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Mitochondrial EF4 links respiratory dysfunction and cytoplasmic translation in *Caenorhabditis elegans*

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ABSTRACT

How animals coordinate cellular bioenergetics in response to stress conditions is an essential question related to aging, obesity and cancer. Elongation factor 4 (EF4/LEPA) is a highly conserved protein that promotes protein synthesis under stress conditions, whereas its function in metazoans remains unknown. Here, we show that, in *Caenorhabditis elegans*, the mitochondria-localized CeEF4 (referred to as mtEF4) affects mitochondrial functions, especially at low temperature (15 °C). At worms' optimum growing temperature (20 °C), *mtef4* deletion leads to self-brood size reduction, growth delay and mitochondrial dysfunction. Transcriptomic analyses show that *mtef4* deletion induces retrograde pathways, including mitochondrial biogenesis and cytoplasmic translation reorganization. At low temperature (15 °C), *mtef4* deletion reduces mitochondrial translation and disrupts the assembly of respiratory chain supercomplexes containing complex IV. These observations are indicative of the important roles of mtEF4 in mitochondrial functions and adaptation to stressful conditions.

1. Introduction

Mitochondria are ancient bacterial symbionts which possess their own genome (mitochondrial DNA, mtDNA) and translation machinery. The mitochondrial translation, which synthesizes the proteins encoded by mtDNA, is involved in regulating the respiration chain function, oxygen consumption, and mtDNA maintenance [1–3]. Some reports have shown that the mutations of translation elongation factors are usually linked to disease. mtEFTu, mtEFG1 and mtEFTs are elongation factors involved in mitochondrial translation [4]. Mutations in mtEFG1 lead to a significant global translational defect and severe hepato(encephalo) pathy [2,5,6]. mtEFTu mutations also result in a severe decrease in mitochondrial protein synthesis [5,7]. In addition, mtEFTs mutations lead to encephalomyopathy or hypertrophic cardiomyopathy [8]. More interestingly, mitochondrial translation inhibition has been identified as the mechanism of tigecycline-mediated leukemia lethality, and inhibition of mitochondrial translation could be a therapeutic strategy to treat human acute myeloid leukemia [9]. These results demonstrate that mitochondrial translation elongation factors, which influence the accuracy and rate of mitochondrial translation, have an important impact on health.

mtef4 is the mitochondrial homolog of *Escherichia coli lepA*, as well as yeast *guf1*, which are translation elongation factors. LEPA can promote back-translocation of the ribosome along the mRNA *in vitro*, and the cryo-electron microscopy-derived structure shows that LEPA interacts indirectly with the back-translocated tRNA in the A-site region [10]. It was first proposed to affect the fidelity of protein synthesis in *E. coli* and yeast [11,12]. Nevertheless, *in vitro* and *in vivo* analyses showed that LEPA does not affect the accuracy of translation in *E. coli* [13,14]. In contrast, LEPA/GUF1 can promote protein synthesis under stress conditions, such as low temperatures and high Mg^{2+} concentrations [11, 13]. Consistent with the roles of LEPA in translation, *lepA* mutation affects bacterial growth under stressful conditions, including potassium

Abbreviations: C. elegans, *Caenorhabditis elegans*; WT, wild-type; EF4, elongation factor 4; mtEF4, mitochondrial EF4; mtDNA, mitochondrial DNA; BP, base pairs; ETC, electron transport chain; mtEFTu, mtEFG1 and mtEFTs, mitochondrial elongation factor Tu, G1 and Ts; MMP, mitochondria membrane potential; L1, larvae stage 1; L3, larvae stage 3; L4, larvae stage 4; RNA-Seq, RNA-Sequencing; DEGs, differentially expressed genes; qPCR, quantitative polymerase chain reaction; BNG, blue native gel; IGA, in gel activity; sDR, solid diet restriction; CFU, colony-forming units; NGM, nematode growth medium; TCA, trichloroacetic acid; ND1, NADH dehydrogenase, subunit 1; CTB-1, cytochrome b subunit I; CO1, cytochrome c oxidase subunit I; ATP6, ATP synthase F0 subunit 6; RPL, ribosomal proteins of the large subunit; AAK-2, AMP-activated protein kinase α subunit; SOD-2, superoxide dismutase 2; CLK-1, clock (biological timing) abnormality 1; DAF-16, abnormal dauer formation protein 16; EAT-2, EATing: abnormal pharyngeal pumping; HSF-1, heat shock transcription factor 1; ISP-1, the Rieske iron-sulfur protein; FOXO, forkhead box O transcription factor; PKC, protein kinase C; TRP, transient receptor potential; TRPA-1, transient receptor potential cation channel, subfamily A, member 1; eIF2α, eukaryotic translation initiation factor 2 alpha; GCN2, general control nonderepressible 2; TOR, target of rapamycin; ATM, Ataxia-telangiectasia mutated; PKR, RNA activated protein kinase; FSTR-1, faster 1; FSTR-2, faster 2; CEH-23, *C. elegans* homeobox 23; HIF-1, hypoxia-inducible factor-1

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tellurite, high ionic conditions, and low temperatures, but has no obvious effects under normal survival conditions [13,14]. Similar to *lepA* in *E. coli*, no effect is observed in the null mutation of *guf1* under optimal yeast survival conditions, while mitochondrial dysfunctions and yeast growth defects have been found under suboptimal conditions, such as low and high temperatures and non-fermentable carbon starvation [11]. However, the roles of mtEF4 in mitochondrial translation and the adaptation of mitochondria to stressors in metazoans have not been characterized.

Here, we examined the functions of mtEF4 in *Caenorhabditis elegans* and found that it played roles in mitochondrial functions, worm growth and stress adaptation. We showed that mtEF4 was localized to the mitochondria, similar to GUF1 in yeast. Although there were no obvious phenotypes, *mtef4* deletion resulted in slight developmental delays and a decrease in self-brood size under normal culturing conditions. In addition, we observed mitochondrial dysfunction and the attenuation of cytoplasmic translation in *mtef4*-deleted worms. Further study showed that *mtef4* deletion significantly reduced mitochondrial translation and disrupted the assembly of ETC supercomplexes at low temperatures. Lifespan screening was subsequently performed in specific metabolic conditions, such as low temperature, diet restriction and starvation, to explore the roles of mtEF4 in aging. However, loss of mtEF4 did not affect lifespan under various conditions. Collectively, our data demonstrated an adaptive role for mtEF4 in mitochondrial functions, especially under suboptimal conditions.

2. Materials and methods

2.1. C. elegans strains and maintenance conditions

Conditions for growth, maintenance and genetic manipulation of *C. elegans* were as described previously [15]. The wild-type (WT) strain was N2 Bristol. *mtef4(ok3023)*, *isp-1(qm150)*, *eat-2(ad1116)*, and SD1347 *ccIs4251* were obtained from the Caenorhabditis Genetics Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). *mtef4(tm4178)* was obtained from the Japanese National Bioresource Project for *C. elegans*. Both *mtef4(tm4178)* and *mtef4(ok3023)* were outcrossed four times against N2. The two alleles were confirmed by single-worm genomic PCR. For low temperatures (15 °C), starvation and sDR, experiments were beginning from L4, adult D6 and adult D4, respectively.

2.2. Plasmid construction and worm microinjection

DNA fragments encoding *mtef4* with a mutation in the stop codon and the 1.6-kb upstream region as the promoter were amplified from the genome by PCR. The primer sequences were as follows: Forward: 5′-cgggatccggtttccgaaaaattgacaag-3′; Reverse: 5′-ggggtaccatactaggag gactaggaggactaggaggctttctcttaagaacatttagga-3′.

The PCR product was inserted into the p95.75 plasmid at the *Nco*I/*Kpn*I site and confirmed by sequencing. The translational expression plasmid $P_{zk1236.1}$*::zk1236.1::gfp* was injected into N2 worms with the pRF4 plasmid as a marker. Roller worms were used to perform experiments. To determine the subcellular localization of ZK1236.1, roller worms were stained with 10 μM MitoTracker Red CMXRos (Invitrogen, USA) on NGM plates for 16–18 h and then directly observed with an Olympus laser-scanning confocal microscope (Olympus Fluoview FV500, Japan).

2.3. Mitochondrial morphology analysis

To examine mitochondrial morphology, we crossed *mtef4* mutants with *ccIs4251* worms, which carry mitochondria- and nuclear-targeted GFP under the control of the *myo-3* promoter [16]. Young adult worms were mounted on a 2% agarose pad, immobilized with 10 mM Levamisol (Sigma, USA), and analyzed under an Olympus laser-scanning confocal microscope.

2.4. ATP detection

ATP was quantified using the luciferin–luciferase method following the protocol of the ATP Bioluminescence Assay Kit CLS II (Roche, CH). Approximately 50 worms were picked into 100-μl ddH_2O, washed three times and boiled for 15 min. After centrifugation at 13,000 rpm for 30 min at 4 °C, the supernatant was transferred to a new tube and protein content was determined using the BCA kit (Pierce, USA). The standard curve of ATP level was prepared according to the kit protocol. Samples were diluted to place the measured value in the confidence interval. The luminescence of 5 μl of each sample was assayed in a luminometer (Perkin Elmer, USA) with 50 μl ATP detection buffer.

2.5. Developmental rate analysis

Adult worms were allowed to lay eggs for 2–3 h. After 24 h, unhatched eggs were examined. For the larval developmental assay, synchronized L1 worms were placed on NGM plates seeded with OP50, and the worms that failed to reach the young adult stage after 60 h were scored.

2.6. Self-brood size assay

Synchronous L3 animals were placed onto NGM plates seeded with OP50. After 36 h, 10–20 worms were transferred to a new plate (one worm per plate). The worms were transferred daily to new plates for about 5 days and live progeny were counted two days after removal of the mother worm.

2.7. Oxidative stress analysis

Oxidative stress analysis experiments were performed at 20 °C, as described previously [17]. Briefly, after growing on NGM plates for at least two generations at 20 °C, adult worms were placed on NGM plates to lay eggs for 8–12 h. At least 30 adult D6 worms were then transferred to plates containing 8 mM paraquat. For oxidative stress analysis at high temperature, worms were first cultured on plates at 20 °C until L4 and then transferred to 25 °C. After 3 days, worms were transferred to plates containing 8 mM paraquat. Worms were transferred every week, and the day of transferring to paraquat plates was recorded as $t = 0$. Censored animals including bagged, ruptured, and those crawled off the plates were omitted from the analysis.

2.8. RNA extraction, cDNA synthesis, and qPCR

Total RNA was isolated from at least 1000 worms using TRIzol reagent (Invitrogen, USA), and 2 μg of total RNA were reverse transcribed into single-strand cDNA in 25-μl reaction buffer using Molony murine leukemia virus reverse transcriptase (Promega, USA) and oligo(dT) (Promega, USA) as the primer. qPCR reactions were performed with SYBR Green Master Mix (Takara, Japan) and using the ABI StepOne plus Real-time PCR System (Applied Biosystems, USA). Target genes were normalized with the housekeeping gene *ama-1*. The primer sequences are available upon request.

2.9. RNA deep-sequencing analysis

For RNA-Seq experiments, young adult worms were harvested and washed three times with M9 buffer. Total RNA was isolated using TRIzol reagent and the integrity was examined using an agarose gel. The mRNA-Sequencing libraries were prepared according to the protocol of mRNA-Seq 8 sample-prep Kit (Illumina, USA). The reads were

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