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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

obtained from the Illumina GAII system (Illumina, USA). The quality of RNA-Seq data was checked using the FastQC program (www.bioinformatics.babraham.ac.uk/projects/fastqc). Bowtie 0.12.7 [18], Tophat 1.3.3 [19] and Cufflinks 1.2.0 [20] were used to analyze the data and identify differentially expressed genes (DEGs). The DAVID online tool was used to analyze DEGs [21,22].

2.10. Cytoplasmic translation assay

The cytoplasmic translation assay using ^{35}S-methionine incorporation was performed as described previously [23]. Briefly, OP50 bacteria were cultured in LB containing ^{35}S-methionine (PerkinElmer, USA) for 12 h and then concentrated 10-fold. Each sample (about 1000 young adult worms) was mixed with 100 μl of concentrated bacteria and incubated for 3 h at 20 °C with shaking. All samples were subsequently washed three times with M9 buffer and incubated in non-radioactive OP50 for 30 min. Worms were then washed twice with M9 buffer and protein extracts were prepared by trichloroacetic acid (TCA) precipitation. Protein concentrations were measured using the BCA kit (Pierce, USA) and ^{35}S radioactivity was measured by liquid scintillation (PerkinElmer, USA). ^{35}S-methionine incorporation was calculated by normalizing ^{35}S counts to protein.

2.11. Mitochondria isolation and organelle translation assay

Briefly, WT and *mtef4(tm4178)* worms were cultured on 15-cm plates until reaching the adult D1 stage, or transferred to 15 °C from L4 for 48 h until reaching the adult D1 stage. Worms were then collected, resuspended in MSME (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EDTA, pH to 7.4 with KOH), and homogenized in a glass Potter/Elvehjem tissue grinder with a Teflon pestle. After adding fatty acid-free bovine serum albumin (BSA, 1%), the homogenate was centrifuged (1100 ×*g*, 10 min, 4 °C) and then re-centrifuged (11,000 ×*g*, 10 min, 4 °C). The mitochondrial pellet was resuspended in 100 μl MSME and protein quantity was determined using the BCA kit. An organelle translation assay was performed as described previously with some modifications [24]. Briefly, 200 μg of mitochondria were incubated in mitochondrial incubation buffer (25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 1 mM $MgCl_2$, 0.05 mM EDTA, 10 mM Tris–HCl and 10 mM K_2HPO_4, pH 7.4) containing 10 mM glutamate, 2.5 mM malate, 1 mM ADP, 1 mg/ml fatty acid-free BSA, 100 μg/ml emetine, 100 μg/ml cycloheximide and 0.2 mM amino acids (minus methionine) (Promega, USA). The translation mixture (95 μl) was pre-warmed at 20 °C for 5 min. Next 5 μl of EasyTag™ L-methionine (0.5 mCi/ml) (PerkinElmer, USA) was added and translation was allowed to proceed for 60 min at 20 °C. After mitochondrial translation, 5 μl of ddH2O were added to another 95 μl of translation mixture as a control. Mitochondria were then washed three times with MSEM buffer containing 200 mM methionine, and the mitochondria pellet was finally resuspended in SDS-PAGE loading buffer and 25-μg mitochondrial proteins were subjected to 15–20% SDS-PAGE. The control was stained with Coomassie brilliant blue, and the labeled mitochondrial translational products were detected using the Typhoon Trio scanner (GE Healthcare, Germany).

2.12. Blue native gel (BNG) electrophoresis and in gel activity (IGA)

Mitochondria were isolated from worms as described above. BNG and IGA staining were performed as described previously [25,26]. Mitochondria (400 μg) were subjected to membrane solubilization, and Digitonin (Sigma, USA) was used as the detergent to observe ETC supercomplexes. The complexes and supercomplexes were identified as described previously [27].

2.13. Mitochondrial DNA quantification

mtDNA was quantified using qPCR. Briefly, about 20 young adult worms were collected and lyzed by standard protocol [28]. Primers (for Mito 1 and *ama-1*) and qPCR conditions were adapted from the previous paper [29]. Each comparison pools at least 3 biological repeats.

2.14. Oxygen consumption rate (OCR) measurement at 15 °C and 20 °C

WT and *mtef4(tm4178)* worms were transferred to 15 °C from L4 for 48 h until reaching the adult D1 stage, or cultured on NGM plates at 20 °C until young adult or adult D1. Worms then were harvested, washed 3 times with M9 buffer and rotated in M9 buffer for 30 min allowing worms to digest bacteria in their intestine. Between 50 and 100 washed worms were seeded into each well of the Seahorse XF-24 cell culture plates (Seahorse Bioscience, USA). Measurements were taken under basal conditions for OCR. The results normalized to the number of worms counted per well. The experiment was repeated three times under these conditions.

2.15. ETC enzymatic activity determination

Mitochondrial fractions from WT and *mtef4(tm4178)* worms under both 15 °C and 20 °C were prepared as above. The activities of mitochondrial complex IV and complex V from isolated mitochondria were measured spectrophotometrically at 550 nm and 340 nm, respectively, by using Mitochondria Complex IV Activity Assay Kit (GENMED SCIENTIFICS INC., USA) following the protocol of the manufacturer. Briefly, the purified mitochondria was resuspended in the buffer (320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris–HCl, pH 7.4) at a concentration of 1 mg/ml and placed on ice. 100 μl protein was used for enzymatic activity assay for complex IV and complex V in water baths at 25 °C and 30 °C, respectively. The activities were calculated according to the formula given in the protocols.

2.16. Lifespan analysis

Worm lifespan experiments were performed at 20 °C as described previously unless noted [23]. 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. Then, at least 90 L4/young adult worms were transferred to NGM plates. Worms were moved every other day to new plates during the first week and every week after the developing time. For lifespan experiments at 15 °C and 25 °C, worms were transferred to the corresponding temperature from L4. Experiments were beginning from adult D6 in starvation condition and from adult D4 in sDR. For sDR, we used ultraviolet-killed OP50 at 5×10^9 or 5×10^8 colony-forming units (cfu)/ml to feed worms. For conditions of superoxide, 0.1 mM paraquat was contained in the NGM plate and worms were transferred to the plates at L1 at 20 °C or a high temperature 25 °C. Also, 50 μM 2′fluoro-5′deoxyuridine (FUDR, Sigma, USA) was added to plates to prevent progeny from developing, and the day young adults formed was recorded as t = 0 for lifespan analysis. Censored animals including bagged, ruptured, and those that crawled off the plates were omitted from the lifespan analysis.

2.17. Statistical analysis

All data in this article are represented as means ± SD, with the exception of Supplementary Table S2, which shows means ± SE. Graphs were generated using GraphPad Prism (GraphPad software Inc., USA). Statistical analysis was performed using SigmaPlot (Systat Software, USA). The significance of differences between groups was determined by *t*-test or one-way ANOVA. The survival data were analyzed using the log-rank test. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. mtEF4 is localized to the mitochondrion in C. elegans

GUF1 in yeast is located in the mitochondrial matrix [11,30], while the location of its *C. elegans* homolog ZK1236.1 has not been verified. ZK1236.1 was predicted to be located in the mitochondria using the TargetP 1.1 Server with the mTP score of 0.736, with the N-terminal 22 amino acids functioning as a mitochondria-signaling peptide [31]. To confirm the above results, we constructed a translational expression plasmid $P_{zk1236.1}$*::zk1236.1::gfp* for expressing ZK1236.1::GFP in N2 worms. ZK1236.1::GFP was widely expressed in the intestine, pharyngeal bulbs and vulva (Fig. 1a). We then found that ZK1236.1::GFP co-localized with MitoTracker Red CMXRos (Invitrogen, USA) in vulva muscle and intestinal cells (Fig. 1b). At this time, we renamed *zk1236.1* as *C. elegans mtef4*. These findings suggested that mtEF4 in *C. elegans*, similar to GUF1 in yeast, was localized to the mitochondria.

3.2. Loss of mtEF4 disrupts mitochondrial morphology

There are two alleles for the *mtef4* mutant: namely, *tm4178* and *ok3023*. In the *tm4178* allele, the coding sequence for the mitochondrial targeting peptide is deleted (Fig. 2a). The *ok3023* allele contains a large deletion in the fifth exon, and the exons left have been disrupted by a frame-shift mutation (Fig. 2a). Both alleles are presumed to be null mutations. The two alleles *tm4178* and *ok3023* showed 263- and 508-bp deletions, respectively (Fig. 2b). Note that the *tm4178* allele also contained a 1-bp insertion. Mitochondrial morphology reflects mitochondrial functions, such as mitochondria membrane potential (MMP), respiratory and ATP production. We investigated mitochondrial morphology in muscle cells, in which mitochondria form tubular structures. A previous study showed that *mtef4* RNAi causes mitochondrial fragmentation, while no detailed information was available in *C. elegans* [32]. Confocal microscopy showed that mitochondrial fragmentation occurred in *mtef4*-deleted worms (Fig. 2c, d). However, like other mitochondrial mutants, such as *isp-1* and *clk-1*, the ATP level did not change in *mtef4*-deleted worms (Supplementary Fig. S1).

3.3. Loss of mtEF4 induces self-brood size reduction and developmental delay

Having established that *mtef4* was required for normal mitochondrial morphology (Fig. 2), we explored whether *mtef4* mutants exhibit phenotypes associated with mitochondrial dysfunction, such as slower developmental rate, reduced self-brood size and altered adaptation to oxidative stress [17]. We first examined embryonic and larval development. Few eggs did not hatch into first-stage larvae (L1) within 24 h in WT worms, while unhatched eggs accounted for 15.2% and 7.4% in *mtef4(tm4178)* and *mtef4(ok3023)* worms, respectively (Fig. 3a, Table 1). We next found that 20.8% and 7.1% of hatched *mtef4(tm4178)* and *mtef4(ok3023)* worms, respectively, didn't reach the young adult stage or older within 60 h (Fig. 3a, Table 1). We did not observe stage-specific developmental delay in mutants, such as the L1 or starvation-induced dauer diapause. We found a developmental delay in *mtef4* mutants, and self-brood sizes of *mtef4(tm4178)* and *mtef4(ok3023)* decreased to 1/3 and 1/17, respectively, compared to the WT (Fig. 3b). Thus, *mtef4*-deletion mutants exhibited a general slowing of developmental processes. Because *mtef4(ok3023)* had a very small brood size and we had difficulty obtaining a sufficient number of worms, only *mtef4(tm4178)* was used for subsequent experiments, such as RNA isolation, cytoplasmic translation and mitochondria isolation. We also found that *mtef4*-mutant worms showed an improved mean survival rate compared to WT worms when exposed to 8 mM paraquat, a superoxide-inducing agent (Fig. 3c). Taken together, the above results indicated that mitochondrial functions were disrupted in *mtef4* mutants.

3.4. RNA-Sequencing reveals retrograde pathways in mtef4-deleted worms

Mitochondrial dysfunctions induce retrograde pathways to regulate metabolic activities for normal growth. However, different mitochondrial mutants activate distinct pathways to affect behavioral rates and lifespan in *C. elegans* [29]. Therefore, it is important to identify retrograde pathways induced by *mtef4* deletion. We used RNA-Sequencing (RNA-Seq) to identify differentially expressed genes (DEGs) in the activated pathways and biological processes in *mtef4* mutant worms. The quality of RNA-Seq data derived from the samples of WT and *mtef4(tm4178)* were high based on FastQC (Supplementary Fig. S2a, b).

Further differential gene expression analysis identified 142 DEGs in *mtef4(tm4178)* worms, including 97 upregulated and 45 downregulated genes (Fig. 4a, Supplementary Table S1). To verify the RNA-Seq results, we used quantitative polymerase chain reaction (qPCR) to measure the mRNA levels of 18 genes selected from the up- and down-regulated groups. The results verified RNA-Seq output with a R^2 of 0.7620 (Fig. 4b, Supplementary Fig. S2c). And the upregulated genes were enriched in mitochondrial respiration, while downregulated genes included those involved in cytoplasmic translation and development (Fig. 4c, Table 2). In *C. elegans*, 12 proteins, including ND1-6 and ND4L

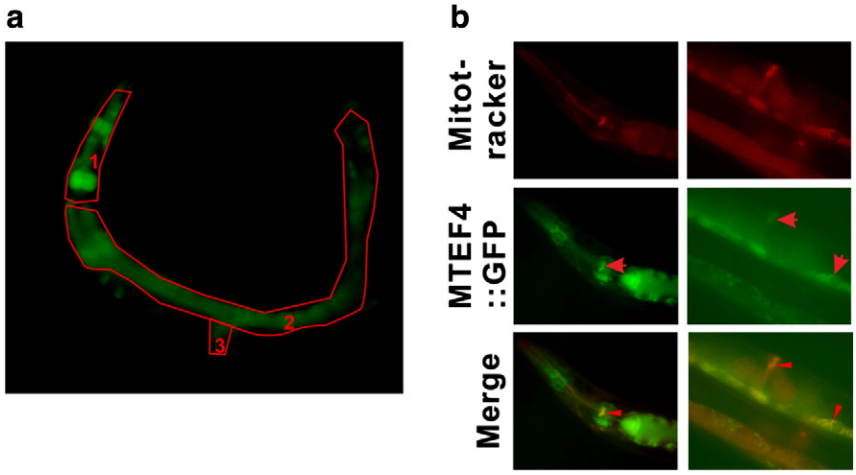


Fig. 1. mtEF4 localizes to the mitochondria. (a) An overview of mtEF4::GFP expression in the head and pharyngeal bulbs (1), intestine (2) and vulva (3). (b) mtEF4::GFP co-localized with the mitochondria marker MitoTracker red. Top: MitoTracker red; middle: mtEF4::GFP; bottom: the merged image. Arrows in (b) showed mtEF4::GFP localization, and arrowheads showed the strong expression points where mtEF4::GFP co-localized with MitoTracker red.

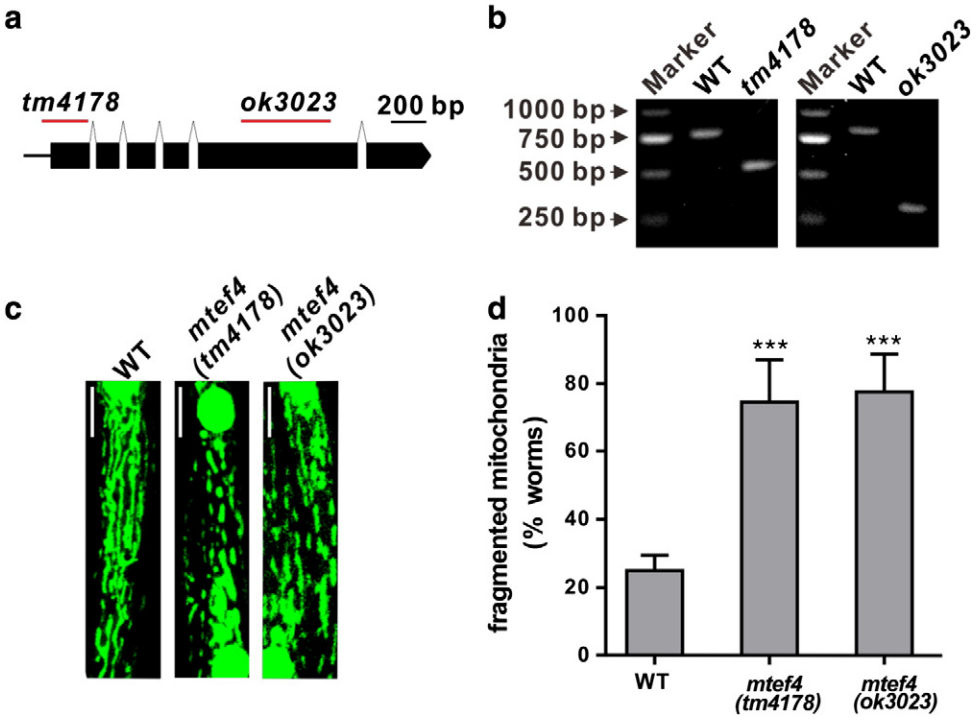


Fig. 2. Mitochondria are fragmented in *mtef4* mutants at 20 °C. (a) The panel was the schematic representation of the *mtef4* gene. Black boxes indicate exons, and black lines represent introns and the 5′ untranslated region. (b) Two different primer sets detected the 263- and 508-bp deletions in *tm4178* and *ok3023* alleles, respectively, using single worm PCR. (c) Representative images of mitochondrial morphology in the WT (*ccls4251*) and mutants (*mtef4*; *ccls4251*). The scale bar represents 10 μm. (d) Quantification of mitochondrial fragmentation in *mtef4* mutants. At least 30 worms were analyzed in each experiment (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

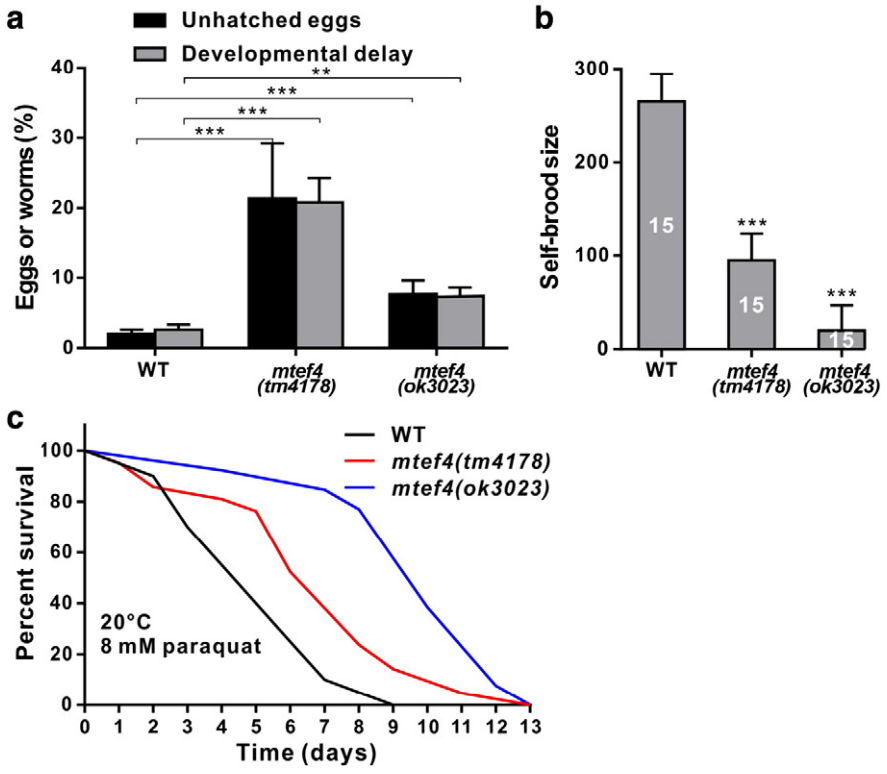


Fig. 3. Loss of mtEF4 in *C. elegans* delays development and reduces self-brood size at 20 °C. (a) More eggs unhatched within 24 h and delayed larva development from L1 to young adult in *mtef4* mutants. (b) Loss of mtEF4 resulted in decreased self-brood size. The results are presented as means ± SD. The mean total self-brood size was 270 for N2, 94 for *mtef4(tm4178)*, and 17 for *mtef4(ok3023)*. (c) *mtef4*-mutant worms showed increased survival rates when exposed to 8 mM paraquat. The mean was 4.9 days for WT, 6.4 days for *mtef4(tm4178)* ($P = 0.0129$ VS WT) and 9.8 days for *mtef4(ok3023)* ($P < 0.001$ VS WT) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

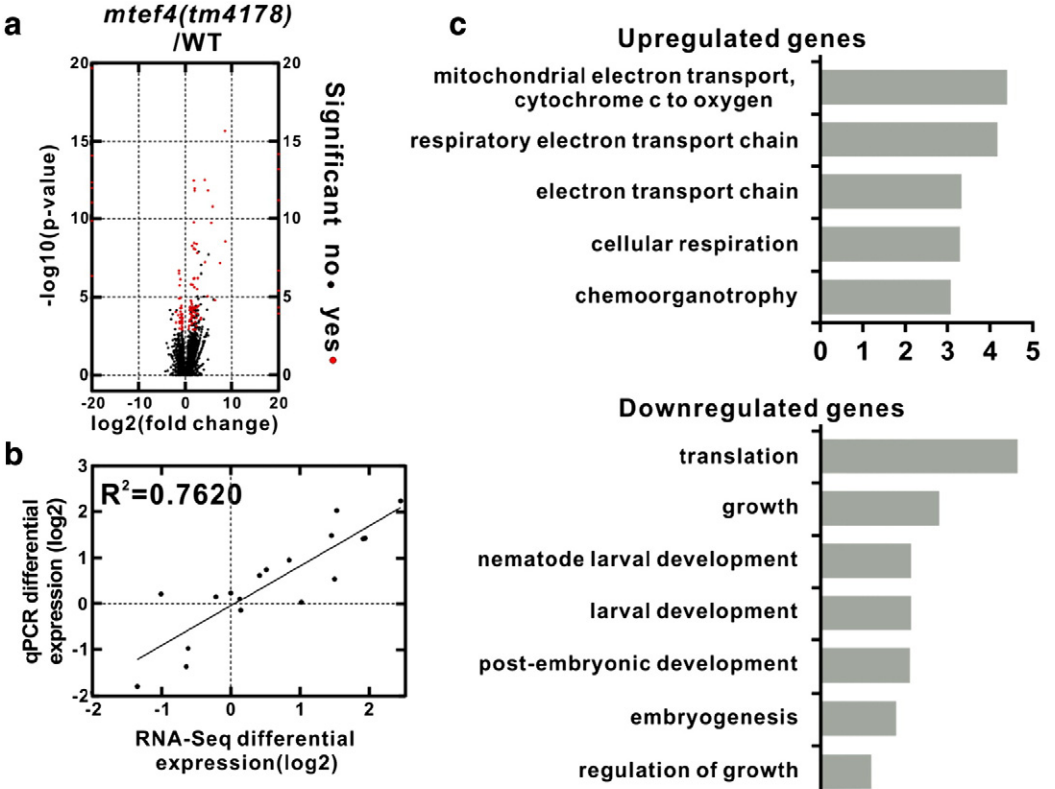
Table 1
Embryogenesis and development rate in mutants at 20 °C.

	Eggs development (24 h after laying eggs)			Larval development (60 h after L1)		
	Unhatched eggs	Hatched eggs	Ratio of unhatched eggs	L4 or before L4	Young adult or older	Ratio of worms L4 or before L4
WT	20	1011	2.0%	18	680	2.6%
mtef4(tm4178)	154	639	19.4%	189	720	20.8%
mtef4(ok3023)	41	513	7.4%	33	432	7.1%

subunits of complex I, CTB-1 subunit of complex III, COI-III subunits of complex IV and ATP6 subunit of complex V, are encoded by mtDNA [33]. 5 out of the 12 genes, *atp-6*, *ctb-1*, *coI*, *coII* and *coIII*, were upregulated upon *mtef4* deletion (Fig. 4d, Table 2). Interestingly, we also observed decreased cytoplasmic ribosomal biogenesis in *mtef4(tm4178)* worms. There are a total of 84 cytoplasmic ribosomal protein genes in *C. elegans* [34]. Among them, 13 genes (*rpl-14*, *rpl-17*, *rpl-22*, *rpl-34*, *rpl-37*, *rpl-39*, *rpl-41*, *rpl-7*, *rps-13*, *rps-20*, *rps-21*, *rps-26* and *Y37E3.8*) were downregulated (Fig. 4c, Table 2). RNA-Seq data increased our understanding of how *mtef4* mutants adapt to mitochondrial dysfunctions and suggested that mitochondrial biogenesis and cytoplasmic translation may be involved in this process.

3.5. mtEF4 is critical for mitochondrial protein synthesis and the assembly of ETC supercomplexes at low temperature 15 °C

Our RNA-Seq data revealed a large number of downregulated genes related to the cytoplasmic ribosome (Fig. 4c, Table 2). Therefore, we examined whether cytoplasmic translation was influenced by *mtef4* mutation. An overall decrease in cytoplasmic translation in *mtef4(tm4178)* worms was observed based on the ^{35}S-methionine incorporation assay (Fig. 5a). Because LEPA/GUF1 protein is known to affect mitochondrial translation at low temperatures in both *E. coli* and yeast (16 °C and 15 °C, comparing to their optimal temperatures 37 °C and 30 °C, respectively) [11,13], we examined mitochondrial translation under



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Genes	All	Mitochondrial-encoded	Ribosome proteins
DEGs	142	5	13
Up	97	5	0
Down	45	0	13

Fig. 4. Loss of mtEF4 increases mitochondrial biogenesis but suppresses cytoplasmic ribosome biogenesis at 20 °C. (a) Volcano plots showing the results of RNA-Seq; red dots represented 142 DEGs. (b) Confirmation of RNA-Seq results by qPCR with randomly selected genes ($R^2 = 0.7620$). (c) Functional classification of genes changed by mutation of the *mtef4* gene. Up- and downregulated genes were enriched respectively using online DAVID bioinformatics tools; differences with a $P < 0.001$ are shown. (d) Summary of differently expressed genes; 97 genes were upregulated and 45 downregulated. Among these genes, 13 encoding cytoplasmic ribosomal proteins were downregulated. Seven mitochondrial ETC genes were also affected. The five mitochondria-encoded genes were upregulated, while the two nuclear-encoded genes were downregulated (for details of the RNA-Seq results, see Supplementary Table S1).

Table 2
List of the 13 downregulated genes encoding ribosome proteins and 5 upregulated genes encoded by mtDNA.

Gene name	Fold change (*mtef4*/WT)	*P*-value	Gene description (concise)
(Downregulated genes encoding cytoplasmic ribosomal proteins)			
rpl-14	0.61	1.32E−04	60S ribosomal protein L14
rpl-17	0.62	4.95E−04	60S ribosomal protein L17
rpl-22	0.56	3.40E−05	60S ribosomal protein L22
rpl-34	0.61	1.21E−03	60s ribosomal protein L34
rpl-37	0.50	1.28E−04	60S ribosomal protein L37
rpl-39	0.39	3.17E−07	60s ribosomal protein L39
rpl-41	0.49	1.13E−05	60S ribosomal protein L41
rpl-7	0.66	8.46E−04	60S ribosomal protein L7
rps-13	0.54	4.92E−05	40S ribosomal protein S13
rps-20	0.54	1.99E−04	40S ribosomal protein S20
rps-21	0.40	1.22E−05	40S ribosomal protein S21
rps-26	0.48	7.53E−07	40s ribosomal protein S26
Y37E3.8	0.64	9.00E−04	60s ribosomal protein L15/L27
(Upregulated mtDNA-encoded genes)			
atp6	2.20	1.16E−04	ATP synthase F0 subunit 6
ctb-1	2.83	5.32E−09	Cytochrome b
cox3	1.90	3.55E−04	Cytochrome c oxidase subunit III
cox1	3.64	3.61E−13	Cytochrome c oxidase, subunit I
cox2	2.47	4.50E−05	Cytochrome c oxidase, subunit II

either normal condition (20 °C) or at low temperature (15 °C) in *C. elegans*. In contrast to translation in the cytoplasm, mitochondrial translation was increased in *mtef4(tm4178)* at the worm's optimal temperature of 20 °C. However, lack of mtEF4 decreased mitochondrial translation significantly at 15 °C (Fig. 5b). The increased mitochondrial translation in *mtef4(tm4178)* worms at 20 °C may be due to the high levels of mRNA, which was revealed in the RNA-Seq results (Table 2). To confirm this, mtDNA copy number was normalized to nDNA copy number and the transcript levels of mitochondria-encoded genes were determined under both normal and low temperature conditions. Consistent with this concept, mtDNA and mRNA levels of *ctb-1* and *nd5* were increased in *mtef4(tm4178)* at normal temperatures (Supplementary Fig. S3a, b, Table 2). In addition, low temperature induced higher levels of mtDNA in WT worms (Supplementary Fig. S3a), but not in *mtef4*-mutant worms (Supplementary Fig. S3). These findings suggested that (at low temperatures) the roles of mtEF4 in mitochondrial protein synthesis were dependent on altered mitochondrial ribosomal activity.

To further assess the roles of mtEF4 in mitochondrial functions and the effects of mitochondrial translation on ETC complexes, we measured the activities of ETC complexes using blue native gel (BNG) and subsequent in gel activity (IGA) analysis. Low temperature did not affect the activities of complex I and complex IV in WT worms (Fig. 5c, d).

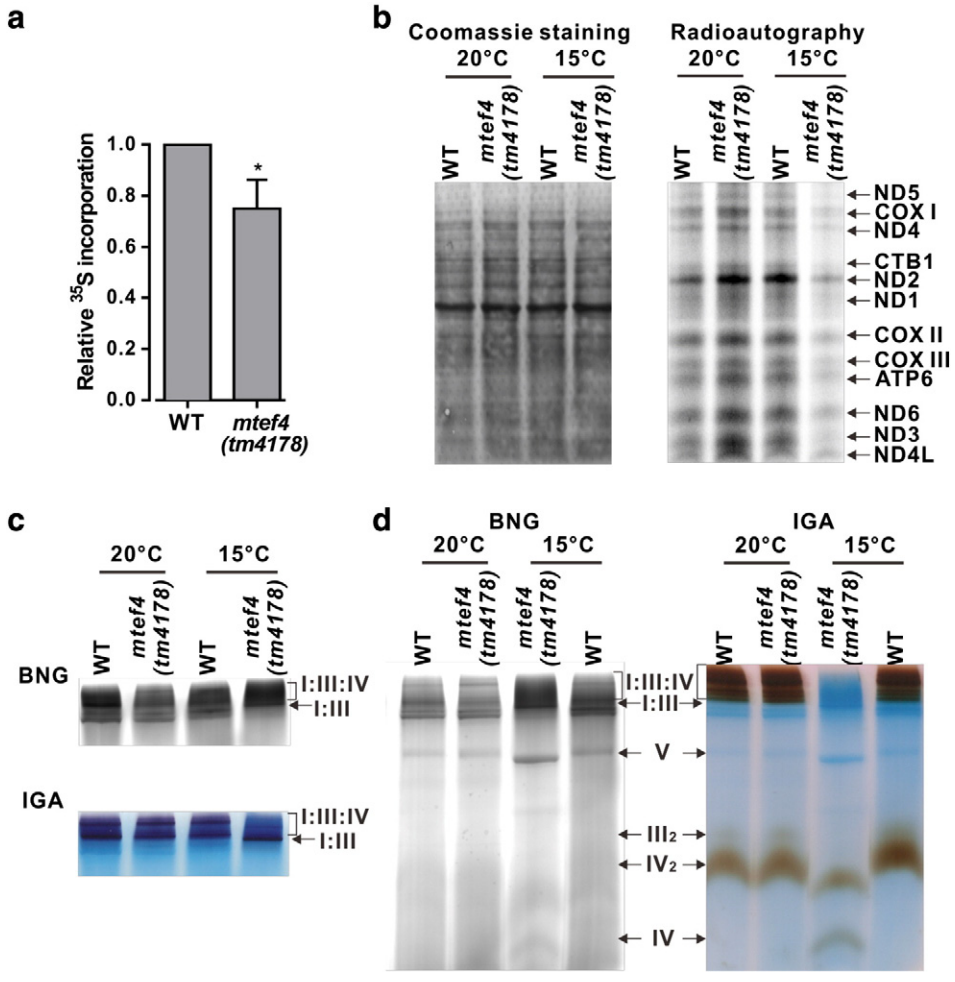

Fig. 5. Loss of *mtef4* suppresses cytoplasmic translation but improves mitochondrial translation. (a) Cytoplasmic translation was assayed using the ^{35}S-methionine incorporation method at 20 °C. Compared to WT worms, *mtef4(tm4178)* worms showed a 25% decrease in cytoplasmic translation ($P = 0.018$). (b) Mitochondrial translation from WT and *mtef4(tm4178)* worms under normal and low temperatures (20 °C and 15 °C, respectively). Loss of mtEF4 increased mitochondrial translation at 20 °C, but significantly decreased mitochondrial translation at 15 °C. (c, d) The organization of respiratory complexes in WT and *mtef4(tm4178)* worms. Representative digitonin-based BNG shows one of two similar independent gels. There was no difference at 20 °C. At 15 °C, several supercomplex bands containing complex IV disappeared from the *mtef4(tm4178)* mutant, as shown in the complex I IGA gel (c) and complex IV IGA gel (d) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

However, the total activity of complex I had a slight decrease and the supercomplex of I:III:IV was disrupted in *mtef4(4178)* worms at 15 °C, while the I:III supercomplex remained intact (Fig. 5c). This finding was confirmed by assaying complex IV activity using the IGA method, which showed that supercomplexes containing complex IV had no activity (Fig. 5d). Under this condition, we observed more single complex IV and single complex V (Fig. 5d). These findings suggested that, at low temperatures, the lack of mtEF4 induced disruption of supercomplexes containing complex IV (Fig. 5d). We also determined oxygen consumption rate (OCR) using Seahorse XF instrument (Seahorse Bioscience, USA) and found a slight decrease in *mtef4(tm4178)* worms at normal temperature (20 °C). In addition, low temperature (15 °C) did not affect OCR in WT worms significantly, but decreased that in *mtef4(tm4178)* worms (Supplementary Fig. S4a). We also performed direct enzymatic activity assays of ETC complex IV and complex V. As shown in Fig. S4b, the enzymatic activity of complex IV was decreased significantly at 15 °C, with no difference at 20 °C. In addition, low temperature or *mtef4* deletion did not affect activity of complex V (Fig. S4c). These results are consistent with a previous study, in which the activity of complex IV (but not complex III) decreased significantly under stress conditions in *guf1*-deleted yeast [11]. The opposite regulation of mitochondrial and cytoplasmic translations at 20 °C reflected an overall rearrangement of the cellular material and energy distribution. At low temperature (15 °C), *mtef4* was involved in regulating mitochondrial translation to affect the assembly of ETC supercomplexes.

3.6. Ablation of mtEF4 does not affect lifespan under various conditions

Mitochondrial functions and cytoplasmic translation are known to be associated with lifespan [35,36]. It has been reported that knockdown of *rps-26*, which is downregulated in *mtef4*-deleted worms (Table 2), prolongs lifespan [23]. Thus, we hypothesized that loss of *mtef4*, which decreased cytoplasmic translation and increased stress resistance (Figs. 2, 3), would extend lifespan. However, the lifespan of the two mutants did not differ from that of WT animals (Fig. 6a). We then examined the lifespan of mutants under low temperature (15 °C), in which *mtef4(tm4178)* exhibited severe mitochondrial defects. However, *mtef4* mutation did not affect lifespan at low temperature (Fig. 6b). Then we asked if *mtef4* plays roles in lifespan determination under higher temperature (25 °C) and found no difference (Supplementary Fig. S5a). Next, we explored whether mtEF4 was involved in lifespan extension by diet restriction. Diet restriction is known to increase lifespan in many species, including mammals, and different diet restriction methods affect lifespan in *C. elegans via* different but overlapping pathways [37]. Solid diet restriction (sDR) [38] and starvation [39,40] are two diet restriction regimens. The lifespan extension of sDR depends on *daf-16*, *aak-2* and the mitochondria-targeted gene *clk-1*, which affects mitochondrial oxidative phosphorylation. Lifespan extension by starvation depends on *hsf-1* and *eat-2*. In addition, *eat-2* is a genetic approach to reduce food intake and mimics diet restriction. Furthermore, lifespan extension by *eat-2* is mediated by *pha-4* and *clk-1*. Besides the diet restriction conditions, we also examined the roles of *mtef4* in lifespan extension in another mitochondrial mutant, *isp-1*. Because loss of *mtef4* increased oxidative stress resistance (8 mM paraquat, Fig. 3c), we further asked if *mtef4* plays roles in lifespan extension by superoxide (induced by 0.1 mM paraquat). Superoxide and *isp-1* increase lifespan by the same mechanism, while superoxide, *clk-1* and *eat-2* are mechanistically distinct and additive [41]. Unexpectedly, loss of mtEF4 did not affect lifespan under the above conditions (Fig. 6c, Supplementary Fig. S5).

4. Discussion

We used the model organism *C. elegans* to examine the expression and mitochondrial location of mtEF4 protein, and used the *mtef4* mutants to determine its roles in mitochondrial functions and worm growth. Further transcriptomic analyses revealed that knockout of *mtef4* induced retrograde pathways and altered the physiological status to sustain normal growth. Interestingly, decreased mitochondrial functions by *mtef4* deletion did not affect aging under various conditions, whereas other mitochondrial mutants, such as *isp-1*, *clk-1* and *sod-2*, increased lifespan. Overall, we have shown that *mtef4* is required for adaptation to stress conditions, which represents the complex interactions between genes and environmental factors.

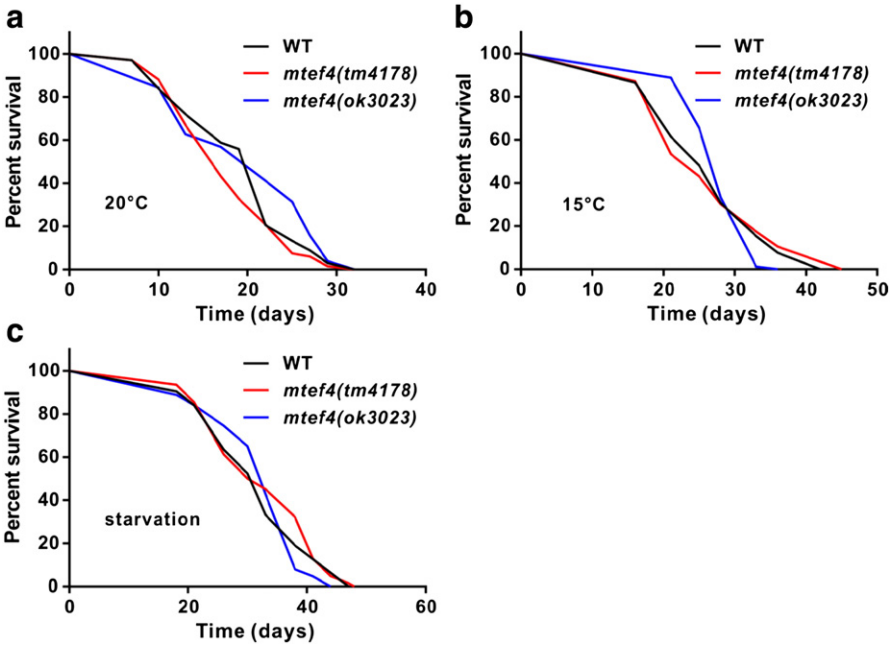

Fig. 6. *mtef4* mutation does not affect lifespan. Lifespans of *mtef4* mutants were not altered under normal conditions at 20 °C (a, mean lifespan was 19.3 days for WT, 18.0 days for *mtef4(tm4178)*, and 20.2 days for *mtef4(ok3023)*), at 15 °C (b, mean lifespan was 29.5 days for WT, 28.8 days for *mtef4(tm4178)*, and 30 days for *mtef4(ok3023)*) or under starvation conditions at 20 °C (c, mean lifespan was 31.8 days for WT, 32.8 days for *mtef4(tm4178)*, 32.3 days for *mtef4(ok3023)*) (for details of lifespan experiments, see Supplementary Table S2).

GUF1 is localized to the mitochondria in yeast, and we also confirmed the mitochondrial localization of its homolog mtEF4 in *C. elegans*. As a conserved mitochondrial protein, mtEF4 is widely expressed in the intestine, pharyngeal bulbs and vulva (Fig. 1). However, a previous DNA microarray showed that the *mtef4* transcript is enriched in germlines, suggesting that it may also be expressed in germlines [42]. Although the GFP-tagged mtEF4 protein was hardly visible in germlines, we did observe defects during embryogenesis in the *mtef4* mutants (Fig. 3a, b, Table 1).

In yeast, mitochondrial mutants induce a robust transcriptional response to increase mitochondrial biogenesis, enhance heat shock resistance and remodel metabolism [43–46]. In *C. elegans*, two well-known mitochondrial mutants, *clk-1* and *isp-1*, are known to invoke different but overlapping retrograde pathways [29,47,48]. However, an increase in mitochondrial biogenesis is a common response to respiration inhibition in yeast and *C. elegans* [29,49]. In agreement with these findings, we observed an increased mitochondrial biogenesis in *mtef4*-mutant worms *via* RNA-Seq and qPCR (Fig. 4, Supplementary Fig. S3a, b, Table 2). The mitochondrial biogenesis, which resulted in increased mtDNA and transcripts of mitochondria-encoded genes, may contribute to increased mitochondrial translation under normal conditions.

Reduced cytoplasmic translation is thought to protect against mitochondrial stress in yeast, fly and *C. elegans* [50–52]. Attenuation of cytoplasmic translation reduces the level of mitochondria-targeted proteins, decreases the folding load on mitochondrial chaperones, and has an overall benefit to the mitochondria. Our transcriptomic analyses and subsequent experiments also showed that mutation of *mtef4* reduced cytoplasmic translation, which was not induced by energy limitation since there was no decrease in ATP levels in *mtef4(tm4178)* young adult worms (Fig. 5a, Supplementary Fig. S1). Therefore, cytoplasmic translation may be a retrograde pathway that protects against mitochondrial dysfunctions by *mtef4* deletion. But how mitochondrial dysfunctions affect cytoplasmic translation is unclear. Previous reports suggested that the phosphorylation of the translation initiation factor (eIF2α) by the kinase General Control Non-derepressible 2 (GCN2) or RNA activated Protein Kinase (PKR) is a possible pathway [50]. Based on our results of decreased cytoplasmic ribosome biogenesis, we also proposed Target of rapamycin (TOR) may participate in the process [46,53]. A relation between TOR and mitochondrial function has been described in mammalian cells in which perturbation of mitochondrial function leads to inhibition of TOR kinase activity [54]. Importantly, TOR inhibition decreases translation and the expression of ribosomal proteins by *sfp1* [55,56]. It is currently unclear how GCN2 and TOR sense mitochondrial dysfunction, but amino acid and ROS, both of which are regulated by mitochondrial functions and in turn affect GCN2 and TOR [57–60], may be involved in the process. Although the details remain to be clarified, a previous study showed a cytoplasmic pathway for ROS-induced Ataxia-telangiectasia mutated (ATM) to regulate TOR signaling [61] and interestingly, GCN2 in *C. elegans* was required for the induction of *pha-4* and the lifespan extension by TOR inactivation [62].

How mtEF4 is regulated under unfavorable conditions is unclear. mtEF4 does not appear to be regulated at the transcriptional level, because the mRNA levels of *mtef4* did not change at low temperatures and under starvation conditions (Supplementary Fig. S3c). mtEF4 In yeast, GUF1 protein was increased under suboptimal temperatures (low or high temperatures), although the mRNA level was not determined [11]. It is possible that mtEF4 production is regulated at the post-transcriptional level. It is also possible that mitochondrial functions are affected by the distribution of mtEF4 in *C. elegans*. This is observed in *E. coli*, in which LEPA attaches to the membrane under normal conditions and is released in the cytoplasm to restore arrested ribosomes under stress [13]. Based on the conservation of LEPA, GUF1 and mtEF4, it is reasonable to assume that GUF1 and mtEF4 also move between the mitochondrial matrix and inner membrane to adapt to stressful conditions. Future studies should focus on the intramitochondrial localization of mtEF4 in *C. elegans*.

mtEF4 was thought to affect lifespan because of its roles in mitochondrial functions and cytoplasmic translation under normal or stressful conditions. However, in our study, it had no effects on lifespan under various conditions, including low temperature (Figs. 5c, d, 6). These findings suggested that mitochondrial dysfunction, attenuation of cytoplasmic translation and low temperature invoke specific pathways to extend lifespan, and are not simply a passive consequence of the above conditions. Indeed, *clk-1* and *isp-1* are known to affect lifespan through specific molecules. *fstr-1/2* is specific for lifespan extension by *clk-1*, and *ceh-23* for lifespan extension of *isp-1* [29,48]. In addition, *hif-1* is the common message for both *isp-1* and *clk-1* [47]. As for the attenuation of cytoplasmic translation, not all translation perturbations exert their effects on lifespan in the same way. The transcription factor DAF-16/FOXO is required for lifespan extension by inhibiting translation initiation factors. But reducing the levels of ribosome proteins extends lifespan independent of *daf-16* [23]. Even low temperature, which was previously considered as a passive thermodynamic process to extend lifespan, requires a complex genetic program for lifespan extension. A report shows that TRPA-1, a cold-sensitive TRP channel, detects low temperature, and then induces calcium influx and a calcium-sensitive PKC that signals to DAF-16/FOXO to extend lifespan [63]. Thus, it will be interesting to identify differences in the retrograde pathways in various mitochondrial mutants and understand how the retrograde pathways regulate lifespan.

Taken together, our data provided some clues about the relationship between mitochondrial dysfunction and cytoplasmic translation in *C. elegans*. We showed that lack of mtEF4 caused severe mitochondrial dysfunctions under unfavorable conditions, which were mainly manifested by the disruption of ETC supercomplexes containing complex IV, while mild mitochondrial dysfunctions under normal conditions. The mild mitochondrial dysfunctions caused by lack of mtEF4 induced retrograde pathways to influence cytoplasmic translation and mitochondria biogenesis. But how mtEF4 deletion leads to the mitochondrial dysfunctions and what factors invoke the retrograde pathways remain to be investigated further. For future work, we will try to find mitochondrial parameters and cytoplasmic pathways connecting mitochondrial dysfunctions and cytoplasmic translation. In addition, we also intend to investigate the roles of mtEF4 in the assembly of supercomplexes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2014.05.353.

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