Loss of an uncharacterized mitochondrial methionine tRNA-synthetase induces mitochondrial unfolded protein response in *Caenorhabditis elegans*

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Abstract

Aminoacyl-tRNA synthetases (aaRSs) are essential for translation, as they charge tRNA molecules with their corresponding amino acids. Alterations in aaRSs can significantly disrupt both cytosolic and mitochondrial translation. Through a forward genetic screen for mitochondrial unfolded protein response (UPR^{mt}) activators in *C. elegans*, we identified a missense mutation (P447V) in the previously uncharacterized gene Y105E8A.20, which encodes a dually localized methionine tRNA synthetase (MetRS). Here, we characterize the UPR^{mt} induction by Y105E8A.20, which we call *mars-2*, and demonstrate that the P447V allele is a loss-of-function mutation. Furthermore, we show impaired *mars-2* activity in the mitochondria triggers UPR^{mt}.

24   This strain provides a valuable tool for studying mitochondrial translation and understanding

25   how aaRSs are involved in mitochondrial homeostasis.

26

Introduction

28   Mitochondria are multifaceted organelles involved in a wide range of essential processes,

29   including energy production, intracellular signaling, and programmed cell death[1,2]. This semi-

30   autonomous organelle has its own genome. In *Caenorhabditis elegans*, the mitochondrial

31   genome encodes 36 genes, including essential protein subunits of the mitochondrial respiratory

32   complexes, mitochondrial rRNAs, and mitochondrial tRNAs[3]. The mitochondrial genome is

33   transcribed as a single polycistronic RNA, which is subsequently processed and then mRNAs

34   are translated within mitochondria by specialized mito-ribosomes[4]. While mitochondrial tRNAs

35   are encoded within the mitochondria, they must be charged with their corresponding amino

36   acids to locally translate mitochondrial DNA encoded proteins. This charging process is carried

37   out by aminoacyl tRNA synthetases (aaRSs), which are encoded by the nuclear genome and

38   imported into the mitochondria[5]. aaRSs are highly specialized enzymes involved in covalently

39   attaching amino acids to their cognate tRNAs. Given their house-keeping function across

40   eukaryotes, bacteria, and archaea[6,7], mutations in aaRS genes are linked to Charcot-Marie-

41   Tooth (CMT) disease[8–12], tumor formation[13–15], and death[16]. Recent studies show that mutations

42   in aaRS can alter tRNA levels, which in turn regulate the production of tRNA-derived fragments

43   and contribute to diverse disease phenotypes[17].

44

45   Although mitochondrial aaRS mutations are associated with several diseases[18–20], the

46   mechanisms by which these mutations affect mitochondrial function and contribute to

47   pathological phenotypes remains largely unknown. Dysfunctional aaRS activity can directly

48   affect mitochondrial protein synthesis causing mitochondrial stress[21,22]. Mitochondrial quality

49   control pathways, including the mitochondrial unfolded protein response (UPR[mt]) are activated

50   in response to mitochondrial stress[23]. UPR[mt] upregulates nuclear-encoded chaperones and

51   proteases to preserve mitochondrial functionality[24,25]. In *C. elegans*, this response is mediated

52   by the transcription factor ATFS-1, which in healthy cells is imported to the mitochondria[26].

53   However when mitochondria are stressed, a decrease in mitochondrial protein import disrupts

54   ATFS-1 mitochondrial import[27], resulting in ATFS-1 nuclear accumulation and subsequent

55   transcription of UPR[mt]-associated genes. Transcriptional reporters, such as the *hsp-6p::GFP*

56   strain (one of the UPR[mt]-associated genes), have been developed to monitor UPR[mt] activation[28].

57

58   This study reveals a compelling link between mitochondrial protein translation fidelity and the

59   activation of the mitochondrial unfolded protein response. We demonstrate a missense mutation

60   in a previously uncharacterized mitochondrial methionine tRNA synthetase gene in *C. elegans*

61   that activates UPR[mt]. Using CRISPR-Cas9, we confirmed the causality of this point mutation

62   and demonstrated that it represents a loss-of-function mutation. Furthermore, we mutated the

63   mitochondrial targeting sequence (MTS) and found that the absence of MARS-2 in the

64   mitochondria is sufficient to induce UPR[mt]. These findings not only enhance our understanding

65   of mitochondrial quality control but also identify MARS-2 as a potential target for developing

66   therapeutic strategies to modulate UPR[mt] in conditions involving mitochondrial dysfunction.

67

68

Results

70   **P447 is a highly conserved amino acid residue in MARS-2**

71   In a previous study[29], we used *hsp-6p::GFP* strain (Figure 1A) to perform a forward genetic

72   screen aimed at identifying inducers of UPR[mt] (Figure 1B). This screen uncovered a missense

73   mutation (P447V) in the gene *Y105E8A.20*, which encodes a methionine tRNA synthetase in *C.*

74   *elegans* (mpt139 strain shown in Figure 1C and 1D) along with other non-causal mutations

75   introduced through EMS mutagenesis. Phylogenetic analysis of protein sequences from

76   cytoplasmic and mitochondrial methionine tRNA synthetases (MARS1 and MARS2,

77   respectively) across various species revealed that Y105E8A.20 protein in *C. elegans* – hereafter

78   referred as MARS-2 – shares greater similarity with MARS-2 proteins compared to MARS-1

79   proteins in other species (Figure 1E). Furthermore, protein sequence alignment using CLUSTAL

80   tool established that the 447th amino acid residue is highly conserved across species (Figure

81   1F).

82

83   **P447V mutation in MARS-2 induces mitochondrial unfolded protein response**

84   To confirm the role of the P447V mutation in activating UPR^{mt}, we introduced this missense

85   mutation into the *zcIs13 V* background (*hsp-6p::GFP* strain) using CRISPR-Cas9 gene editing.

86   Two independent clones carrying the desired mutation were recovered and validated through

87   Sanger sequencing. Fluorescent imaging of whole animal revealed that the P447V mutant

88   exhibited a 7-fold increase in UPR^{mt} reporter activation compared to wildtype animals (Figure 2A

89   and 2B), confirming the mutation's causative role in inducing UPR^{mt}.

90

91   **P447V mutation in MARS-2 induces mitochondrial unfolded protein response via ATFS-1**

92   **signaling**

93   Previous studies have shown that ATFS-1 plays a central role in inducing UPR^{mt} in *C.*

94   *elegans*[26,27]. To determine whether the P447V mutation in MARS-2 activates UPR^{mt} through this

95   pathway, we performed RNAi-mediated knockdown of *atfs-1* in the mutant strains. This

96   completely suppressed reporter activation (Figure 3A and 3B), confirming that UPR^{mt} activation

97   in the mutant strains occurs via the canonical ATFS-1 pathway. Interestingly, animals harboring

98   the P447V mutation arrested upon *atfs-1* knockdown (Figure 3A). This is likely due to their

99   inability to mount a sufficient stress response in the absence of ATFS-1, consistent with other

100   reports that ATFS-1 is protective against multiple stressors[30]. Collectively, these findings

101   establish the P447V mutation in *mars-2* gene as a driver of UPR^{mt} activation via ATFS-1.

102

103   **P447V mutation in MARS-2 is a loss-of-function mutation**

104   Next, we asked what effect this mutation has on the protein. InterPro domain analysis identified

105   the P447V mutation within the anticodon-binding domain of the MARS-2 protein, a region critical

106   for recognizing the anticodon loop of methionine tRNA. Given that mutations in the anticodon-

107   binding domain of other aaRSs are often associated with reduced enzymatic activity[19,31], we

108   hypothesized that P447V mutation in *mars-2* represents a loss-of-function mutation. To test this,

109   we performed RNAi targeting Y105E8A.20 transcripts in wildtype and mutant strains. RNAi in

110   wildtype animals induced UPR^{mt} (Figure 4A and 4B), mimicking the effect of the P447V

111   mutation. Furthermore, RNAi in P447V mutant animals resulted in an even greater activation of

112   UPR^{mt} and exacerbated growth defects (Figure 4A and 4B), consistent with P447V being a loss-

113   of-function mutation.

114

115   **Mitochondrial loss of MARS-2 activates robust mitochondrial unfolded protein response**

116   The *mars-2* gene encodes for two isoforms: isoform A (406 aa) and isoform B (524 aa). Analysis

117   using MitoFates software predicts a mitochondrial targeting sequence (MTS) within the N-

118   terminal region of isoform B, absent in isoform A. To determine whether loss of *mars-2*

119   mitochondrial function specifically underpins UPR^{mt} activation, we generated a mitochondrial-

120   specific loss-of-function mutant, *mpt227* by replacing the first methionine with alanine using

121   CRISPR-Cas9 gene editing. This results in mutants that lack the mitochondrial isoform but

122   retained the shorter, likely cytoplasmic isoform. Consistent with a previous study where RNAi

123   knockdown of several mitochondrial ARS genes induced UPR^{mt} [32], we found that the

124   mitochondrial-specific loss-of-function mutants (labelled as *mars-2[ΔMTS]*) exhibited robust

125   UPR^{mt} (Figure 5A and 5B). Moreover, we concluded that P447V is likely a hypomorphic

126 mutation in *mars-2*, which compromises its mitochondrial function but remains a viable strain, in

127 contrast to the severe growth defects and developmental arrest observed in the mitochondrial-

128 specific loss-of-function mutants (Figure 5A and 5C). These findings suggest that mitochondrial

129 isoform of *mars-2* is essential in maintaining mitochondrial homeostasis.

130

131 ## Discussion

132 Our study demonstrates that the P447V mutation in the *mars-2* gene is a loss-of-function

133 mutation that induces UPR^{mt}. One reasonable possibility is that the mutation disrupts the

134 function of mitochondrial methionine-tRNA synthetase. Supporting this, we found that preventing

135 MARS-2 from entering mitochondria also strongly activates UPR^{mt} (Figure 5A and 5B). As the

136 P447V mutation occurs in a highly conserved anticodon binding domain of the enzyme (Figure

137 1F), we hypothesize it reduces methionine tRNA charging, thereby affecting mitochondrial

138 protein production. Given that defects in mitochondrial translation are known to trigger UPR^{mt}

139 [33,34], P447V mutation in *mars-2* may induce UPR^{mt} by disrupting mitochondrial protein

140 production. In the future, this can be further tested by measuring charged methionine levels and

141 assessing mitochondrial protein production in P447V mutants. Additionally, our phylogenetic

142 analysis of MARS-2 in *C. elegans* reveals functional similarity with mitochondrial methionine

143 tRNA-synthetases from other species (Figure 1E).

144

145 In this study, we did not investigate the effects of P447V mutation on the cytoplasmic isoform of

146 *mars-2* in *C. elegans*. Considering the functional role of *let-65* (*mars-1*) as the cytoplasmic

147 methionyl-tRNA synthetase[35], it is plausible that any deleterious effects of P447V mutation in the

148 cytoplasmic isoform of *mars-2* are mitigated due to functional redundancy. Interestingly, UPR^{mt}

149 activation in P447V mutants was restricted to the intestine (Figure 1C, 2A, 3A, 4A, and 5A). This

150 tissue-specific response parallels findings in humans, where mitochondrial aaRS mutations

151 predominantly affect high-energy-demand tissues such as muscles and the nervous system[36].

152 The intestinal specificity observed in these mutants may reflect intrinsic differences in

153 mitochondrial dynamics or stress sensitivity between tissues.

154

155 While this study establishes a strong link between the P447V mutation in *mars-2* and UPR^{mt}

156 activation, the precise mechanism by which mitochondrial MetRS dysfunction triggers UPR^{mt}

157 remain to be explored. The mutant strains developed in this study provide a valuable tool for

158 mitochondrial biology and the pathological consequences of aaRS mutations.

159

Materials and methods

161 <u>Worm strains</u> – All the strains used in this study are listed in the table below:

162

Genotype	Source	Strain name
C. elegans wildtype	CGC	N2
zcIs13[hsp-6p::GFP] V	CGC	GL347
mptsi1, zcIs13 V; mpt139	Held et al (2024)[29]	MRP649
mars-2(mpt213[P447V]) I	This study	MRP1019
mars-2(mpt215[P447V]) I; zcIs13 V	This study	MRP1021
mars-2(mpt216[P447V]) I; zcIs13 V	This study	MRP1022
mars-2(mpt227[ΔMTS]) I +/-; zcIs13 V	This study	MRP1042
mars-2(mpt228[ΔMTS]) I +/-; zcIs13 V	This study	MRP1043

163

164 <u>Worm maintenance</u> – All worms were raised at 20 °C on nematode growth media (NGM)

165 seeded with OP50 *Escherichia coli* bacteria.

166

167   CRISPR/Cas9 gene editing – CRISPR was performed as previously described[37] using Alt-R

168   S.p. Cas9 Nuclease V3 (IDT #1081058), tracrRNA (IDT #1072532) and using *dpy-10* as a co-

169   injection marker[38]. The crRNA sequence used for introducing P447V mutation in *mars-2* was

170   TCAATCAAGTCAACCATGGA and ssODN sequence:

171   TGAAAGAAGCGAATCGATTATTTCAATCAAGTCAATCATGGAAAGAAATTGATGAAAAACGGC

172   TGAAAAGTCTACTTTT. The crRNA sequence used for introducing ΔMTS mutation in *mars-2*

173   was 5' – CGCTTTTCATGAATCCTTGG – 3' and ssODN sequence: 5' –

174   cgattgattaattcactttttttttgcgcttttcGCTAATCCATGGAGATTTTTCGTGAGAAAATCGAGTACATTTG

175   TCACTTC– 3'. The Cas9::tracrRNA::crRNA complexes and repair templates were microinjected

176   into the zcls13 strain to obtain *mpt215* and *mpt227*, respectively. The *dpy-10* co-injection

177   marker was then outcrossed using a wildtype (N2, RRID: WBStrain00000001) strain.

178

179   Genetic crosses – The genetic crosses were performed by crossing 5 larval stage 4 (L4)

180   hermaphrodites of a strain to 15 heterozygous males of another strain (these were first obtained

181   by crossing L4 hermaphrodites of the strain with wildtype males). An appropriate number of F1

182   generation L4 hermaphrodites were cloned out of the cross plate and allowed to have self-

183   progeny. An appropriate number of F2 progeny were cloned and genotyped for allele of interest

184   once they had progeny. The genotyping was performed using IDT primers and NEB enzymes

185   below:

186   For genotyping P447V mutation in *mars-2,* Forward – GGAAATGGTCGAGGAGAGCAGAG

187   Reverse –CCTCCAGAATAGCTTCCAAATCGGG and NcoI cuts wildtype amplicon only. For

188   genotyping ΔMTS mutation in *mars-2,* Forward –

189   CAGGAATTGAAGGAGCTAAATTCTGCAAAG Reverse –

190   CGAAATTAGGGATTATCAGACGCAAGTTCC and NcoI-HF cuts mutant amplicon only.

191

192   Fluorescence microscopy – Zeiss Axio Zoom V16 stereo zoom microscope was used for

193   imaging fluorescent UPR^{mt} reporter in whole animals. The worms were mounted on 2% agarose

194   pads on microscopic slides and immobilized using 1 µL of 100 mM levamisole (ThermoFisher

195   #AC187870100). A coverslip was used to avoid the worms from drying.

196

197   Image analysis – The brightfield and fluorescent animal images were analyzed using the Fiji

198   application. The outline of each worm was traced as the region of interest on the brightfield

199   snapshot. The 'Measure' feature under 'Analyze' tab was used for measuring the area of the

200   animal and its fluorescence intensity (calculated by summing all the pixel values within the

201   region of interest and dividing by the total number of pixels in that area) in the fluorescence

202   snapshot.

203

204   RNA interference –RNAi was performed by shifting embryos to NGM plates seeded with RNAi

205   bacteria, as described previously[39]. *E. coli* HT115 carrying specific RNAi clones were grown

206   overnight from a single colony in 2 mL of LB supplemented with 50 µg/mL ampicillin. To make

207   16 RNAi plates, 50 ml of LB supplemented with 50 µg/ml ampicillin and inoculated with 500 µL

208   of the overnight culture, then incubated while shaking at 37 °C for 4–5 hours (to an OD$_{550\text{-}600}$ of

209   ~0.8). Next, to induce expression of the double stranded RNA, cultures were supplemented with

210   an additional 50 ml of LB supplemented with 50 µg/ml ampicillin and 4 mM IPTG and then

211   continued to incubate while shaking at 37 °C for 4 hours. Following incubation, bacteria were

212   pelleted by centrifugation at 3900 rpm for 6 min. Supernatant was decanted and pellets were

213   gently resuspended in 4 ml of LB supplemented with 8 mM IPTG. 250 µl of resuspension was

214   seeded onto standard NGM plates containing 1 mM IPTG. Plates were left to dry overnight and

215   then used within 2 weeks. Bacterial RNAi strains were from Ahringer RNAi Feeding Library,

216   grown from single colony and identity confirmed by Sanger sequencing. *Y105E8A.20*

217   (Y105E8A.20), *atfs-1* (ZC376.7).

218

219   <u>Statistical analysis</u> – For whole animal fluorescence analysis, a sample size of 24 was used in

220   the study and each animal was considered a biological replicate. The p-values under 0.05 were

221   considered significant while p-values above 0.05 were considered non-significant (ns). All

222   statistical analysis was performed using Prism 10 and the information on specific statistical test

223   can be found in the figure legend.

224

Data availability

226   Strains are available upon request. The authors affirm that all data necessary for confirming the

227   conclusions of the article are present within the article and figures.

228

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236

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239

Conflict of interest

241   The authors declare no conflict of interest.

242

Author contributions

Bharat Vivan Thapa – Writing - original draft, Investigation, Methodology, Validation, Formal analysis, Visualization, Conceptualization

Mohit Das – Investigation, Methodology, Validation, Formal analysis, Visualization, Conceptualization

James P. Held – Conceptualization, Investigation, Methodology, Writing - review & editing

Maulik R. Patel – Supervision, Writing - review & editing, Funding acquisition, Project administration, Conceptualization

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References

1. Suomalainen, A. & Nunnari, J. Mitochondria at the crossroads of health and disease. *Cell* **187**, 2601–2627 (2024).

2. Chen, W., Zhao, H. & Li, Y. Mitochondrial dynamics in health and disease: mechanisms and potential targets. *Signal Transduct. Target. Ther.* **8**, 333 (2023).

3. Okimoto, R., Macfarlane, J. L., Clary, D. O. & Wolstenholme, D. R. The mitochondrial genomes of two nematodes, Caenorhabditis elegans and Ascaris suum. *Genetics* **130**, 471–498 (1992).

4. Ojala, D., Montoya, J. & Attardi, G. tRNA punctuation model of RNA processing in human mitochondria. *Nature* **290**, 470–474 (1981).

5. Sissler, M., González-Serrano, L. E. & Westhof, E. Recent Advances in Mitochondrial Aminoacyl-tRNA Synthetases and Disease. *Trends Mol. Med.* **23**, 693–708 (2017).

264   6. Giegé, R. & Springer, M. Aminoacyl-tRNA Synthetases in the Bacterial World. *EcoSal*

265   *Plus* **7**, 10.1128/ecosalplus.ESP-0002–2016 (2016).

266   7. Wolf, Y. I., Aravind, L., Grishin, N. V. & Koonin, E. V. Evolution of aminoacyl-tRNA

267   synthetases--analysis of unique domain architectures and phylogenetic trees reveals

268   a complex history of horizontal gene transfer events. *Genome Res.* **9**, 689–710

269   (1999).

270   8. Boczonadi, V., Jennings, M. J. & Horvath, R. The role of tRNA synthetases in

271   neurological and neuromuscular disorders. *FEBS Lett.* **592**, 703–717 (2018).

272   9. Wei, N., Zhang, Q. & Yang, X.-L. Neurodegenerative Charcot-Marie-Tooth disease as

273   a case study to decipher novel functions of aminoacyl-tRNA synthetases. *J. Biol.*

274   *Chem.* **294**, 5321–5339 (2019).

275   10.   Jordanova, A. *et al.* Disrupted function and axonal distribution of mutant tyrosyl-

276   tRNA synthetase in dominant intermediate Charcot-Marie-Tooth neuropathy. *Nat.*

277   *Genet.* **38**, 197–202 (2006).

278   11.   Antonellis, A. *et al.* Glycyl tRNA synthetase mutations in Charcot-Marie-Tooth

279   disease type 2D and distal spinal muscular atrophy type V. *Am. J. Hum. Genet.* **72**,

280   1293–1299 (2003).

281   12.   Latour, P. *et al.* A major determinant for binding and aminoacylation of tRNA(Ala)

282   in cytoplasmic Alanyl-tRNA synthetase is mutated in dominant axonal Charcot-Marie-

283   Tooth disease. *Am. J. Hum. Genet.* **86**, 77–82 (2010).

284   13.   Kim, D., Kwon, N. H. & Kim, S. Association of aminoacyl-tRNA synthetases with

285   cancer. *Top. Curr. Chem.* **344**, 207–245 (2014).

286   14.   Arif, A. *et al.* Two-site phosphorylation of EPRS coordinates multimodal

287   regulation of noncanonical translational control activity. *Mol. Cell* **35**, 164–180 (2009).

288   15.   Han, J. M. *et al.* Leucyl-tRNA synthetase is an intracellular leucine sensor for the

289   mTORC1-signaling pathway. *Cell* **149**, 410–424 (2012).

290   16.   Shin, S.-H. *et al.* Implication of leucyl-tRNA synthetase 1 (LARS1) over-

291   expression in growth and migration of lung cancer cells detected by siRNA targeted

292   knock-down analysis. *Exp. Mol. Med.* **40**, 229–236 (2008).

293   17.   Torres, A. G., Reina, O., Stephan-Otto Attolini, C. & Ribas de Pouplana, L.

294   Differential expression of human tRNA genes drives the abundance of tRNA-derived

295   fragments. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 8451–8456 (2019).

296   18.   Tang, X. *et al.* Mitochondrial tRNASer(UCN) variants in 2651 Han Chinese

297   subjects with hearing loss. *Mitochondrion* **23**, 17–24 (2015).

298   19.   Scheper, G. C. *et al.* Mitochondrial aspartyl-tRNA synthetase deficiency causes

299   leukoencephalopathy with brain stem and spinal cord involvement and lactate

300   elevation. *Nat. Genet.* **39**, 534–539 (2007).

301   20.   Edvardson, S. *et al.* Deleterious mutation in the mitochondrial arginyl-transfer

302   RNA synthetase gene is associated with pontocerebellar hypoplasia. *Am. J. Hum.*

303   *Genet.* **81**, 857–862 (2007).

304   21.   Maffezzini, C. *et al.* Mutations in the mitochondrial tryptophanyl-tRNA synthetase

305   cause growth retardation and progressive leukoencephalopathy. *Mol. Genet.*

306   *Genomic Med.* **7**, e654 (2019).

307   22.   Konovalova, S. & Tyynismaa, H. Mitochondrial aminoacyl-tRNA synthetases in

308   human disease. *Mol. Genet. Metab.* **108**, 206–211 (2013).

309   23.   Zhao, Q. *et al.* A mitochondrial specific stress response in mammalian cells.

310   *EMBO J.* **21**, 4411–4419 (2002).

311   24.   Melber, A. & Haynes, C. M. UPRmt regulation and output: a stress response

312   mediated by mitochondrial-nuclear communication. *Cell Res.* **28**, 281–295 (2018).

313   25.   Shpilka, T. & Haynes, C. M. The mitochondrial UPR: mechanisms, physiological

314   functions and implications in ageing. *Nat. Rev. Mol. Cell Biol.* **19**, 109–120 (2018).

315   26.   Nargund, A. M., Pellegrino, M. W., Fiorese, C. J., Baker, B. M. & Haynes, C. M.

316   Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation.

317   *Science* **337**, 587–590 (2012).

318   27.   Haynes, C. M., Fiorese, C. J. & Lin, Y.-F. Evaluating and responding to

319   mitochondrial dysfunction: the mitochondrial unfolded-protein response and beyond.

320   *Trends Cell Biol.* **23**, 311–318 (2013).

321   28.   Yoneda, T. *et al.* Compartment-specific perturbation of protein handling activates

322   genes encoding mitochondrial chaperones. *J. Cell Sci.* **117**, 4055–4066 (2004).

323   29.   Held, J. P. *et al.* Germline status and micronutrient availability regulate a somatic

324   mitochondrial quality control pathway via short-chain fatty acid metabolism. *bioRxiv*

325   2024.05.20.594820 (2024) doi:10.1101/2024.05.20.594820.

326   30.   Soo, S. K., Traa, A., Rudich, P. D., Mistry, M. & Van Raamsdonk, J. M. Activation

327   of mitochondrial unfolded protein response protects against multiple exogenous

328   stressors. *Life Sci. Alliance* **4**, e202101182 (2021).

329   31.   James, P. A. *et al.* Severe childhood SMA and axonal CMT due to anticodon

330   binding domain mutations in the GARS gene. *Neurology* **67**, 1710–1712 (2006).

331   32.   Zheng, T. *et al.* Cytoplasmic and mitochondrial aminoacyl-tRNA synthetases

332   differentially regulate lifespan in Caenorhabditis elegans. *iScience* **25**, 105266 (2022).

333   33.   Wang, G., Fan, Y., Cao, P. & Tan, K. Insight into the mitochondrial unfolded

334   protein response and cancer: opportunities and challenges. *Cell Biosci.* **12**, 18

335   (2022).

336   34.   Houtkooper, R. H. *et al.* Mitonuclear protein imbalance as a conserved longevity

337   mechanism. *Nature* **497**, 451–457 (2013).

338   35.   Alriyami, M. Z., Jones, M. R., Johnsen, R. C., Banerjee, Y. & Baillie, D. L. let-65 is

339   cytoplasmic methionyl tRNA synthetase in C. elegans. *Meta Gene* **2**, 819–830 (2014).

340   36.   Antonellis, A. & Green, E. D. The Role of Aminoacyl-tRNA Synthetases in

341   Genetic Diseases. *Annu. Rev. Genomics Hum. Genet.* **9**, 87–107 (2008).

342   37.   Dokshin, G. A., Ghanta, K. S., Piscopo, K. M. & Mello, C. C. Robust Genome

343   Editing with Short Single-Stranded and Long, Partially Single-Stranded DNA Donors

344   in Caenorhabditis elegans. *Genetics* **210**, 781–787 (2018).

345   38.   Paix, A., Folkmann, A., Rasoloson, D. & Seydoux, G. High Efficiency, Homology-

346   Directed Genome Editing in Caenorhabditis elegans Using CRISPR-Cas9

347   Ribonucleoprotein Complexes. *Genetics* **201**, 47–54 (2015).

348   39.   Held, J. P. *et al.* A tRNA processing enzyme is a key regulator of the

349   mitochondrial unfolded protein response. *eLife* **11**, e71634 (2022).

350

351   **Figures**

352   **Figure 1**: Phylogenetic analysis show that P447 is a highly conserved amino acid residue

353   across species. (A) Gene transcription diagram depicting the expression of *hsp-6p::GFP*

354   transcriptional reporter. (B) Schematic for the forward genetic screen to identify activators of

355   mitochondrial unfolded protein response. (C) Representative brightfield and corresponding

356   fluorescent micrographs showing UPR^mt reporter (*hsp-6p::GFP*) activation in day 2 (D2) adult

357   wildtype and mutant (*mpt139)* animals. (D) Scatter plot showing fluorescence intensity

358   quantification of the UPR^mt reporter in wildtype and mutant animals normalized to the reporter

359   intensity in wildtype animals (n = 24, mean and SD shown, unpaired t-test with Welch's

360   correction). (E) Phylogenetic tree illustrating evolutionary relationships between species based

361   on their mitochondrial and cytoplasmic methionine tRNA synthetase protein sequences

362   (Y105E8A.20 highlighted in red). (F) Pictorial depiction of the two isoforms of MARS-2 protein,

363   along with the conserved proline residue (P447) in the anticodon binding domain of the protein

364   in *C. elegans*. Scale bar – 200 μm, GFP – green fluorescent protein, MTS – mitochondrial

365   targeting sequence, F.C. – Fold change, ns – non-significant, * – P < 0.05, ** – P < 0.01, *** – P

366   < 0.001, **** – P < 0.0001

367

368   **Figure 2**: P447V is the causal mutation in MARS-2 that induces mitochondrial unfolded protein

369   response.

370   (A) Representative brightfield and corresponding fluorescent micrographs showing UPR^mt

371   reporter (*hsp-6p::GFP*) activation in day 2 (D2) adult wildtype and *mars-2[P447V]* animals. (C)

372   Scatter plot showing fluorescence intensity quantification of the UPR^mt reporter in wildtype and

373   *mars-2[P447V]* animals normalized to the reporter intensity in wildtype animals (n = 24, mean

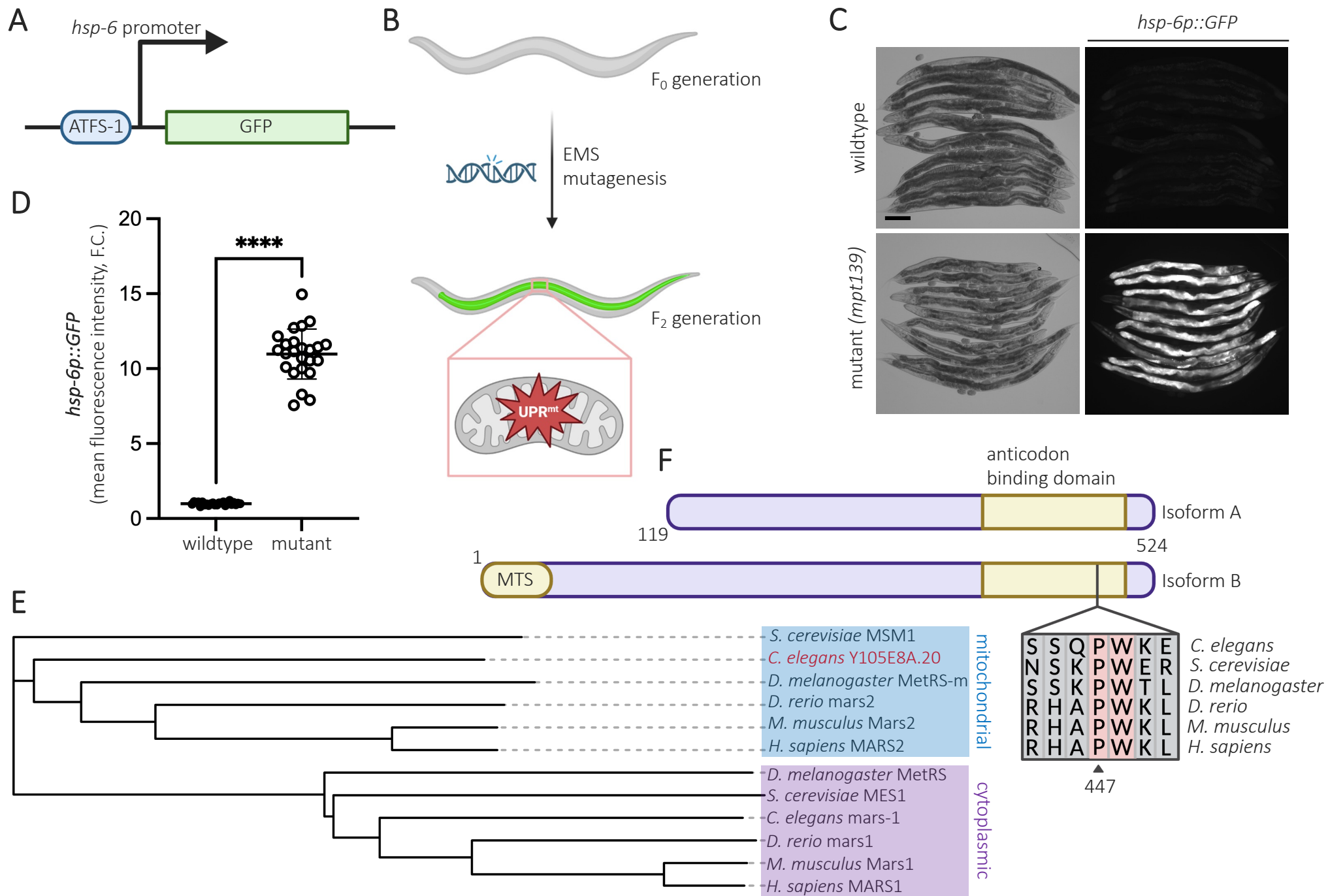374   and SD shown, unpaired t-test with Welch's correction). Scale bar – 200 μm.

375

376   **Figure 3**: MARS-2(P447V)-mediated mitochondrial unfolded protein response is ATFS-1

377   dependent. (A) Representative brightfield and corresponding fluorescent micrographs showing

378   UPR^mt reporter activation in D2 adult wildtype and *mars-2[P447V]* animals upon control and

379   *atfs-1* RNAi. (B) Scatter plot showing fluorescence intensity quantification of the UPR^mt reporter

380   in wildtype and *mars-2[P447V]* animals upon control and *atfs-1* RNAi normalized to the reporter

381   intensity in wildtype animals on control RNAi (n = 18 in *mars-2[P447V]* on *atfs-1* RNAi, n=24

382   others, mean and SD shown, two-way ANOVA with Sidak's multiple comparison test). Scale bar
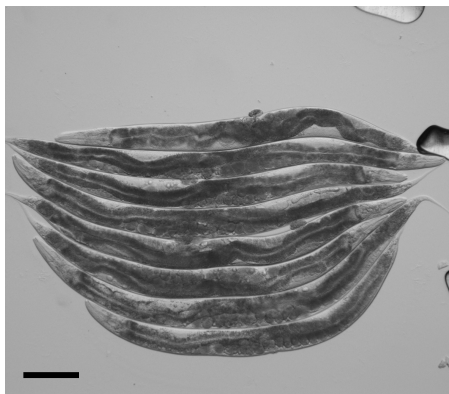
383   – 200 µm.

384

385   **Figure 4**: P447V mutation in MARS-2 is a loss-of-function mutation. (A) Representative

386   brightfield and corresponding fluorescent micrographs showing UPR^{mt} reporter activation in D2

387   adult wildtype and *mars-2[P447V]* animals upon control and *mars-2* RNAi. (G) Scatter plot

388   showing fluorescence intensity quantification of the UPR^{mt} reporter in wildtype and *mars-*

389   *2[P447V]* animals upon control and *mars-2* RNAi normalized to the reporter intensity in wildtype

390   animals on control RNAi (n = 24, mean and SD shown, two-way ANOVA with Sidak's multiple

391   comparison test). Scale bar – 200 µm.

392

393   **Figure 5**: Loss of mitochondrial MARS-2 isoform by disrupting MTS activates mitochondrial

394   unfolded protein response. (A) Representative brightfield and corresponding fluorescent

395   micrographs showing UPR^{mt} reporter activation in D2 adult wildtype, *mars-2[P447V],* and *mars-*

396   *2[ΔMTS]* animals. (B) Scatter plot showing fluorescence intensity quantification of the UPR^{mt}

397   reporter in wildtype, *mars-2[P447V]*, and *mars-2[ΔMTS]* animals normalized to the reporter

398   intensity in wildtype animals (n = 24, mean and SD shown, one-way ANOVA with Dunnett's

399   multiple comparison test) (C) Scatter plot depicting outline area quantification of wildtype, *mars-*

400   *2[P447V]*, and *mars-2[ΔMTS]* animals (n = 24, mean and SD shown, one-way ANOVA with

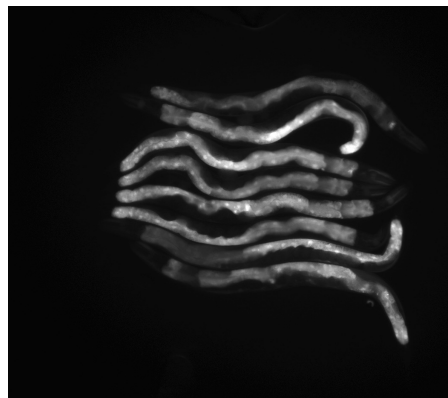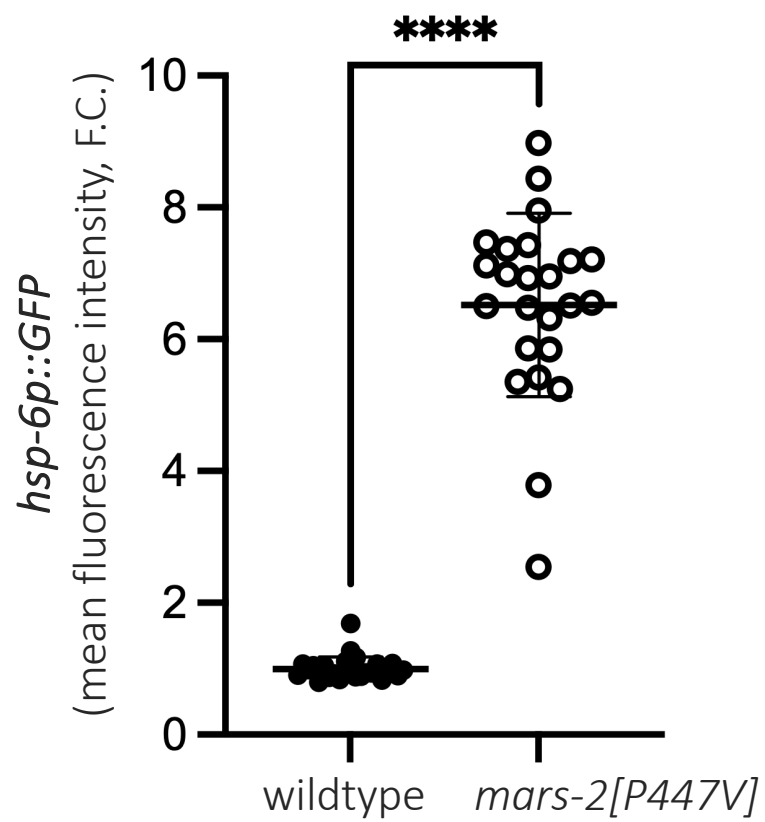401   Dunnett's multiple comparison test). Scale bar – 200 µm.

A

hsp-6 promoter
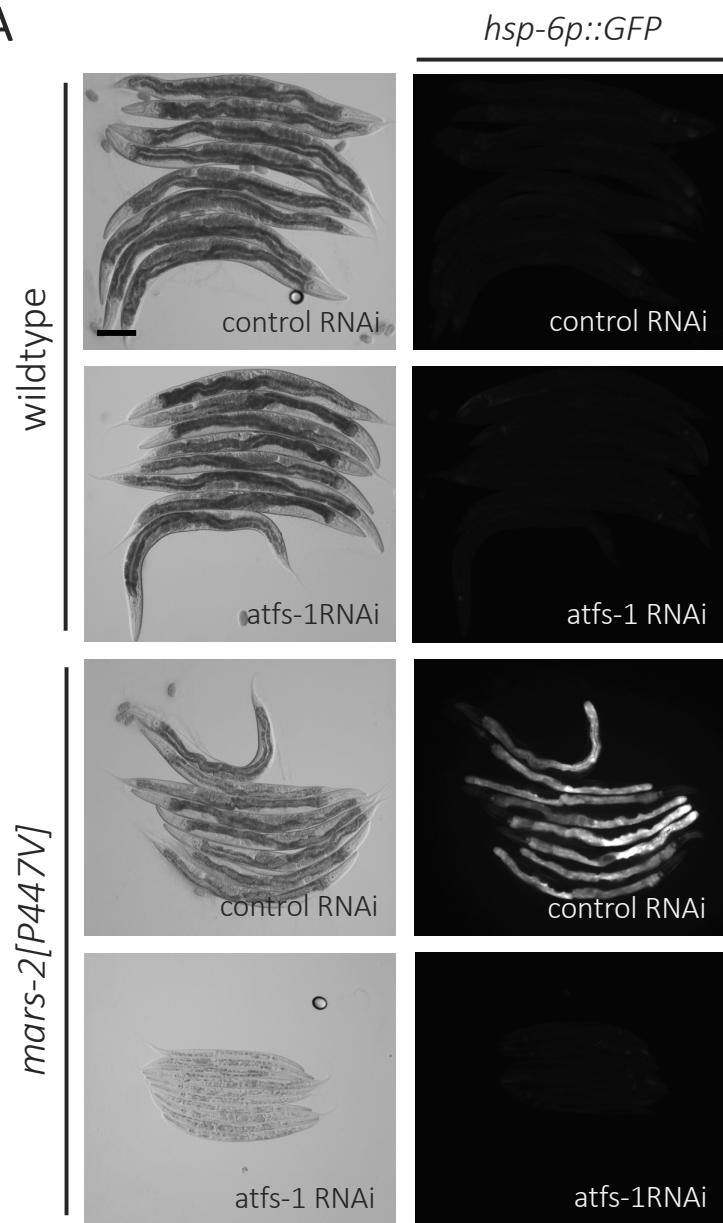
ATFS-1   GFP

B

F₀ generation

EMS mutagenesis

F₂ generation

UPRᵐᵗ

C

hsp-6p::GFP

wildtype

mutant (mpt139)

D

hsp-6p::GFP (mean fluorescence intensity, F.C.)

****

wildtype   mutant

F

anticodon binding domain

119   524   Isoform A

1   MTS   Isoform B

447

S	S	S	Q	P	W	K	E	*C. elegans*
N	S	S	K	P	W	E	R	L	*S. cerevisiae*
N	S	S	K	P	W	T	L	L	*D. melanogaster*
S	R	H	A	A	P	W	K	L	L	*D. rerio*
R	R	H	A	A	P	W	K	L	L	*M. musculus*
R	H	A	P	W	K	L	L	*H. sapiens*

E

S. cerevisiae MSM1
C. elegans Y105E8A.20
D. melanogaster MetRS-m
D. rerio mars2
M. musculus Mars2
H. sapiens MARS2

mitochondrial

D. melanogaster MetRS
S. cerevisiae MES1
C. elegans mars-1
D. rerio mars1
M. musculus Mars1
H. sapiens MARS1

cytoplasmic

A

wildtype

mars-2[P447V]

hsp-6p::GFP

B

hsp-6p::GFP
(mean fluorescence intensity, F.C.)

****

wildtype   mars-2[P447V]

A

hsp-6p::GFP

wildtype
- control RNAi | control RNAi
- atfs-1RNAi | atfs-1 RNAi

mars-2[P447V]
- control RNAi | control RNAi
- atfs-1 RNAi | atfs-1RNAi

B

- ● control RNAi
- ● *atfs-1* RNAi

hsp-6p::GFP
(mean fluorescence intensity, F.C.)

ns

****

wildtype   *mars-2[P447V]*

A

wildtype

hsp-6p::GFP

control RNAi

control RNAi

mars-2 RNAi

mars-2 RNAi

mars-2[P447V]

control RNAi

control RNAi

mars-2 RNAi

mars-2RNAi

B

● control RNAi ● *mars-2* RNAi

**

****

hsp-6p::GFP
(mean fluorescence intensity, F.C.)

25

20

15

10

5

0

-5

wildtype

mars-2[P447V]

A

wildtype

mars-2[P447V]

mars-2[ΔMTS]

hsp-6p::GFP

B

mars-2[ΔMTS]

mars-2[P447V]

wildtype

****

****

hsp-6p::GFP
(mean fluorescence intensity, F.C.)

C

mars-2[ΔMTS]

mars-2[P447V]

wildtype

****

****

Worm area
(mean worm area, F.C.)