normal aging. The mitoUPR is a conserved
274 pathway that facilitates restoration of mitochondrial homeostasis after internal or external
275 stresses. In this work, we demonstrate a crucial role for the mitoUPR transcription factor ATFS-1
276 in the genetic response to external stressors which ultimately promotes survival of the
277 organism.

278

279 **ATFS-1 is not required for normal longevity**

280 A number of studies have directly or indirectly examined the role of the mitoUPR and or ATFS-1
281 in longevity. In these studies, mitoUPR activation was typically measured using a mitoUPR
282 reporter strain that expresses GFP under the promoter of *hsp-6*, which is a target gene of ATFS-
283 1 and the mitoUPR.

284

285 A relationship between the mitoUPR and longevity was initially supported by the
286 observation that disruption of the mitochondrial electron transport chain (ETC) by RNAi
287 knockdown of the cytochrome c oxidase-1 (*cco-1*) gene resulted in both increased lifespan [36]
288 and activation of the mitoUPR [8, 37]. Since then, other lifespan-extending mutations have also
289 been shown to activate the mitoUPR, including three long-lived mitochondrial mutants, *clk-1,*
290 *isp-1* and *nuo-6* [7].

291

292 To explore this relationship in more comprehensive manner, Runkel *et al.* compiled a list
293 of genes that activate the mitoUPR and looked at their effect on lifespan. Of the 99 genes
294 reported to activated the mitoUPR, 58 resulted in increased lifespan, while only 7 resulted in
295 decreased lifespan [38]. Bennet *et al.* performed an RNAi screen to identify RNAi clones that
296 increase expression of a reporter of mitoUPR activity (*hsp-6p::GFP*) and measured the effect of

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297 a selection of these clones on lifespan [13]. Of the 19 mitoUPR-inducing RNAi clones that they
298 tested, 10 RNAi clones increased lifespan, while 6 decreased lifespan [13]. Using a similar
299 approach to screen for compounds that activate a mitoUPR reporter strain (*hsp-6p::GFP*),
300 metolazone was identified as a compound that activates the mitoUPR, and extends lifespan in
301 an ATFS-1-dependent manner [39]. Combined these results indicate that there are multiple
302 genes or interventions which activate the mitoUPR and extend longevity, but there are also
303 examples in which these phenotypes are uncoupled.
304
305 Multiple experiments including the present study have also looked at the effect of the
306 mitoUPR on lifespan directly by either increasing or decreasing the expression of components
307 of the mitoUPR. Knocking down *atfs-1* expression using RNAi does not decrease wild-type
308 lifespan [7, 13, 40], nor do deletions in the *atfs-1* gene decrease wild-type lifespan (**Fig. 6**; [7,
309 13]). Thus, despite activation of the mitoUPR being correlated with longevity, ATFS-1 is not
310 required for normal lifespan in a wild-type animal.
311
312 **ATFS-1 mediates lifespan extension in long-lived mutants**
313 While ATFS-1 is dispensable for wild-type lifespan, ATFS-1 is required for lifespan extension of
314 multiple long-lived mutants. Longevity can be extended by disrupting mito-nuclear protein
315 balance through knocking down the expression of mitochondrial ribosomal protein S5 (*mrsp*-5),
316 which also increases the expression of mitoUPR target gene *hsp-6*. The magnitude of the
317 lifespan extension caused by *mrsp-5* RNAi is decreased by knocking down key mitoUPR
318 component genes *haf-1* or *ubl-5* [11]. In the long-lived mitochondrial mutant *nuo-6,* deletion of
319 *atfs-1* completely reverts the long lifespan to wild-type length, and treatment with *atfs-1* RNAi
320 has similar effects [7]. In mitochondrial mutant *isp-1* worms, knocking down the a key initiatior
321 of mitoUPR, *ubl-5,* decreases their long lifespan but has no effect on the lifespan of wild-type
322 worms [8]. In contrast it has been reported that knockdown of *atfs-1* using RNAi does not
323 decrease *isp-1* lifespan [13]. However, it is possible that in the latter study that the magnitude
324 knockdown was not sufficient to have effects on lifespan, as we and others have found that life-
325 long exposure to *atfs-1* RNAi prevents larval development of *isp-1* worms [7, 41]. Similarly,

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326 differing results have been obtained for the requirement of the mitoUPR in the extended
327 lifespan resulting from *cco-1* knockdown. While it has been reported that mutation of *atfs-1*
328 does not decrease lifespan of worms treated with *cco-1* RNAi, despite preventing activation of
329 mitoUPR reporter [13], a subsequent study showed that *atfs-1* RNAi decreases the extent of
330 lifespan extension resulting from *cco-1* RNAi [40]. While differing results have been observed in
331 some cases, overall, these studies suggest a role of ATFS-1 and the mitoUPR in mediating the
332 lifespan extension in a subset of long-lived mutants.
333
334 Despite the fact that long-lived mutants with chronic activation of the mitoUPR depend
335 on ATFS-1 for their long lifespan, our current results using the constitutively active *atfs-1(et15)*
336 and *atfs-1(et17)* mutants, as well as previous results using constitutively active *atfs-1* mutants
337 (*et17* and *et18*) show that constitutive activation of ATFS-1 in wild-type worms results in
338 decreased lifespan [13]. This may be partially due to activation of ATFS-1 increasing the
339 proportion of damaged mtDNA when heteroplasmy exists [42]. Consistent with this finding,
340 overexpression of the mitoUPR target gene *hsp-60* also leads to a small decrease in lifespan
341 [43]. In contrast, overexpression of a different mitoUPR target gene, *hsp-6,* is sufficient to
342 increase lifespan [44]. It has also been shown that a hypomorphic reduction-of-function
343 mutation allele of *hsp-6* (*mg583*) can also increase lifespan, while *hsp-6* null mutations are
344 thought to be lethal [45]. Combined these results indicate that chronic activation of the
345 mitoUPR is mildly detrimental for lifespan, but that modulation of specific target genes can be
346 beneficial.
347
348 **ATFS-1 is necessary for stress resistance in wild-type animals**
349 While ATFS-1 is not required for longevity in wild-type animals, it plays a significant role in
350 protecting animals against exogenous stressors. Disrupting *atfs-1* function decreases
351 organismal resistance to oxidative stress, heat stress, osmotic stress, and anoxia (**Fig. 4**).
352 Additionally, we previously determined that inhibiting *atfs-1* in long-lived *nuo*-6 worms
353 completely prevented the increased resistance to oxidative stress, osmotic stress and heat
354 stress typically observed in that mutant [7], and that disruption of *atfs-1* in Parkinson's disease

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355 mutants *pdr-1* and *pink-1* decreased their resistance to oxidative stress, osmotic stress, heat
356 stress, and anoxia [46]. Combined, these results demonstrate that ATFS-1 is required for
357 resistance to multiple types of exogenous stressors.
358
359 Even though ATFS-1 is required for the upregulation of stress response genes in
360 response to bacterial pathogens (**Fig. 3**), deletion of *atfs-1* (*gk3094* mutation) did not impact
361 bacterial pathogen resistance. Similarly, another *atfs-1* deletion mutation (*tm4919)* was found
362 not to affect survival during exposure to *P. aeruginosa* [14]. Knocking down *atfs-1* through
363 RNAi inconsistently decreased survival on *P. aeruginosa* (e.g. Fig.3a versus Fig.3h in [14]). While
364 the effect of *atfs-1* disruption on bacterial pathogen resistance was variable, decreasing the
365 expression of a downstream ATFS-1 target gene, *hsp-60,* by RNAi caused a robust decrease in
366 organismal survival on *P. aeruginosa* [43]. As we have previously found that disrupting *atfs-1*
367 induces upregulation of other protective cellular pathways [7] and others have observed a
368 similar phenomenon when a mitoUPR downstream target, *hsp-6,* is disrupted [47], it is possible
369 that the upregulation of other stress pathways may compensate for the inhibition of the
370 mitoUPR, ultimately yielding wild-type or increased levels of resistance to bacterial pathogens,
371 and hiding the normal role of the mitoUPR in resistance to bacterial pathogens.
372
373 **Activation of ATFS-1 enhances resistance to exogenous stressors**
374 In this work, we show that constitutive activation of ATFS-1 (*atfs-1(et15)* and *atfs-1(et17)*
375 mutants) is sufficient to increase resistance to multiple different exogenous stressors, including
376 acute oxidative stress, osmotic stress, anoxia and bacterial pathogens. Previous studies have
377 shown that activating the mitoUPR, either through *spg-7* RNAi or through a constitutively active
378 *atfs-1(et15)* mutant, decreased risk of death after anoxia-reperfusion [15], and that
379 constitutively active *atfs-1(et18)* mutants also have increased resistance to *P. aeruginosa* [14].
380 Overexpression of the mitoUPR target gene *hsp-60* also increases resistance to *P. aeruginosa*
381 [43]. These results support a clear role for ATFS-1 in surviving external stressors.
382
383

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384 **ATFS-1 upregulates target genes of multiple stress response pathways**

385 In exploring the mechanism by which ATFS-1 and the mitoUPR modulate stress resistance, we
386 found that activation of ATFS-1, through either a mutation that mildly impairs mitochondrial
387 function (*nuo-6*) or through a mutation that constitutively activates ATFS-1 (*atfs-1(et15)*),
388 causes upregulation of genes involved in multiple stress response pathways including the ER-
389 UPR pathway, the Cyto-UPR pathway, the DAF-16-mediated stress response pathway, the SKN-
390 1-mediated oxidative stress response pathway, the HIF-mediated hypoxia response pathway,
391 the p38-mediated innate immune response pathway and antioxidant genes (**Fig. 2**). These
392 findings are supported by earlier work demonstrating a role for ATFS-1 in upregulating genes
393 involved in innate immunity. Pellegrino *et al.* reported a 16% (59/365 genes) overlap between
394 genes upregulated by activation of the mitoUPR through treatment with *spg-7* RNAi and genes
395 upregulated by exposure a bacterial pathogen [14]. A connection between the mitoUPR and the
396 innate immunity pathway was also suggested by the finding that overexpression of a mitoUPR
397 downstream target, *hsp-60,* increases expression of three innate immunity genes: T24B8.5,
398 C17H12.8 and K08D8.5 [43]. Our results clearly indicate that the role of ATFS-1 in stress
399 response pathways is not limited to the innate immunity, but extends to multiple stress
400 response pathways, thereby providing a mechanistic basis for the effect of ATFS-1 on resistance
401 to stress.

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402 **Conclusions**

403 The mitoUPR is required for animals to survive exposure to exogenous stressors, and activation
404 of this pathway is sufficient to enhance resistance to stress (**Table S4**). In addition to
405 upregulating genes involved in restoring mitochondrial homeostasis, the mitoUPR increases
406 stress resistance by upregulating the target genes of multiple stress response pathways.
407 Although increased stress resistance has been associated with long lifespan, and multiple long-
408 lived mutants exhibit activation of the mitoUPR, constitutive activation of ATFS-1 shortens
409 lifespan while increasing resistance to stress, indicating that the role of ATFS-1 in stress
410 resistance can be experimentally dissociated from its role in longevity. Overall, this work
411 highlights the importance of the mitoUPR in not only protecting organisms from internal stress,
412 but also improving organismal survival upon exposure to external stressors.

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413 **Materials and Methods**

414

415 **Strains**

416 *C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center (CGC): N2 (wild-
417 type), *nuo-6(qm200), atfs-1(gk3094), nuo-6(qm200);atfs-1(gk3094), atfs-1(et15), atfs-1(et17),*
418 *ife-2 (ok306), clk-1(qm30), sod-2(ok1030), eat-2(ad1116), osm-5(p813), isp-1(qm150), daf-*
419 *2(e1370),* and *glp-1(e2141).* Strains were maintained at 20°C on nematode grown medium
420 (NGM) plates seeded with OP50 *E. coli*.

421

422 **Gene expression in response to stress**

423 *<u>Stress treatment</u>*: Young adult worms were subject to different stress before mRNA was
424 collected. For heat stress, worms were incubated at 35°C for 2 hours and 20°C for 4 hours. For
425 oxidative stress, worms were transferred to plates containing 4 mM paraquat and 100 µM FUdR
426 for 48 hours. For ER stress, worms were transferred to plates containing 5 µg/mL tunicamycin
427 for 24 hours. For osmotic stress, worms were transferred to plates containing 300 mM NaCl for
428 24 hours. For bacterial pathogen stress, worms were transferred to plates seeded with
429 *Pseudomonas aeruginosa* strain PA14 for 4 hours. For anoxic stress, worms were put in BD Bio-
430 Bag Type A Environmental Chambers (Becton, Dickinson and Company, NJ) for 24 hours and left
431 to recover for 4 hours. For unstressed control conditions, worms were collected at the young
432 adult stage and at Day 1 adult stage. For Day 2 adult control, worms were transferred NGM
433 plates containing 100µM FUdR and collected 2 days later.

434

435 *<u>RNA isolation</u>*: RNA was harvested as described previously [48]. Plates of worms were washed
436 three times using M9 buffer to remove bacteria and resuspended in TRIZOL reagent. Worms
437 were frozen in a dry ice/methanol bath and then thawed three times and left at room
438 temperature for 15 minutes. Chloroform was mixed into the tubes and mixture was left to sit at
439 room temperature for 3 minutes. Tubes were then centrifuged at 12,000 g for 15 minutes at
440 4°C. The upper phase containing the RNA was transferred to a new tube, mixed with
441 isopropanol, and allowed to sit at room temperature for 10 minutes. Tubes were centrifuged at

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442 12,000 g for 10 minutes at 4°C. The RNA pellet was washed with 75% ethanol and resuspended
443 in RNAse-free water.
444
445 *Quantitative RT-PCR:* mRNA was converted to cDNA using a High-Capacity cDNA Reverse
446 Transcription kit (Life Technologies/Invitrogen) as described previously [49]. qPCR was
447 performed using a PowerUp SYBR Green Master Mix kit (Applied Biosystems) in a Viia 7 RT-PCR
448 machine from Applied Biosystems. All experiments were performed with least three biological
449 replicates collected from different days. mRNA levels were normalized to act-3 levels and then
450 expressed as a percentage of wild-type. Primer sequences are as follows:
451 *gst-4* (CTGAAGCCAACGACTCCATT, GCGTAAGCTTCTTCCTCTGC),
452 *hsp-4* (CTCGTGGAATCAACCCTGAC, GACTATCGGCAGCGGTAGAG),
453 *hsp-6* (CGCTGGAGATAAGATCATCG, TTCACGAAGTCTCTGCATGG),
454 *hsp-16.2* (CCATCTGAGTCTTCTGAGATTGTT, CTTTCTTTGGCGCTTCAATC),
455 *sod-3* (TACTGCTCGCACTGCTTCAA, CATAGTCTGGGCGGACATTT),
456 *sod-5* (TTCCACAGGACGTTGTTTCC, ACCATGGAACGTCCGATAAC),
457 *nhr-57* (GACTCTGTGTGGAGTGATGGAGAG, GTGGCTCTTGGTGTCAATTTCGGG),
458 *gcs-1* (CCACCAGATGCTCCAGAAAT, TGCATTTTCAAAGTCGGTC),
459 *trx-2* (GTTGATTTCCACGCAGAATG, TGGCGAGAAGAACACTTCCT),
460 *Y9C9A.8* (CGGGGATATAACTGATAGAATGG, CAAACTCTCCAGCTTCCAACA),
461 *T24B8.5* (TACACTGCTTCAGAGTCGTG, CGACAACCACTTCTAACATCTG),
462 *clec-67* (TTTGGCAGTCTACGCTCGTT, CTCCTGGTGTGTCCCATTTT),
463 *dod-22* (TCCAGGATACAGAATACGTACAAGA, GCCGTTGATAGTTTCGGTGT),
464 *ckb-2* (GCATTTATCCGAGACAGCGA, GCTTGCACGTCCAAATCAAC),
465 *act-3* (TGCGACATTGATATCCGTAAGG, GGTGGTTCCTCCGGAAAGAA).
466

467 **RNA sequencing and bioinformatic analysis**

468 RNA sequencing was performed previously [50, 51] and raw data is available on NCBI GEO:
469 GSE93724 [51], GSE110984 [7]. Bioinformatic analysis for this study was used to determine

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470 differentially expressed genes and identify the degree and significance of overlaps between
471 genes sets.
472
473 *Determining differentially expressed genes:* Samples were processed using an RNA-seq pipeline
474 based on the bcbio-nextgen project (https://bcbio-nextgen.readthedocs.org/en/latest/). We
475 examined raw reads for quality issues using FastQC
476 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) in order to ensure library
477 generation and sequencing data were suitable for further analysis. If necessary, we used
478 cutadapt http://code.google.com/p/cutadapt/ to trim adapter sequences, contaminant
479 sequences such as polyA tails, and low quality sequences from reads. We aligned trimmed
480 reads to the Ensembl build WBcel235 (release 90) of the *C. elegans* genome using STAR [52].
481 We assessed quality of alignments by checking for evenness of coverage, rRNA content,
482 genomic context of alignments (for example, alignments in known transcripts and introns),
483 complexity and other quality checks. To quantify expression, we used Salmon [53] to find
484 transcript-level abundance estimates and then collapsed down to the gene-level using the R
485 Bioconductor package tximport [54]. Principal components analysis (PCA) and hierarchical
486 clustering methods were used to validate clustering of samples from the same batches
487 and across different mutants. We used the R Bioconductor package DESeq2 [55] to find
488 differential expression at the gene level. For each wildtype-mutant comparison, we identified
489 significant genes using an FDR threshold of 0.01. Lastly, we included batch as a covariate in the
490 linear model for datasets in which experiments were run across two batches.
491
492 *Venn diagrams:* Weighted Venn diagrams were produced by inputting gene lists into BioVenn
493 (https://www.biovenn.nl/). Percentage overlap was determined by dividing the number of
494 genes in common between the two gene sets by the gene list with the smaller gene list.
495
496 *Significance of overlap and enrichment:* The significance of overlap between two gene sets was
497 determined by comparing the actual number of overlapping genes to the expected number of
498 overlapping genes based on the sizes of the two gene sets (expected number = number of

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499 genes in set 1 X number of genes in set 2/number of genes in genome detected). Enrichment
500 was calculated as the observed number of overlapping genes/the expected number of
501 overlapping genes if genes were chosen randomly.
502
503 **Resistance to stress**
504 For acute oxidative stress, young adult worms were transferred onto plates with 300 µM
505 juglone and survival was measured every 2 hours for a total of 10 hours. For chronic oxidative
506 stress, young adult worms were transferred onto plates with 4 mM paraquat and 100 µM FUdR
507 and survival was measured daily until death.

508 For heat stress, young adult worms were incubated in 37°C and survival was measured every 2
509 hours for a total of 10 hours. For osmotic stress, young adult worms were transferred to plates
510 containing 450 mM or 500 mM NaCl and survival was measured after 48 hours.

511 For anoxic stress, plates with young adult worms were put into BD Bio-Bag Type A
512 Environmental Chambers for 75 hours and survival was measured after a 24-hour recovery
513 period.

514 Two different bacterial pathogenesis assays involving *P. aeruginosa* strain PA14 were
515 performed. In the slow kill assay worms are thought to die from intestinal colonization of the
516 pathogenic bacteria, while in the fast kill assay worms are thought to die from a toxin secreted
517 from the bacteria [25]. The slow kill assay was performed as described previously [14, 27]. In
518 the first protocol [14], PA14 cultures were grown overnight and seeded to center of a 35-mm
519 NGM agar plate. Plates were left to dry overnight, and then incubated in 37°C for 24 hours.
520 Plates were left to adjust to room temperature before approximately 40 L4 worms were
521 transferred onto the plates. The assay was conducted 25°C and plates were checked twice a day
522 until death. In the second protocol [27], overnight PA14 culture were seeded to the center of a
523 35-mm NGM agar plate containing 20 mg/L FUdR. Plates were incubated at 37°C overnight,
524 then at room temperature overnight before approximately 40 day three adults were
525 transferred onto these plates. The assay was conducted 20°C and plates were checked daily
526 until death. The fast kill pathogenesis assay was performed as described previously [25]. PA14

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527 cultures were grown overnight and seeded to Peptone-Glucose-Sorbitol (PGS) agar plates.
528 Seeded plates were left to dry for 20 minutes at room temperature before incubation at 37°C
529 for 24 hours and then at 23°C for another 24 hours. Approximately 30 L4 worms were
530 transferred onto the plates and were scored as dead or alive at 2, 4, 6, 8 and 24 hours. Fast kill
531 plates were kept at 23°C in between scoring timepoints.
532

533 **Lifespan**

534 All lifespan assays were performed at 20°C. Lifespan assays included FUdR to limit the
535 development of progeny and the occurrence of internal hatching. Based on our previous
536 studies, a low concentration of FUdR (25mM) was used to minimize potential effects of FUdR
537 on lifespan [56]. Animals were excluded from the experiment if they crawled off the plate or
538 died of internal hatching of progeny or expulsion of internal organs.
539

540 **Statistical Analysis**

541 To ensure unbiased results, all experiments were conducted with the experimenter blinded to
542 the genotype of the worms. For all assays, a minimum of three biological replicates of randomly
543 selected worms from independent populations of worms on different days were used. For
544 analysis of lifespan, oxidative stress, and bacterial pathogen stress, a log-rank test was used. For
545 analysis of heat stress, repeated measures ANOVA was used. For analysis of osmotic stress and
546 anoxic stress, a one-way ANOVA with Dunnett's multiple comparisons tests was used. For
547 quantitative PCR results we used a two-way ANOVA with Bonferroni post-test. For all bar
548 graphs error bars indicate standard error of the mean and bars indicate the mean.

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554

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556

557 **Author Contributions.** Conceptualization: JVR. Methodology: SKS, AT, MM, JVR. Investigation:
558 SKS, AT, MM, JVR. Analysis: SKS, AT, MM, JVR. Visualization: SKS, AT, MM, JVR. Writing –
559 original draft: SKS, JVR. Writing – review and editing: SKS, AT, JVR. Supervision: JVR.

560

561 **Materials & Correspondence.** Correspondence and material requests should be addressed to
562 Jeremy Van Raamsdonk.

563

564 **Data availability.** RNA-seq data has been deposited on GEO: GSE93724, GSE110984. All other
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566 from the corresponding author on request.

567

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574

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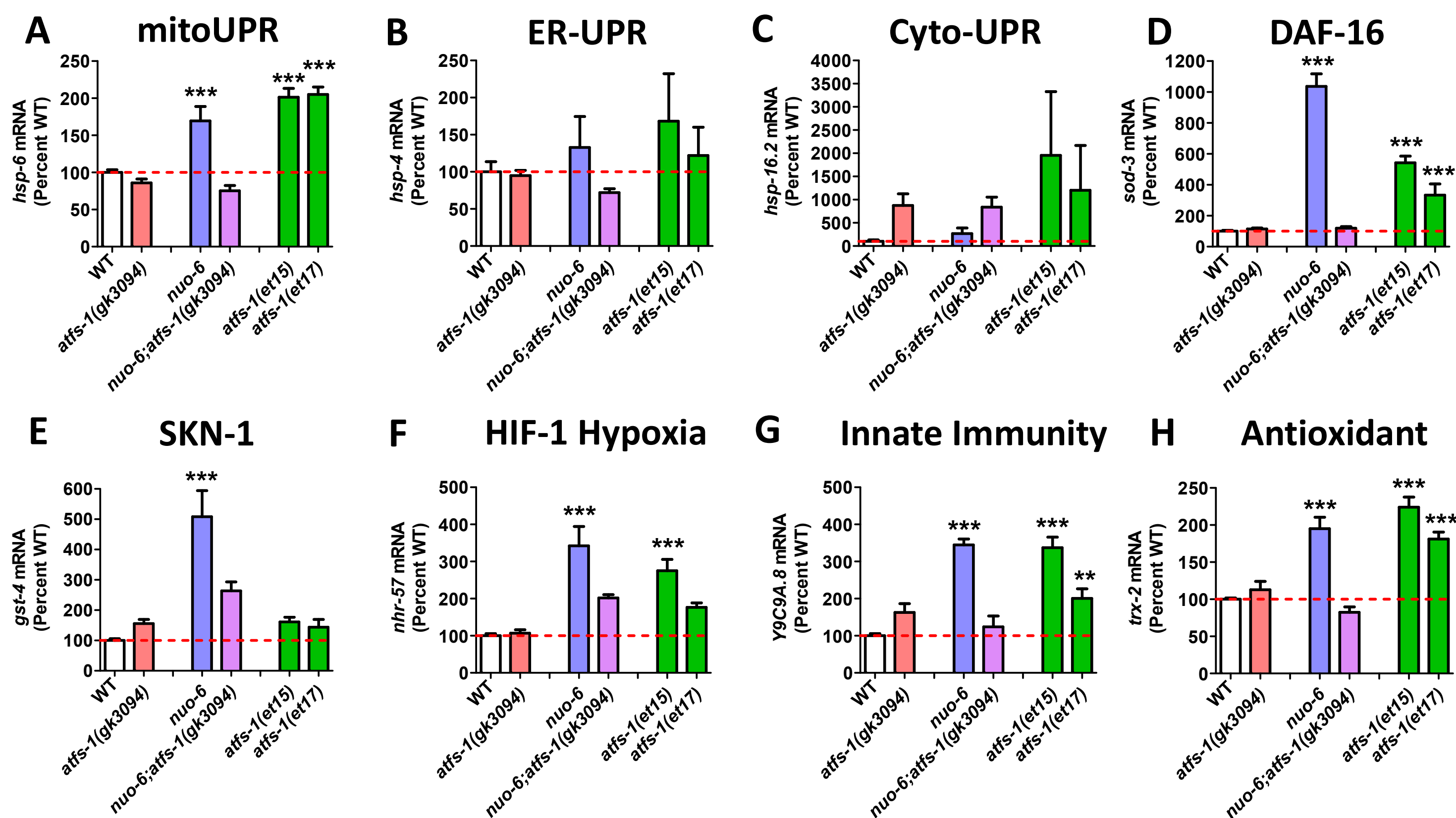

Figure 1. Activation of ATFS-1 upregulates genes from multiple stress response pathways. To determine the role of ATFS-1 in the activation of genes from different stress response pathways, we activated ATFS-1 by mildly impairing mitochondrial function through a mutation in *nuo-6* (blue bars) and then examined the effect of disrupting *atfs-1* using an *atfs-1* deletion mutant *atfs-1(gk3094)* (purple bars). We also examined the expression of these genes in two constitutively active *atfs-1* mutants, *atfs-1(et15)* and *atfs-1(et17)* (green bars). Target genes from the mitochondrial unfolded protein response (mitoUPR) (**A**, *hsp-6*), DAF-16-mediated stress response (**D**, *sod-3*), SKN-1-mediated oxidative stress response (**E**, *gst-4*), HIF-1-mediated hypoxia response (**F**, *nhr-57*), p38-mediated innate immune pathway (**G**, *Y9C9A.8*), and antioxidant defense (**H**, *trx-2*) are all significantly upregulated in *nuo-6* mutants in an ATFS-1-dependent manner. Target genes from the mitoUPR, DAF-16-mediated stress response, HIF-1-mediated hypoxia response, p38-mediated innate immune pathway and antioxidant defense are also upregulated in constitutive activation of ATFS-1 (**A, D, F, G, H**). In contrast, activation of ATFS-1 by *nuo-6* mutation or *atfs-1* gain-of-function mutations did not significantly affect target gene expression for the endoplasmic reticulum unfolded protein response (**B**, ER-UPR, *hsp-4*) or the cytoplasmic unfolded protein response (**C**, Cyto-UPR, *hsp-16.2*). *atfs-1(gk3094)* is a loss-of-function deletion mutant. *atfs-1(et15)* and *atfs-1(et17)* are constitutively active gain-of-function mutants. Error bars indicate SEM. $^{**}p<0.01$, $^{***}p<0.001$. A full list of genes that are upregulated by ATFS-1 activation can be found in **Table S2**.

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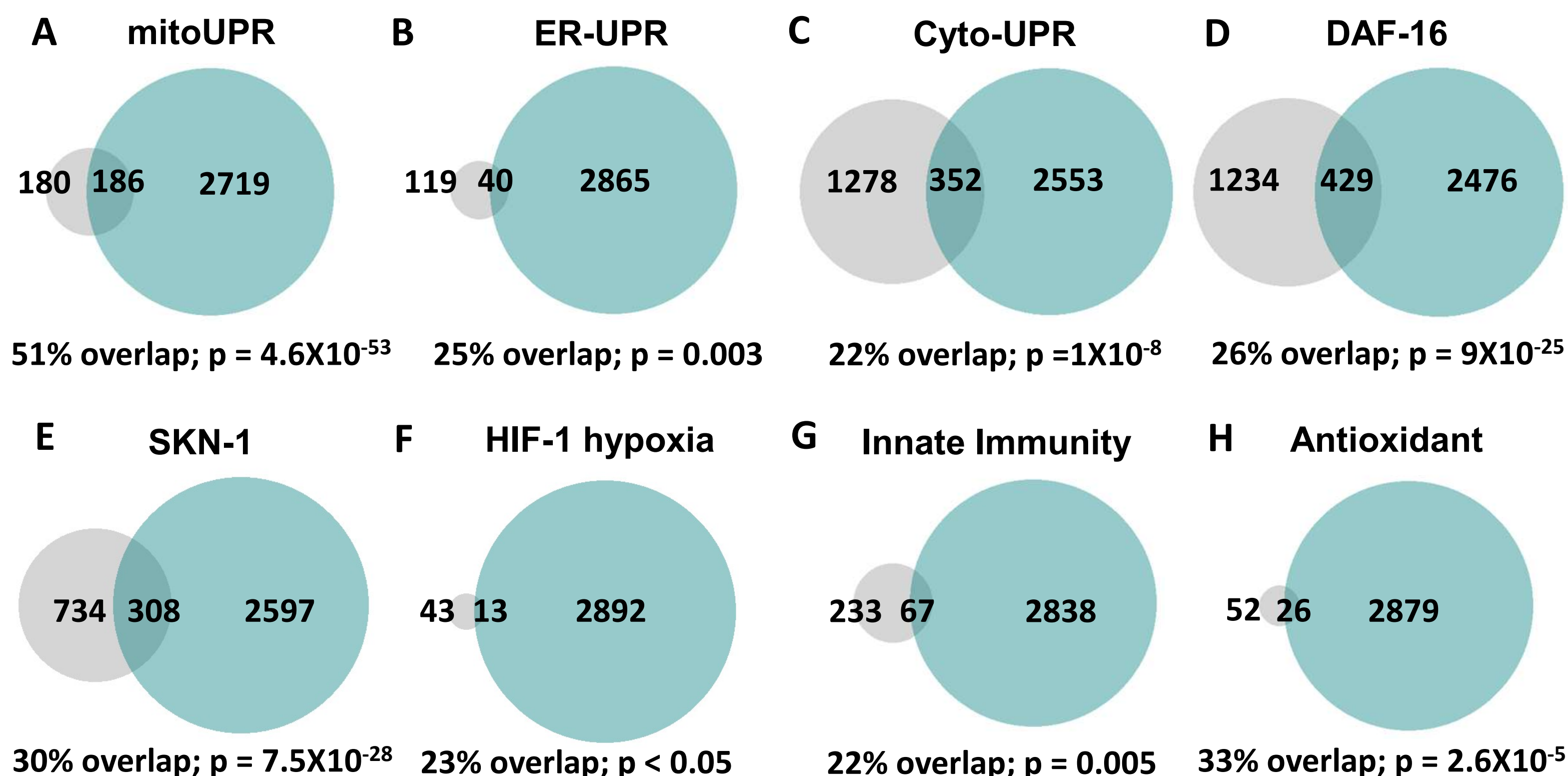

Figure 2. Constitutive activation of ATFS-1 results in upregulation of genes from multiple stress response pathways. Genes that are upregulated by activation of ATFS-1 were compared to previous published lists of genes involved in different stress response pathways, including the mitochondrial unfolded protein response (**A**, mitoUPR), the endoplasmic reticulum unfolded protein response (**B**, ER-UPR), the cytoplasmic unfolded protein response (**C**, Cyto-UPR), the DAF-16-mediated stress response (**D**), the SKN-1-mediated oxidative stress response (**E**), the HIF-1-mediated hypoxia response (**F**), the p38-mediated innate immune response (**G**), and antioxidant genes (**H**). In every case, there was a significant degree of overlap ranging from 22%-51%. Grey circles indicate genes that are upregulated by activation of the stress response pathway indicated. Turquoise circles indicate genes that are upregulated in the *atfs-1(et15)* constitutively active gain-of-function mutant. The numbers inside the circles show how many genes are upregulated. The percentage overlap is the number of overlapping genes as a percentage of the number of genes upregulated by the stress response pathway. mitoUPR = mitochondrial unfolded protein response. ER-UPR = endoplasmic reticulum unfolded protein response. Cyto-UPR = cytoplasmic unfolded protein response. DAF-16 = DAF-16-mediated stress response pathway. SKN-1 = SKN-1-mediated oxidative stress response pathway. HIF-1 = HIF-1-mediated stress response pathway. Innate immunity = p38-mediated innate immunity pathway. Antioxidant = antioxidant genes. Stress pathway gene lists and sources can be found in **Table S3**.

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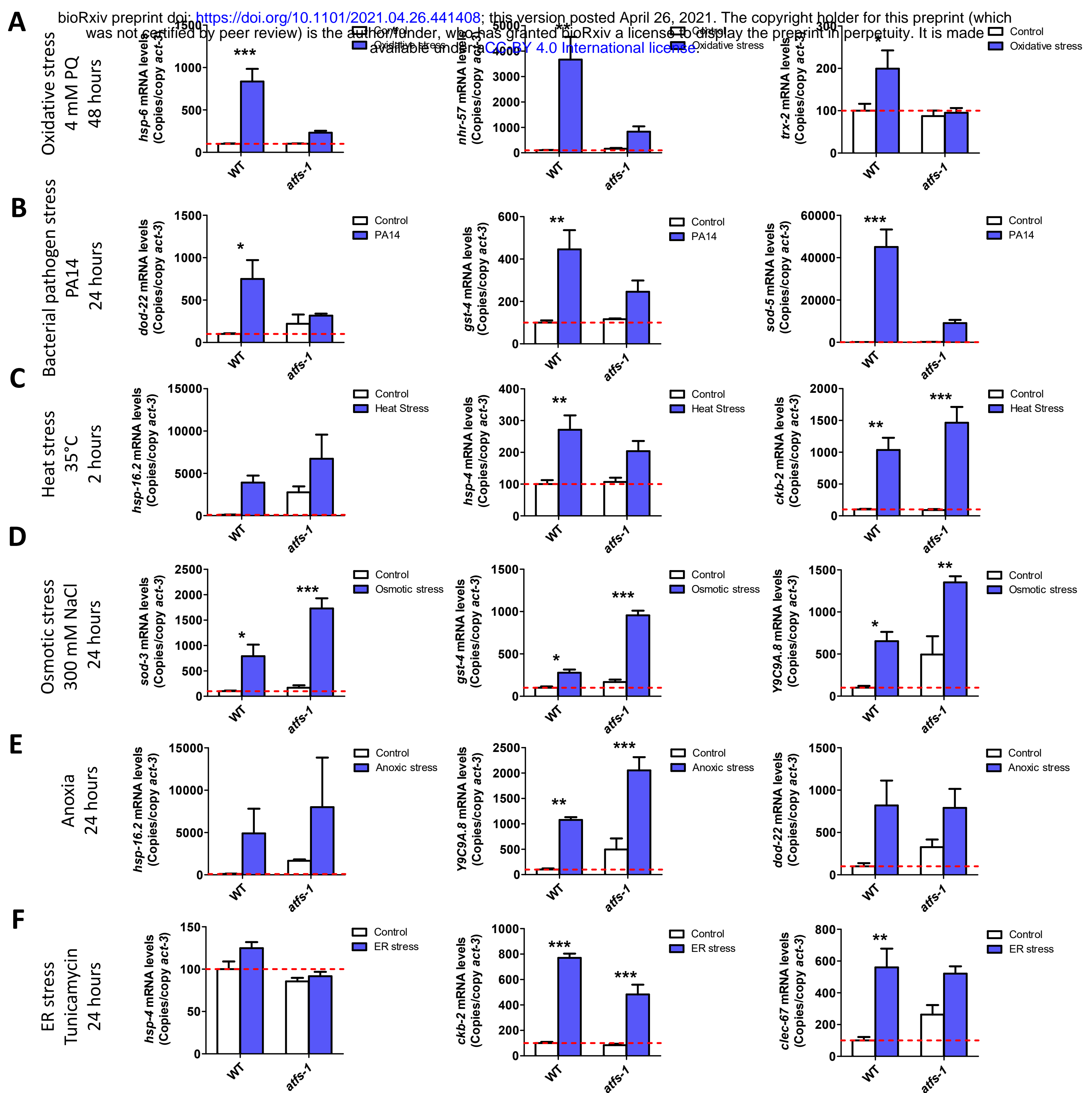

Figure 3. ATFS-1 is required for upregulation of stress response genes after exposure to oxidative stress or bacterial pathogen stress. To determine the role of ATFS-1 in responding to different types of stress, we compared the upregulation of stress response genes in wild-type and *atfs-1(gk3094)* loss-of-function deletion mutants after exposure to different stressors. **A.** Exposure to oxidative stress (4 mM paraquat, 48 hours) caused a significant upregulation of *hsp-6, nhr-57* and *trx-2* in wild-type worms that was prevented by the disruption of *atfs-1.* **B.** Exposure to bacterial pathogen stress (PA14, 24 hours) resulted in an upregulation of *dod-22, gst-4* and *sod-5* in wild-type worms that was prevented by the *atfs-1* deletion. **C.** Exposure to heat stress (35°C, 2 hours) increased the expression of *hsp-16.2, hsp-4* and *ckb-2* in both wild-type and *atfs-1* worms. **D.** Exposure to osmotic stress (300 mM, 24 hours) caused an upregulation of *sod-3, gst-4* and *Y9C9A.8* in wild-type worms and to a greater magnitude in *atfs-1* mutants. **E.** Anoxia (24 hours) resulted in the upregulation of *hsp-16.2, Y9C9A.8* and *dod-22* in both wild-type and *atsf-1* worms. **F.** Exposing worms to endoplasmic reticulum stress (tunicamycin, 24 hours) increased the expression of *ckb-2* and *clec-67* in both wild-type and *atfs-1* worms. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001.

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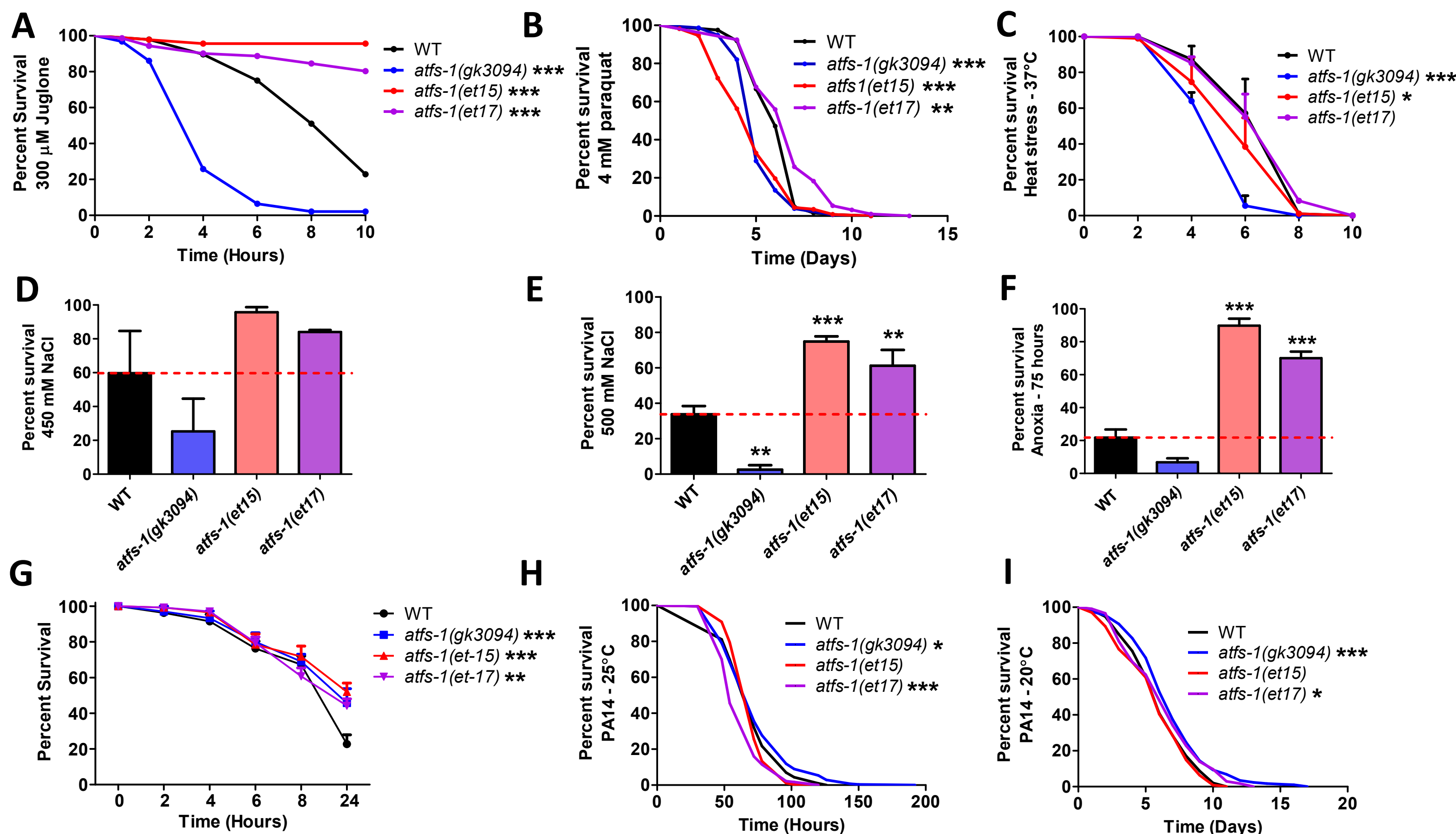

Figure 4. Constitutive activation of ATFS-1 increases resistance to multiple external stressors. To determine the role of ATFS-1 in resistance to stress, the stress resistance of an *atfs-1* loss-of-function mutants (*atfs-1(gk3094)*) and two constitutively active *atfs-1* gain-of-function mutants (*atfs-1(et15), atfs-1(et17)*) was compared to wild-type worms. **A.** Activation of ATFS-1 enhanced resistance to acute oxidative stress (300 μM juglone), while deletion of *atfs-1* markedly decreased resistance to acute oxidative stress. **B.** Disruption of *atfs-1* decreased resistance to chronic oxidative stress (4 mM paraquat). *atfs-1(et17)* mutants showed increased resistance to chronic oxidative stress, while *atfs-1(et15)* mutants had decreased resistance. **C.** Resistance to heat stress (37°C) was not enhanced by activation of ATFS-1, while deletion of *atfs-1* decreased heat stress resistance. **D,E.** Activation of ATFS-1 increased resistance to osmotic stress (450 mM, 500 mM NaCl), while disruption of *atfs-1* decreased osmotic stress resistance. **F.** Constitutively active *atfs-1* mutants show increased resistance to anoxia (75 hours), while *atfs-1* deletion mutants exhibit a trend towards decreased anoxia resistance. **G.** Activation of ATFS-1 increased resistance to *P. aeruginosa* toxin in a fast kill assay. A slow kill assay in which worms die from internal accumulation of *P. aeruginosa* was performed according to two established protocols. **H.** At 25°C **,** *atfs-1(et17)* mutants showed a small decrease in resistance to bacterial pathogens (PA14), while *atfs-1(gk3094)* mutants showed a small increase in resistance. **I.** At 20°C, both *atfs-1(et17)* and *atfs-1(gk3094)* mutants exhibited a small increase in resistance to bacterial pathogens. Error bars indicate SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

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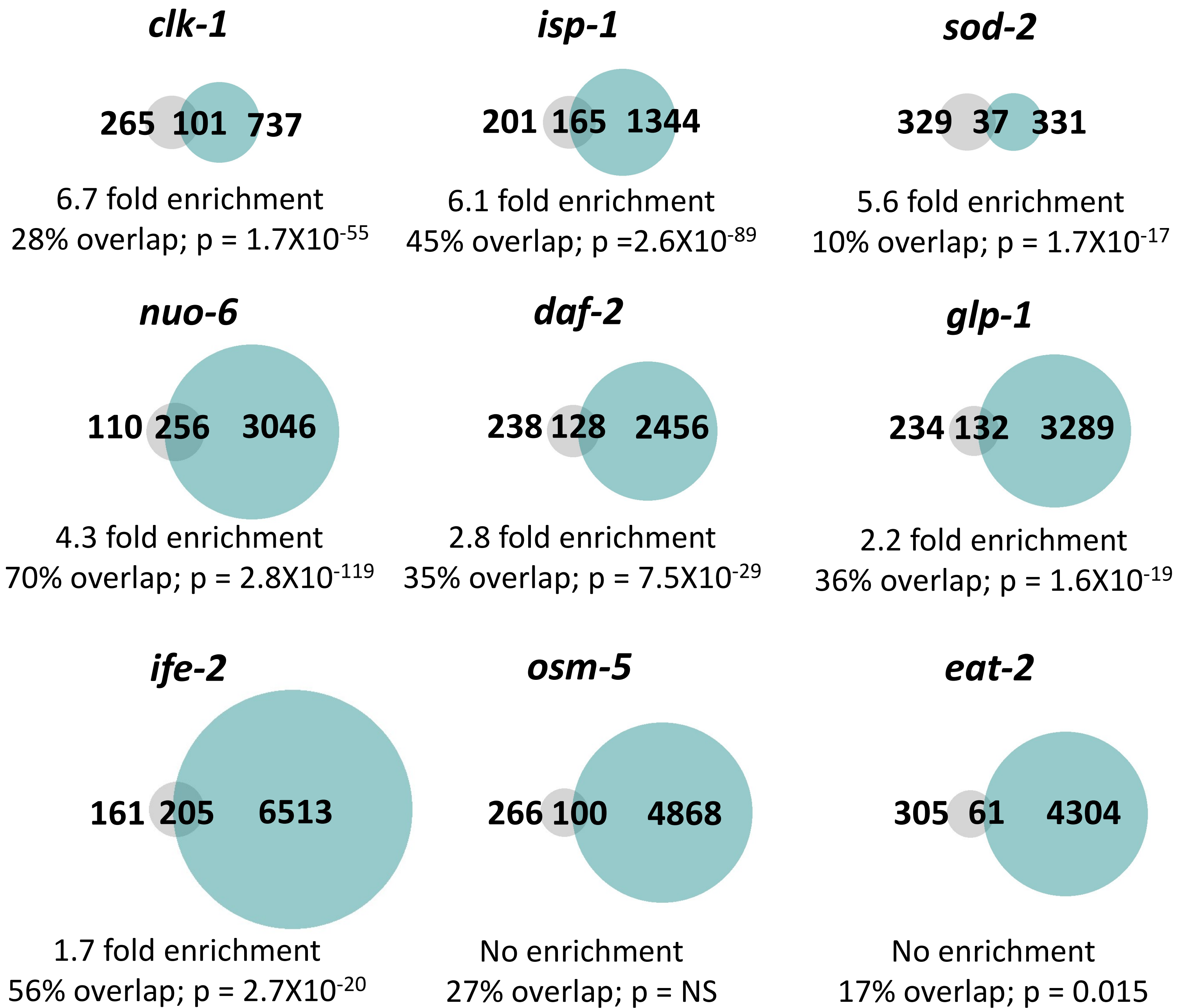

Figure 5. Multiple long-lived mutants from different pathways of lifespan extension show upregulation of ATFS-1-dependent genes. To determine the extent to which long-lived genetic mutants from different pathways of lifespan extension show differential expression of ATFS-1 target genes, we compared genes that are upregulated in nine different long-lived mutants to a published list of *spg-7* RNAi-upregulated, ATFS-1-dependent target genes (Nargund *et al.*, 2012). We found that *clk-1, isp-1, nuo-6, sod-2, daf-2, glp-1* and *ife-2* worms all show a highly significant degree of overlap with genes upregulated by *spg-7* RNAi in an ATFS-1-dependent manner. The grey circles represent the 366 genes that are upregulated by *spg-7* RNAi in an ATFS-1 dependent manner. Turquoise circles are genes that are significantly upregulated in the long-lived mutant indicated as determined from our RNA sequencing data. The number of unique and overlapping genes are indicated. Overlap is calculated as the number of genes in common between the two gene sets divided by the total number of genes that are upregulated by *spg-7* RNAi in an ATFS-1 dependent manner. Enrichment is calculated as the number of overlapping genes observed divided by the number of overlapping genes predicted if genes were chosen randomly.

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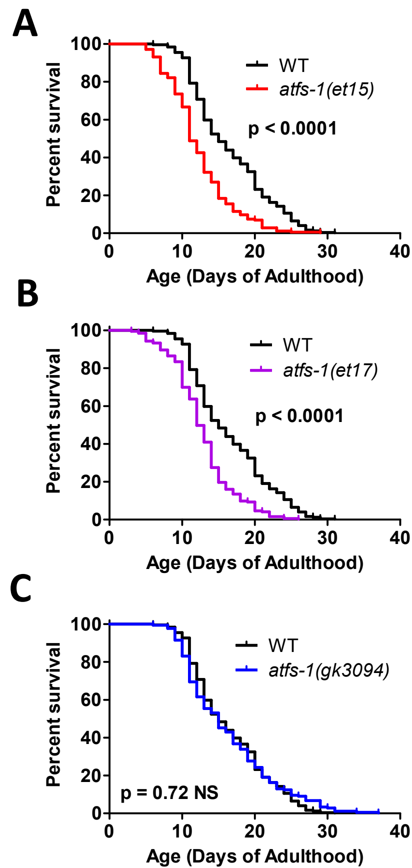

Figure 6. Activation of ATFS-1 does not increase lifespan. To determine the effect of ATFS-1 on aging, we quantified the lifespan of an *atfs-1* deletion mutant and two constitutively active *atfs-1* mutants. **A,B.** Both constitutively active *atfs-1* mutants, *et15* and *et17,* have a significantly decreased lifespan compared to wild-type worms. **C.** Deletion of *atfs-1* does not affect lifespan compared to wild-type worms. *atfs-1(gk3094)* is a loss of function mutant resulting from a deletion. *atfs-1(et15)* and *atfs-1(et17)* are constitutively active gain-of-function mutants.

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Supplementary Figures for:

Mitochondrial unfolded protein response transcription factor ATFS-1 increases resistance to exogenous stressors through upregulation of multiple stress response pathways

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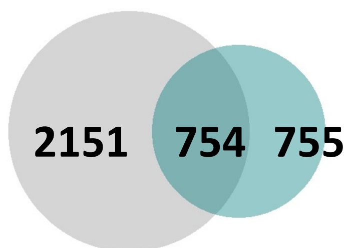
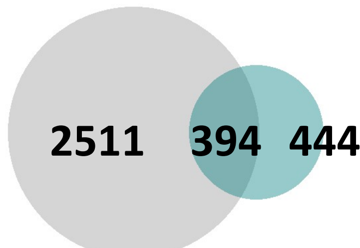
isp-1



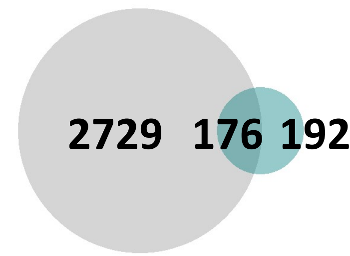
3.5 fold enrichment
50% overlap; $p = 8.9X10^{-267}$

clk-1



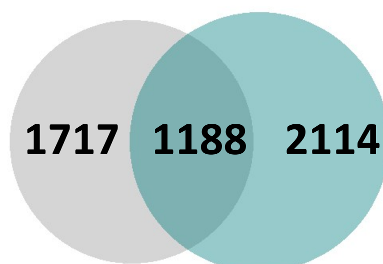
3.3 fold enrichment
47% overlap; $p = 2.5X10^{-121}$

sod-2



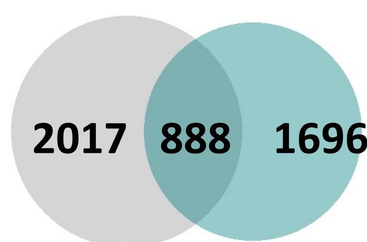
3.4 fold enrichment
48% overlap; $p = 7.6X10^{-55}$

nuo-6



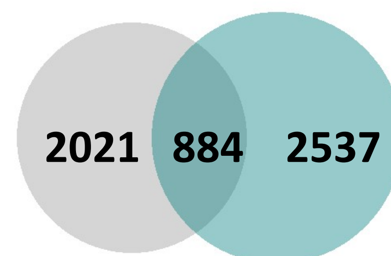
2.5 fold enrichment
41% overlap; $p = 1.6X10^{-272}$

daf-2



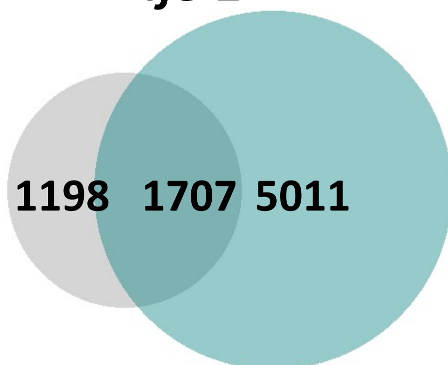
2.4 fold enrichment
34% overlap; $p = 6.3X10^{-175}$

glp-1



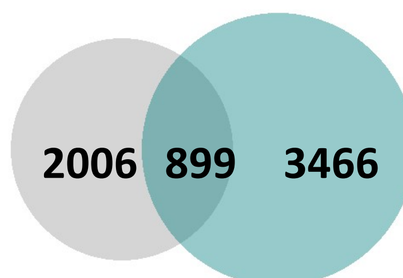
1.8 fold enrichment
30% overlap; $p = 3.1X10^{-89}$

ife-2



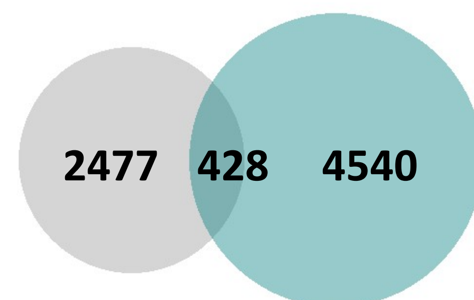
1.8 fold enrichment
59% overlap; $p = 4.9X10^{-213}$

eat-2



1.5 fold enrichment
31% overlap; $p =4.2X10^{-40}$

osm-5



No enrichment
15% overlap; $p = 2.8X10^{-82}$

Figure S1. Multiple long-lived mutants from different pathways of lifespan extension show upregulation of ATFS-1-dependent genes. To determine the extent to which long-lived genetic mutants from different pathways of lifespan extension show differential expression of ATFS-1 target genes, we compared genes that are upregulated in nine different long-lived mutants to genes upregulated in a constitutively active *atfs-1* mutant (*et15*). All of the long-lived mutant worms, except for *osm-5,* show a highly significant degree of overlap with the constitutively active *atfs-1* mutant. The grey circles represent genes that are significantly upregulated in the constitutively active *atfs-1(et15)* mutant. Turquoise circles are genes that are significantly upregulated in the long-lived mutant indicated. The number of unique and overlapping genes are indicated. Overlap is calculated as the number of genes in common between the two gene sets divided by the smaller gene set. Enrichment is calculated as the number of overlapping genes observed divided by the number of overlapping genes predicted if genes were chosen randomly.

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Table S1. Target genes examined for each stress response pathway.

Target Gene	Stress response pathway
hsp-6	Mitochondrial unfolded protein response
hsp-4	ER unfolded protein response
hsp-16.2	Cytoplasmic unfolded protein response
sod-3	DAF-16-mediated stress response pathway
gst-4	SKN-1-mediated stress response pathway
nhr-57	HIF-1-mediated hypoxia pathway
Y9C9A.8	p38-mediated innate immune pathway
trx-2	Antioxidant genes

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Table S4. Effect of modulating ATFS-1 levels and activation on stress resistance, lifespan and expression of stress response genes.

	atfs-1(et15) Gain-of-function	*atfs-1(et17)* Gain-of-function	*atfs-1(gk3094)* Loss-of-function
Acute oxidative stress resistance	↑	↑	↓
Chronic oxidative stress resistance	↓	↑	↓
Heat stress resistance	↓	=	↓
Osmotic stress resistance	↑	↑	↓
Anoxia resistance	↑	↑	↓
Bacterial pathogens resistance	==	↓↑	↑↑
Lifespan	↓	↓	=
hsp-6 expression (mitoUPR)	↑	↑	=
hsp-4 expression (ER-UPR)	=	=	=
hsp-16.2 expression (Cyto-UPR)	↑ (NS)	↑ (NS)	↑ (NS)
sod-3 expression (DAF-16)	↑	↑	=
gst-4 expression (SKN-1)	=	=	=
nhr-57 expression (HIF-1)	↑	=	=
Y9C9A.8 expression (Innate immunity)	↑	↑	=
trx-2 (antioxidant)	↑	↑	=