SLC-25A46 Regulates Mitochondrial Fusion through FZO-1/Mitofusin and is Essential for

Maintaining Neuronal Morphology

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Abstract

Mitochondria are dynamic organelles shaped by sequential fission and fusion events. The

mitochondrial protein SLC25A46 has been identified as a causative gene for mitochondrial

neuropathies. However, the function of SLC25A46 in mitochondrial morphogenesis remains

controversial, with several reports suggesting it acts as a mitochondrial fission factor, while others

propose it as a fusion factor. In this study, employing forward genetics, we identified *slc-25A46*, a

Caenorhabditis elegans orthologue of human SLC25A46, as an essential factor for mitochondrial

fusion. Suppressor mutagenesis screening revealed loss-of-function mutations in *drp-1*, a mitochondrial

fission factor, as suppressors of *slc-25A46*. The phenotype of *slc-25A46* mutants is similar to those of

fzo-1 mutants, wherein the mitochondrial fusion factor Mitofusin is disrupted. Overexpressing

FZO-1/Mitofusin mitigated mitochondrial defects in *slc-25a46* mutants, indicating SLC-25A46

promotes fusion through FZO-1/Mitofusin. Disease model worms carrying mutations associated with

SLC25A46 exhibited mitochondrial fragmentation and accelerated neurodegeneration, suggesting

slc-25A46 maintains neuronal morphology through mitochondrial fusion regulation.

Introduction

Mitochondria are highly dynamic organelles that constantly change their shapes to adapt to the

metabolic states and physiological environments of the cell [1]. The morphological diversities of

mitochondria arise from sequential fission and fusion activities regulated by a family of GTPase

enzymes [1]. In humans, mitochondrial fission is mediated by dynamin-related protein 1 (DRP1), a

GTPase that forms ring-like structures at division sites[2,3]. Conversely, mitochondrial fusion requires

other GTPases, such as Mitofusin 1 and 2 (MFN1 and 2) for outer membrane (OM) fusion and Optic

atrophy 1 (OPA1) for inner membrane (IM) fusion[4-6]. In yeast, the fusion process involves the

participation of another protein called Ugo1, which localizes to OM and coordinates fusion between

the outer and inner membranes by interacting with Mitofusin and OPA1 orthologs [7,8].

A mitochondrial protein SLC25A46, originally identified as a cause of optic atrophy and spastic

paraplegia, shares weak similarities with Ugo1[9]. Like Ugo1, SLC25A46 is localized on the OM,

forms a complex with MFNs and OPA1[10]. Despite these similarities, the exact function of SLC25A46

in mitochondrial dynamics remains controversial. Several studies have reported that acute knockdown

of SLC25A46 in cultured cell lines leads to the formation of large elongated mitochondria[9-11].

Knockdown of SLC25A46 has been shown to cause elongated mitochondria in zebrafish and

Drosophila[12-14].A similar elongated mitochondrial phenotype has been observed in SLC25A46 mutant

mice that have a 46-bp deletion in the exon 8 [15]. Conversely, overexpression of SLC25A46 induces

mitochondrial fragmentation [9] . All of these studies support the function of SLC25A46 as a fission

factor. In contrast, the complete loss of SLC25A46 in the knock-out Hela cell results in the

fragmentation of the mitochondrial network[16]. In a separate study using a knockout mouse model, loss

of SLC25A46 function led to the formation of small mitochondrial fragments in the nervous system[17].

A mutation in bovine SLC25A46 gene is associated with sensorimotor neuropathy, known as Turning

calves syndrome, wherein fragmented aggregated mitochondria were observed[17]. Moreover, a null

mutation in human SLC25A46 gene has been identified as a cause of optic atrophy spectrum

disprder[18]. In patient cells with this mutation, mitochondrial fragmentation was observed[18]. These

phenotypes are similar to those observed in yeast Ugo1 mutants, suggesting that primary role of

SLC25A46 is as a mitochondrial fusion factor.

Caenorhabditis elegans is a widely-used model organism for studying organelle dynamics and

morphogenesis, including the nucleus, synaptic vesicles and mitochondria [19-24]. Disruption of

intracellular transport of mitochondria is observed in *unc-116*, *miro-1* and *trak-1* mutant worms,

where the molecular motor kinesin-1 and its adaptor proteins are mutated[20,21] . Orthologues of these

proteins have similar functions in mammals and flies[24]. Mitochondrial fragmentation has been

reported in *fzo-1* and *eat-3* mutant worms[25]. *fzo-1* and *eat-3* respectively encode MFN1/2 and OPA1

orthologues. Inversely, mitochondrial hyperfusion has been observed in *drp-1* mutants in which a

DRP1 orthologue is disrupted [26 27].

In this study, through forward genetic screening in *C. elegans*, we identified a loss-of-function

mutation in *slc-25A46* gene, a *C. elegans* orthologue of human SLC25A46. The *slc-25A46* mutant

worms exhibited fragmented and small mitochondria, similar to the phenotype observed in worms

lacking *fzo-1/Mitofusin*. Notably, this defect was partially rescued by a loss-of-function mutation in

drp-1 or overexpressing *fzo-1*. These findings suggest that *slc-25A46* is upstream of *fzo-1* and is

essential for mitochondrial fusion, rather than fission. To further investigate the function of SLC25A46,

we introduced pathogenic mutations reported in human SLC25A46 gene into *C. elegans slc-25A46*

gene and examined their effects on mitochondrial morphology and distribution. Our results indicate that

slc-25A46 mutations accelerated the morphological degeneration in neurons. Collectively, these

findings suggest that *slc-25A46* plays a critical role in mitochondrial morphology and neuronal

maintenance.

Results

Isolation of *slc-25A46* mutants

It is established that mitochondria accumulate in dendrites of ciliated sensory neurons, such as

olfactory neurons [28]. To elucidate the molecular mechanisms of mitochondrial morphogenesis in

sensory dendrites, we labeled mitochondria in *C. elegans* PHA neurons (Fig. 1A). GFP was fused to the

N-terminal 54 amino acids of TOMM-20 protein and used as a marker to visualize mitochondria[19,29].

The PHA neuron is a highly polarized neuron characterized by a dendrite with sensory cilia, a cell body

and an axon[30]. We utilized the *flp-15* promoter, which exclusively expresses in PHA neurons[31]. In the

resulting strain, intense GFP fluorescence of mitochondria was observed in both the soma and dendrites

(Fig 1A). In contrast, analyzing signals in the axon was challenging due to the sparse distribution of

mitochondria and the autofluorescence of gut cells.

To identify molecules essential for mitochondrial morphogenesis, we conducted forward genetic

screens through genome-wide ethyl methanesulfonate (EMS) mutagenesis[32]. We isolated a mutant

allele named *jpn15*. In *jpn15* worms, the number of mitochondria was reduced, and their size was

smaller compared to wild type (Fig 1B). Through genetic mapping and genome sequencing, we

identified a nonsense mutation at W127 of the *slc-25A46* gene, which is the *C. elegans* orthologue of

the mammalian SLC25A46 (Fig 1B). Previous RNAi screen of mitochondrial morphology have not

linked the function of s*lc-25A46* with mitochondrial morphogenesis [19]. To confirm that W127Stop

mutation in the *slc-25A46* gene is the causative mutation for the *jpn15* phenotype, we used

CRISPR/Cas9-mediated genome editing to substitute the nonsense mutation in *jpn15* with the *wild-type*

amino acid. We introduced silent mutations and created a restriction enzyme site in the repair template

to prevent subsequent Cas9 cleavage and verify successful genome editing (Fig 1B). The resultant allele,

jpn33, carrying the repaired *slc-25A46*, showed restored mitochondrial number and size, indicating that

the W127Stop mutation in *slc-25A46* locus is responsible for the abnormal mitochondrial morphology

observed in *jpn15* allele (Fig 1B-D). Moreover, we found that *slc-25A46(gk570223)*, obtained from the

million mutation project[33], had a Q310Stop mutation and showed the mitochondrial fragmentation

phenotype (Supplementary Figure S1A).

Next, to determine if mitochondrial fragmentation occurs in other tissues of *slc-25A46(jpn15)*

mutant worms, we observed mitochondria within the body wall muscles. Similar to the observations

in the PHA neuron cell bodies, mitochondria in *slc-25A46(jpn15)* mutants exhibited rounded and

fragmented structures compared to wild type (Supplementary Figure S1B). Prior studies have shown

that cristae structures are degenerated by SLC25A46 mutations in human and mice [10,15,16], whereas a

study has shown that cristae structures are not strongly affected in SLC25A46-knockout mice[17]. We

observed the fine structure of mitochondria in the muscular cells by transmission electron microscopy.

Our observation suggested that cristae structures in mitochondria was not strongly affected in

slc-25A46 mutant worms (Supplementary Figure S1C and D), resembling the findings described in

Duchesne et al.(2017) [17].

Overall, our findings suggest that *slc-25A46* plays a crucial role in maintaining the size and

distribution of mitochondria.

***drp-1* mutation suppress mitochondrial fragmentation in *slc-25A46* mutants**

To understand the function of SLC25A46 in mitochondrial morphogenesis, we performed second

EMS mutagenesis screens and searched for mutations that can suppress the mitochondrial

fragmentation phenotype in the *slc-25A46(jpn15)* mutant. A mutant allele, named *jpn73*, could recover

the mitochondrial network in the cell body of *slc-25A46(jpn15)* mutant (Fig 2A). Mapping and

sequencing revealed that *jpn73* is an allele of *drp-1* mutant. Genome sequencing revealed that exon 1

of the *drp-1* gene, including the start codon, was deleted in *drp-1(jpn73)* allele (Fig 2B). DRP-1 is a *C.*

elegans orthologue of DRP1 that is essential for the mitochondrial fission[26,27]. We next compared the

phenotype of *drp-1(jpn73)* and *slc-25A46(jpn15)* single mutants (Fig 2C). Moreover, we observed

drp-1(tm1108), which is a null allele of *drp-1*, for comparison (Fig 2B). In the dendrite of PHA neuron,

both *drp-1(jpn73)* and *drp-1(tm1108)* mutants had an elongated mitochondrion, indicating

mitochondrial hyperfusion (Fig 2C). The phenotype of *drp-1* was clearly different from mitochondrial

fragmentation phenotype observed in *slc-25A46(jpn15)* (Fig 1 and 2C). These results shows that

SLC-25A46 and DRP-1 have opposite functions in the mitochondrial morphogenesis. As DRP-1 is a

mitochondrial fission factor, it is suggested that SLC-25A46 works as a mitochondrial fusion factor.

slc-25A46* works as a mitochondrial fusion factor in *C. elegans

To study the phenotype of *slc-25A46*, we performed a comparative analysis of mitochondrial

morphology using *C. elegans* mutants with loss-of-function mutations in key genes associated with

mitochondrial dynamics, including *drp-1* (Fig 2), *fzo-1* and *eat-3* (Fig 3A). FZO-1 and EAT-3,

orthologues of MFN1/2 and OPA1 respectively, play important roles in mitochondrial fusion (Fig 3A).

We firstly focused on the morphology of mitochondria within the cell bodies of PHA neurons.

Mitochondria were categorized into tubular, fragmented, and aggregated forms (Fig 3B). Within the

fragmented category, a further distinction was made between weak and strong fragmentation based on

the size of the fragmented mitochondria (Fig 3B). In the cell body of *wild type* and *drp-1(tm1108)*

mutants, we could find a tubular mitochondrial network (Fig 3C and D). No fragmented mitochondria

were observed in *wild type* and *drp-1(tm1108)* mutants. In contrast, the cell body of the *slc-25A46*

(jpn15) mutant contained fragmented and small mitochondria, similar to cell bodies of *fzo-1(tm1133)*

and *eat-3(tm1107)* mutants (Fig 3C and D). However, the degree of fragmentation in

slc-25A46(jpn15) was milder than that in *fzo-1(tm1133)* and *eat-3(tm1107)* mutants (Fig 3D). Next,

we compared the number and morphology of mitochondria in the PHA dendrite. In *slc-25A46(jpn15)*

mutants, there was a significant decrease in both the number and size of mitochondria compared to the

wild type (Figs 3E-G). Similarly, *fzo-1(tm1133)* mutants displayed reductions in both the number and

size of mitochondria. Interestingly, *eat-3(tm1107)* mutants only exhibited a decrease in mitochondrial

size, without abnormal localization. Based on these phenotypes, it is suggested that SLC-25A46 acts

as a fusion factor for mitochondria, similar to FZO-1 and EAT-3. Notably, the phenotype of

slc-25A46 is more similar to that of *fzo-1* than to that of *eat-3*.

Ectopic Expression of FZO-1 partially suppresses defects in *slc-25a46* mutant

The mammalian SLC25A46 has been reported to physically interact with the outer and inner

membrane fusion factors MFN1 and MFN2 (FZO-1 orthologs) [11]. The above genetic experiments

suggested that *slc-25A46* and *fzo-1* work together in mitochondrial morphogenesis. However, the

functional interaction between SLC25A46 and MFNs remains elusive. To investigate the relation

further, we conducted additional genetic experiments. Firstly, we generated *slc-25A46*; *fzo-1* double

mutants and found that the phenotype was not enhanced compared with either *slc-25A46* or *fzo-1*

single mutants (Supplementary Figure S2). We next expressed FZO-1 in the *slc-25A46* mutant (Fig 4).

As a result, the ectopic expression of FZO-1::mCherry in PHA neurons was able to restore both the

number and size of mitochondria in *slc-25A46 (jpn15)* mutant (Fig 4A-C). These results collectively

suggest that *slc-25A46* is functionally upstream of *fzo-1* in the regulation of mitochondrial

morphology.

Ectopic Expression of SLC-25A46 induce mitochondrial fragmentation

Previous studies have shown that overexpression of SLC25A46 induces mitochondrial

fragmentation in vertebrate systems [9]. Thus, we overexpressed SLC-25A46 in *C. elegans* and

observed the mitochondrial morphology (Fig 5A-C). Similar to vertebrate systems, the overexpression

of SLC-25A46 induced mitochondrial fragmentation in the *wild-type* background (Fig 5A-C).

Moreover, we found that the expression of SLC-25A46 did not rescue fragmented mitochondria

phenotype in either the *slc-25A46* or *fzo-1* mutant background. These results suggest that the proper

expression level of SLC-25A46 is essential for mitochondrial fusion, as discussed later.

SLC25A46-associated disease model worms

Human SLC25A46 has been identified as a causative gene for neurodegenerative disorders such as

Charcot-Marie-Tooth disease and optic atrophy [9]. Most of these diseases are caused by autosomal

recessive mutations, and numerous amino acid changes have been reported as disease-causing in

humans. However, the mechanisms underlying disease onset remain unclear. Therefore, we attempted

to validate the effects of disease-causing mutations on mitochondrial morphology and distribution by

introducing these mutations into *C. elegans slc-25A46* gene. Among the human disease-associated

mutations reported to date, we introduced three mutations into *C. elegans slc-25A46* gene by

CRISPR/cas9 (Fig 6A). These residues are embedded in the OM (Fig 6B). Firstly, we observed the

morphology of mitochondria in the cell body of mutant alleles. We found mitochondria are

fragmented in disease-associated *slc-25A46* mutant worms (Fig 6C and D). However, compared with

slc-25A46(jpn15), which is considered to be a null allele, these three mutants showed more subtle

mitochondrial fragmentation in the cell body (Fig. 6C and D). Next, we observed the distribution and

morphology of mitochondria in the PHA dendrite (Fig 6E-G). The size of mitochondria was

statistically smaller in two out of three disease mutant model worms (Fig 6F). While we could not

detect statistical significance, *slc-25A46(E301D)* also tended to have smaller mitochondria. In

contrast, the number of mitochondria was not significantly affected by these disease-associated

mutations (Fig 6G). These data suggest that disease-associated SLC25A46 mutations result in partial

loss of function in the regulation of mitochondrial fusion event.

The neuronal morphology is disrupted by *slc25-a46* mutations

Mutations in the human SLC25A46 gene have been linked to neuronal degeneration [9,10]. In light of

this, we investigated the neuronal morphology in our *slc-25A46* mutant worms. Specifically, we

observed the morphology of PVD neurons, a model neuronal cell often used to study neurodegeneration

and regeneration in *C. elegans* [21,34,35] (Fig 7A). We observed adult stage worms 1 day to 7 days after the

final molting. In 1-day-old adult worms, the overall morphology of the PVD neuron appeared

unaffected by *slc-25A46* mutations, preserving the characteristic menorah structure [36] (Fig 7A and B).

However, we noticed an increased number of bead-like structures along the dendrite, a reported sign of

neuronal degeneration [21 37 34] , in *slc-25A46* mutant worms at day 1 and day 3 (Fig 7B and 7C). At

5-day-old and 7-day-old adult worms, because the number of bead-like structures is increased even in

wild-type worms, no significant difference was detected between *wild type* and *slc-25A46* mutants in

the number of bead-like structures (Fig 7C). However, the morphological defects were more evident in

slc-25A46 mutant worms at day 5, showing an increased ectopic branches (Fig 7B). As a result, the

area occupied by neurites was significantly increased in 5-day-old *slc-25A46* mutants compared to

age-matched wild type worms (Fig 7D). Previous studies have shown that ectopic branches of PVD

neurons increase when neurons are injured and failed to be repaired [34] [35]. These data indicate that

slc-25A46 mutations accelerate morphological degeneration in neurons.

Discussion

SLC-25A46 is essential for mitochondrial fusion

The role of SLC25A46 in multicellular organisms remains controversial. Hyperfusion of

mitochondria has been reported in knockdown zebrafish and Drosophila models [14]. In mammalian

systems, both fragmentation and hyperfusion of mitochondria have been reported when the expression

level of SLC25A46 is perturbed. Most works reporting hyperfusion of mitochondria tend to be based on

the knockdown experiments or an observation in mutant mice considered to be hypomorph, whereas

studies reporting fragmentation of mitochondria tend to be based on the complete knockout. Thus, it is

possible that the manipulation methods or the expression level of SLC25A46 may cause these

differences. We show mitochondrial fragmentation in our *slc-25A46(jpn15)* allele which has an early

stop codon and considered to be an amorph allele. We found that the mitochondrial phenotype of

slc-25A46 mutants is similar to those of *fzo-1* and *eat-3* mutants. FZO-1 is an ortholog of MFN1 and 2

whereas EAT-3 is an ortholog of OPA1, both are essential factors for mitochondrial fusion. Consistent

with the role of SLC-25A46 in mitochondrial fusion, suppressor mutagenesis screening on *slc-25A46*

mutant background shows that a *drp-1* mutation, which disrupt the function of mitochondrial fission

factor, can antagonize the phenotype of *slc-25A46*. These genetic data suggest that *slc-25A46* is

required for the mitochondrial fusion, rather than fission. On the other hand, it has been reported that

fragmentation of mitochondria is induced by the overexpression of SLC25A46 in mammalian cells and

zebrafish[9]. Consistent with these observations, we found overexpression of SLC-25A46 on *wild-type*

background induces mitochondrial fragmentation (Fig 5). These phenomena appears to indicate that

SLC-25A46 promotes mitochondrial fission. However, we have found, on the other hand, that the phenotypes of *slc-25A46* mutants cannot be rescued by the overexpression of SLC-25A46 (Fig 5). These data would show that proper expression levels of SLC-25A46 are essential for the maintenance of mitochondrial morphology, rather than indicate SLC-25A46 is a factor essential for the mitochondrial fission.

SLC-25A46 regulates mitochondrial fusion through FZO-1

 Previous studies have shown physical interactions between SLC25A46 and MFN1 and 2 [10]. However, the functional relation between SLC25A46 and MFNs has been elusive. Taking advantage of worm genetics, we show that mitochondrial fusion mediated by *slc-25A46* is accomplished through the function of FZO-1, a worm MFN. The phenotype of *slc-25A46*; *fzo-1* double mutants are similar to those of single mutants, suggesting that SLC-25A46 and FZO-1 work in the same pathway in the mitochondrial fusion process. Moreover, overexpression of FZO-1 could partially suppress the phenotype of *slc-25A46*, indicating that SLC-25A46 is genetically upstream of FZO-1. It has been shown that FZO-1 is the GTPase that directly induces mitochondrial membrane fusion. Our genetic data suggests that SLC-25A46 helps the activity of FZO-1 in the mitochondrial fusion process. It would be interesting to biochemically test whether SLC-25A46 can enhance the GTPase activity of FZO-1.

Worm models for mitochondria-associated disorders

The mitochondrial phenotypes in model worms suggest that disease mutations induce weak loss of function effects on the function of SLC-25A46. This is consistent with the genotype of patients; most SLC25A46 mutations cause neurological disorders in an autosomal recessive manner[9]. On the other hand, mitochondrial hyperfusion has been reported in patient cells [9]. Further analysis is needed to

determine the extent to which disease worm models reflect human symptoms and whether our model

worms can be used to search for treatment methods for mitochondrial diseases. In addition to

mitochondrial defects, we found acceleration of neurodegeneration in *slc-25A46* mutant worms. While

we have not noticed morphological defects in the PHA neuron, we found morphological defects in the

PVD neuron which is a widely used model to observe neurodegeneration in C. elegans. It has been

shown that PVD neurons show morphological defects in *mtx-1* mutant worm in which mitochondrial

transport is reduced[21]. Because PVD neuron is the largest neuron in *C. elegans* and has more complex

dendrite structures, the morphology of PVD neuron may be more sensitive to mitochondrial defects

than PHA neuron.

Limitation of this study

While worm genetics suggest that SLC-25A46 is a mitochondrial fusion factor, we cannot exclude a

possibility that worm SLC-25A46 and mammalian SLC25A46 have different functions in the

mitochondrial morphogenesis. To show that the relation between SLC-25A46 and FZO-1 is conserved

in mammalian cells, experiments similar to those done in this study, such as rescue of

SLC25A46-knockout cells by the expression of MFN1 and 2 or analyzing of SLC25A46- and DRP1-

double knockout cells, need to be repeated in mammalian systems. For that purpose, using complete

knockout cells (i.e. null mutant), rather than knockdown cells or partial knockout cells (i.e.

hypomorphic mutant), is required.

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grammar and improve English writing. After using this tool, the authors reviewed and edited the content

as needed and take full responsibility for the content of the publication.

Experimental Procedures

Plasmid preparation

Plasmids used in this study are detailed in Supplementary Table S1. Restriction enzymes were

purchased from New England Biolabs (Ipswich, MA, USA). Plasmids encoding *odr-1p::gfp* and

tomm-20(1-54aa)::gfp were obtained from Kang Shen lab. The *flp-15* promoter (*flp-15p*), a PHA

neuron-specific promoter, was previously described [31]. The DNA fragment encoding *flp-15p* was

inserted between the SphI and AscI restriction enzyme sites of the *tomm-20(1-54)::gfp* plasmid. The

plasmid encoding *flp-15p::slc-25A46::(GGGGS)3::mCherry* was constructed based on the plasmid

encoding *flp-15p::tomm-20(1-54)::gfp*. The region encoding *tomm-20(1-54)::gfp* was replaced with

slc-25A46::(GGGGS)3::mCherry. Total worm cDNA from the N2 strain was prepared as previously

described [38]. PCR primers are listed in Supplementary Table S2. *slc-25A46* cDNA was amplified by

PCR using KOD FX neo DNA polymerase (TOYOBO, Tokyo Japan) with worm cDNA as the template.

The slc-25A46_F_NheI and slc-25A46_R_KpnI were employed. The resulting fragment was replaced

with *tomm-20(1-54)* using NheI and KpnI enzymes. Subsequently, the *gfp* sequence was replaced with

(GGGGS)3::mCherry using Gibson assembly. *(GGGGS)3* was inserted as a flexible linker sequence[39].

To generate the plasmid encoding *flp-15p::fzo-1::(GGGGS)3::mCherry,* the *fzo-1* sequence was

amplified from extracted N2 genome DNA by PCR using KOD Plus high-fidelity DNA polymerase

(TOYOBO, Tokyo Japan). The region encoding *slc-25A46* was replaced with the *fzo-1* sequence.

Worm experiments

Worm strains are described in Supplementary Table S3. All *C. elegans* strains were cultured in

standard nematode growth medium (NGM) plates seeded with OP50 *Escherichia coli* at 20□[32]. The

wild-type strain, N2 Bristol, and SD1347 carrying ccIs4251, were obtained from *C. elegans* genetic

center (Minneapolis, MN, USA). The deletion mutant *fzo-1(tm1133)*, *eat-3(tm1107)*, and

drp-1(tm1108) were obtained from National Bioresource Project of Japan (Tokyo Women's Medical

University School of Medicine, Japan). The PVD neuron marker *wyIs592* [*ser-2prom3p::myr-gfp*] is

previously described [36]. For transformation of worms, DNA injection was performed as described [40].

Plasmids encoding *flp-15p::tomm-20(1-54aa)::gfp* and *odr-1p::gfp* were injected to establish

jpnEx15[*flp-15p::tomm-20(1-54aa)::gfp*, *flp-15p::myrTagRFP-T, odr-1p::gfp*]. The

extrachromosomal array was integrated to the genome by UV irradiation. The resultant integration was

named *jpnIs4*. For *slc-25A46::mCherry* and *fzo-1::mCherry* transgenic worms,

flp-15p::slc-25A46::(GGGGS)3::mCherry (20 ng/µl) or *flp-15p::fzo-1::(GGGGS)3::mCherry* (20

ng/µl) plasmid was injected into *jpnIs4* gonads, respectively. pCFJ90 (2.5 ng/µl) and pBlueScript II

KS(-) (80 ng/µl) were used as co-injection markers. Then, worms carrying the extrachromosomal array

were crossed to each mitochondrial mutant.

EMS mutagenesis screening

EMS mutagenesis was performed as described previously [32] [31]. The worm carrying *jpnIs4* was

crossed with N2 for 8 times and used as a starting strain. For suppressor screens, *slc-25A46(jpn15);*

jpnIs4 was used as a starting strain. Synchronized L4 hermaphrodites were mutagenized with 0.1 M

EMS (ethyl methanesulfonate, #M0880-1G, Sigma-Aldrich). Mutagenized worms were picked onto

fresh plates and let them lay eggs. F2 worms were screened under a fluorescent microscopy, and

candidates were identified by the abnormal mitochondrial morphology and localization in PHA neurons.

The candidates were backcrossed to the parent line three times. Mutant locus was determined by

single-nucleotide polymorphism (SNP) mapping[41]. For *jpn15*, whole genome sequencing was

outsourced to Eurofins genomics Japan (Tokyo, Japan). Genomic data was analyzed using the Galaxy

platform as described previously [42] [38]. Reference genome sequence (WBcel235.75.fasta) and gene

annotations (WBcel235.75.gtf) were obtained from WormBase. All of the following sequence analysis

were performed using default parameters predefined in the Galaxy platform. On the Galaxy platform, a

pair of FASTQ files obtained from Eurofins were firstly trimmed with Trim Galore!. Aligning

sequencing reads to the genome sequence was performed using Bowtie 2. Variant search was

performed using FreeBayes. Variants were annotated to genes using Snpeff. By comparing the variant

data and the SNP mapping data, we found a nonsense mutation in *slc-25A46* gene. The mutation was

confirmed by genomic PCR followed by Sanger sequencing using CEQ8000 (Beckman Coulter Inc,

Brea, CA, USA).

For *jpn73*, SNP mapping indicated that the genomic region, which includes *drp-1*, contained the

causative mutation. Therefore, the genomic region of the *drp-1* gene was amplified by PCR from *jpn73*

mutant using drp-1 genome PCR_F (5'-GGCGTTCACAGTCAATCGAAGG-3') and drp-1 genome

PCR_R (5'-GGGAACGGAGCATAGAGATCATACAG-3') primers. The genomic DNA fragment

was separated by electrophoresis using a 1% agarose gel and purified using QIAquick Gel Extraction

Kit (QIAGEN). The genome sequence of *drp-1* was determined by Sanger sequencing using primers

listed in Supplementary Table S2.

Genome editing

Co-CRISPR method was used for genome editing of worms[43]. The single-strand oligo DNA (ssODN)

with *dpy-10 (cn64)* was used as a co-injection marker. Target sequences for guide RNA were inserted to

an expression vector pTK73[44]. We chose two Cas9 target cites in *slc-25A46* sequence for each

experiment.

To correct the *jpn15* mutation, target sites were

*jpn15*_rescue_01, 5'-GAATTCAGACATTCTAGAA-3'

*jpn15*_rescue_02, 5'-CATTCTAGAAAGGAGCAAT-3'

To introduce disease associated mutations, target sites were

*slc-25A46*_disease_01, 5'-GATGAACAATTGTTTCAAA-3'

*slc-25A46*_disease_02, 5'-TTCATCGAATGTATATTCA-3'

Synthesized ssODN (Eurofins genetics Japan) were used as repair templates (Supplementary Table S3).

We introduced restriction enzyme cite as synonymous mutations to guide RNA recognition cites to

prohibit the cleavage of the repair templates and for following genotyping process. All materials were

mixed so that each final concentration became 50 ng/µl, and injected the mixture into worms. *dpy* or *rol*

F1 worms were singled under a stereo microscope (Stemi 508, Carl Zeiss). Recombination was

screened by PCR followed by digestion with each restriction enzyme, and then confirmed by Sanger

sequencing. Obtained lines were crossed with N2 worms three times. All of the plasmids, primers and

ssODNs are listed in Supplementary Table S1 and S2.

Fluorescent microscopy

Fluorescent signals in living worms are observed without fixation, following the previously described

procedure [45]. Worms are mounted on a 0.25mM levamisole (Sigma) that is placed on a 3% agarose pad.

We used a Zeiss Axio Observer microscope equipped with an Objective C-Apochromat 40x (NA 1.2)

and an LSM800 confocal microscope system (Carl Zeiss). The system was controlled using ZEN

software (Carl Zeiss). Airy unit settings were configured to 2, and the Z stack mode was employed to

capture mages. Parameters for resolution in the X-Y direction and thickness in the Z direction were the

optimal values indicated by the ZEN software. Z-projection was performed using the same software.

Mitochondrial size was measured using the ZEN software. Because mitochondria within dendrites have

a thickness in the Z-direction less than the wavelength of light, mitochondrial size was expressed as an

area on the Z-projection image rather than volume.

Rapid cooling procedure and quick freeze substitution for electron microscopy

The cryofixation of worms was conducted using the ultra-rapid cryo-technique as described previously [46].

Briefly, adult animals were picked onto four NGM plates (8-10 animals/plate) with OP50 bacteria and

cultured at 20 °C. On the third day of culture, a mixture of L1-L4 animals was collected using a

cryoprotective solution (10% ethylene glycol in deionized distilled water) and incubated for more than 30

minutes at room temperature. The drop of animals was loaded on small-cut cellulose acetate membrane

(Membrane filter C020A047A, ADVANTEC). After the liquid volume reduction by absorption into the

membrane, the samples were dipped manually into liquid ethane or liquid propane and then transferred

into liquid nitrogen. The samples were further transferred to cryotubes (Cryovial, 2 ml, TOHO) containing

2% OsO_4 and 5% DDW in acetone at -80 °C. The specimens were freeze substituted by the quick freeze

substitution (QFS) method [47], washed five times with acetone and three times with propylene oxide (PO)

and infiltrated with Epon resin/PO in steps of 33% 1 h, 50% 3 h, and 100% resin overnight. After

infiltration, the specimens were embedded in freshly prepared Epon resin and polymerized for 2 days at

60°C.

Electron microscopy

70 nm ultrathin sections were prepared onto Formvar-coated copper slot grids using an ultramicrotome

(EM UC7, Leica). The sections were post-stained for 5 min in 5% uranyl acetate in water at 60°C and

followed by 5 min in lead citrate. Mitochondria in body wall muscles were photographed using a

JEM-2100F electron microscope (JEOL) equipped with TemCam-F216 (TVIPS) at 200 kV and 10,000×.

Statistical analyses and graph preparation

Statistical analyses were performed using Graph Pad Prism version 10.1.1. Statistical methods and

sample size are described in the figure legends. Graphs were prepared using Graph Pad Prism version 9,

exported in the EPS file format and aligned by Adobe Illustrator 2023.

Reference

1.　Giacomello, M., Pyakurel, A., Glytsou, C., and Scorrano, L. (2020). The cell biology of
mitochondrial membrane dynamics. Nat Rev Mol Cell Biol *21*, 204-224.
10.1038/s41580-020-0210-7.
2.　Chappie, J.S., Acharya, S., Liu, Y.W., Leonard, M., Pucadyil, T.J., and Schmid, S.L.
(2009). An intramolecular signaling element that modulates dynamin function in vitro
and in vivo. Mol Biol Cell *20*, 3561-3571. 10.1091/mbc.e09-04-0318.
3.　Antonny, B., Burd, C., De Camilli, P., Chen, E., Daumke, O., Faelber, K., Ford, M.,
Frolov, V.A., Frost, A., Hinshaw, J.E., et al. (2016). Membrane fission by dynamin:

what we know and what we need to know. EMBO J *35*, 2270-2284.
10.15252/embj.201694613.

4. Alexander, C., Votruba, M., Pesch, U.E., Thiselton, D.L., Mayer, S., Moore, A., Rodriguez, M., Kellner, U., Leo-Kottler, B., Auburger, G., et al. (2000). OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. Nat Genet *26*, 211-215. 10.1038/79944.

5. Zuchner, S., Mersiyanova, I.V., Muglia, M., Bissar-Tadmouri, N., Rochelle, J., Dadali, E.L., Zappia, M., Nelis, E., Patitucci, A., Senderek, J., et al. (2004). Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat Genet *36*, 449-451. 10.1038/ng1341.

6. Westermann, B. (2008). Molecular machinery of mitochondrial fusion and fission. J Biol Chem *283*, 13501-13505. 10.1074/jbc.R800011200.

7. Sesaki, H., and Jensen, R.E. (2001). UGO1 encodes an outer membrane protein required for mitochondrial fusion. J Cell Biol *152*, 1123-1134. 10.1083/jcb.152.6.1123.

8. Sesaki, H., and Jensen, R.E. (2004). Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. J Biol Chem *279*, 28298-28303. 10.1074/jbc.M401363200.

9. Abrams, A.J., Hufnagel, R.B., Rebelo, A., Zanna, C., Patel, N., Gonzalez, M.A., Campeanu, I.J., Griffin, L.B., Groenewald, S., Strickland, A.V., et al. (2015). Mutations in SLC25A46, encoding a UGO1-like protein, cause an optic atrophy spectrum disorder. Nat Genet *47*, 926-932. 10.1038/ng.3354.

10. Janer, A., Prudent, J., Paupe, V., Fahiminiya, S., Majewski, J., Sgarioto, N., Des Rosiers, C., Forest, A., Lin, Z.Y., Gingras, A.C., et al. (2016). SLC25A46 is required for mitochondrial lipid homeostasis and cristae maintenance and is responsible for Leigh syndrome. EMBO Mol Med *8*, 1019-1038. 10.15252/emmm.201506159.

11. Steffen, J., Vashisht, A.A., Wan, J., Jen, J.C., Claypool, S.M., Wohlschlegel, J.A., and Koehler, C.M. (2017). Rapid degradation of mutant SLC25A46 by the ubiquitin-proteasome system results in MFN1/2-mediated hyperfusion of mitochondria. Mol Biol Cell *28*, 600-612. 10.1091/mbc.E16-07-0545.

12. Wan, J., Steffen, J., Yourshaw, M., Mamsa, H., Andersen, E., Rudnik-Schoneborn, S., Pope, K., Howell, K.B., McLean, C.A., Kornberg, A.J., et al. (2016). Loss of function of SLC25A46 causes lethal congenital pontocerebellar hypoplasia. Brain *139*, 2877-2890. 10.1093/brain/aww212.

13. Ali, M.S., Suda, K., Kowada, R., Ueoka, I., Yoshida, H., and Yamaguchi, M. (2020). Neuron-specific knockdown of solute carrier protein SLC25A46a induces locomotive

defects, an abnormal neuron terminal morphology, learning disability, and shortened lifespan. IBRO Rep *8*, 65-75. 10.1016/j.ibror.2020.02.001.

14. Suda, K., Ueoka, I., Azuma, Y., Muraoka, Y., Yoshida, H., and Yamaguchi, M. (2018). Novel Drosophila model for mitochondrial diseases by targeting of a solute carrier protein SLC25A46. Brain Res *1689*, 30-44. 10.1016/j.brainres.2018.03.028.

15. Li, Z., Peng, Y., Hufnagel, R.B., Hu, Y.C., Zhao, C., Queme, L.F., Khuchua, Z., Driver, A.M., Dong, F., Lu, Q.R., et al. (2017). Loss of SLC25A46 causes neurodegeneration by affecting mitochondrial dynamics and energy production in mice. Hum Mol Genet *26*, 3776-3791. 10.1093/hmg/ddx262.

16. Schuettpelz, J., Janer, A., Antonicka, H., and Shoubridge, E.A. (2023). The role of the mitochondrial outer membrane protein SLC25A46 in mitochondrial fission and fusion. Life Sci Alliance *6*. 10.26508/lsa.202301914.

17. Duchesne, A., Vaiman, A., Castille, J., Beauvallet, C., Gaignard, P., Floriot, S., Rodriguez, S., Vilotte, M., Boulanger, L., Passet, B., et al. (2017). Bovine and murine models highlight novel roles for SLC25A46 in mitochondrial dynamics and metabolism, with implications for human and animal health. PLoS Genet *13*, e1006597. 10.1371/journal.pgen.1006597.

18. Nguyen, M., Boesten, I., Hellebrekers, D.M., Mulder-den Hartog, N.M., de Coo, I.F., Smeets, H.J., and Gerards, M. (2017). Novel pathogenic SLC25A46 splice-site mutation causes an optic atrophy spectrum disorder. Clin Genet *91*, 121-125. 10.1111/cge.12774.

19. Ichishita, R., Tanaka, K., Sugiura, Y., Sayano, T., Mihara, K., and Oka, T. (2008). An RNAi screen for mitochondrial proteins required to maintain the morphology of the organelle in Caenorhabditis elegans. J Biochem *143*, 449-454. 10.1093/jb/mvm245.

20. Ren, X., Zhou, H., Sun, Y., Fu, H., Ran, Y., Yang, B., Yang, F., Bjorklund, M., and Xu, S. (2023). MIRO-1 interacts with VDAC-1 to regulate mitochondrial membrane potential in Caenorhabditis elegans. EMBO Rep *24*, e56297. 10.15252/embr.202256297.

21. Zhao, Y., Song, E., Wang, W., Hsieh, C.H., Wang, X., Feng, W., Wang, X., and Shen, K. (2021). Metaxins are core components of mitochondrial transport adaptor complexes. Nat Commun *12*, 83. 10.1038/s41467-020-20346-2.

22. Niwa, S., Lipton, D.M., Morikawa, M., Zhao, C., Hirokawa, N., Lu, H., and Shen, K. (2016). Autoinhibition of a Neuronal Kinesin UNC-104/KIF1A Regulates the Size and Density of Synapses. Cell Rep *16*, 2129-2141. 10.1016/j.celrep.2016.07.043.

23. Meyerzon, M., Fridolfsson, H.N., Ly, N., McNally, F.J., and Starr, D.A. (2009). UNC-83 is a nuclear-specific cargo adaptor for kinesin-1-mediated nuclear migration. Development *136*, 2725-2733. 10.1242/dev.038596.

24. Campbell, D., and Zuryn, S. (2024). The mechanisms and roles of mitochondrial dynamics in C. elegans. Semin Cell Dev Biol *156*, 266-275. 10.1016/j.semcdb.2023.10.006.

25. Rolland, S.G., Lu, Y., David, C.N., and Conradt, B. (2009). The BCL-2-like protein CED-9 of C. elegans promotes FZO-1/Mfn1,2- and EAT-3/Opa1-dependent mitochondrial fusion. J Cell Biol *186*, 525-540. 10.1083/jcb.200905070.

26. Scholtes, C., Bellemin, S., Martin, E., Carre-Pierrat, M., Mollereau, B., Gieseler, K., and Walter, L. (2018). DRP-1-mediated apoptosis induces muscle degeneration in dystrophin mutants. Sci Rep *8*, 7354. 10.1038/s41598-018-25727-8.

27. Labrousse, A.M., Zappaterra, M.D., Rube, D.A., and van der Bliek, A.M. (1999). C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol Cell *4*, 815-826. 10.1016/s1097-2765(00)80391-3.

28. Reese, T.S. (1965). Olfactory Cilia in the Frog. J Cell Biol *25*, 209-230. 10.1083/jcb.25.2.209.

29. Kanaji, S., Iwahashi, J., Kida, Y., Sakaguchi, M., and Mihara, K. (2000). Characterization of the signal that directs Tom20 to the mitochondrial outer membrane. J Cell Biol *151*, 277-288. 10.1083/jcb.151.2.277.

30. Inglis PN., O.G., Lerouz MR., Scholey JM. (2006). The sensory cilia of Caenorhabditis elegans (November 27, 2006). WormBook.

31. Niwa, S. (2016). The nephronophthisis-related gene ift-139 is required for ciliogenesis in Caenorhabditis elegans. Sci Rep *6*, 31544. 10.1038/srep31544.

32. Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94. 10.1093/genetics/77.1.71.

33. Thompson, O., Edgley, M., Strasbourger, P., Flibotte, S., Ewing, B., Adair, R., Au, V., Chaudhry, I., Fernando, L., Hutter, H., et al. (2013). The million mutation project: a new approach to genetics in Caenorhabditis elegans. Genome Res *23*, 1749-1762. 10.1101/gr.157651.113.

34. Oren-Suissa, M., Gattegno, T., Kravtsov, V., and Podbilewicz, B. (2017). Extrinsic Repair of Injured Dendrites as a Paradigm for Regeneration by Fusion in Caenorhabditis elegans. Genetics *206*, 215-230. 10.1534/genetics.116.196386.

35. Brar, H.K., Dey, S., Bhardwaj, S., Pande, D., Singh, P., Dey, S., and Ghosh-Roy, A. (2022). Dendrite regeneration in C. elegans is controlled by the RAC GTPase CED-10 and the RhoGEF TIAM-1. PLoS Genet *18*, e1010127. 10.1371/journal.pgen.1010127.

36. Dong, X., Liu, O.W., Howell, A.S., and Shen, K. (2013). An extracellular adhesion molecule complex patterns dendritic branching and morphogenesis. Cell *155*, 296-307. 10.1016/j.cell.2013.08.059.

37. Nakano, J., Chiba, K., and Niwa, S. (2022). An ALS-associated KIF5A mutant forms oligomers and aggregates and induces neuronal toxicity. Genes Cells *27*, 421-435. 10.1111/gtc.12936.

38. Higashida, M., and Niwa, S. (2023). Dynein intermediate chains DYCI-1 and WDR-60 have specific functions in Caenorhabditis elegans. Genes Cells *28*, 97-110. 10.1111/gtc.12996.

39. Trinh, R., Gurbaxani, B., Morrison, S.L., and Seyfzadeh, M. (2004). Optimization of codon pair use within the (GGGGS)3 linker sequence results in enhanced protein expression. Mol Immunol *40*, 717-722. 10.1016/j.molimm.2003.08.006.

40. Mello, C.C., Kramer, J.M., Stinchcomb, D., and Ambros, V. (1991). Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO J *10*, 3959-3970. 10.1002/j.1460-2075.1991.tb04966.x.

41. Davis, M.W., Hammarlund, M., Harrach, T., Hullett, P., Olsen, S., and Jorgensen, E.M. (2005). Rapid single nucleotide polymorphism mapping in C. elegans. BMC Genomics *6*, 118. 10.1186/1471-2164-6-118.

42. Community, G. (2022). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. Nucleic Acids Res *50*, W345-W351. 10.1093/nar/gkac247.

43. Arribere, J.A., Bell, R.T., Fu, B.X., Artiles, K.L., Hartman, P.S., and Fire, A.Z. (2014). Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in Caenorhabditis elegans. Genetics *198*, 837-846. 10.1534/genetics.114.169730.

44. Obinata, H., Sugimoto, A., and Niwa, S. (2018). Streptothricin acetyl transferase 2 (Sat2): A dominant selection marker for Caenorhabditis elegans genome editing. PLoS One *13*, e0197128. 10.1371/journal.pone.0197128.

45. Anazawa, Y., and Niwa, S. (2022). Analyzing the Impact of Gene Mutations on Axonal Transport in Caenorhabditis Elegans. Methods Mol Biol *2431*, 465-479. 10.1007/978-1-0716-1990-2_25.

46. Irdani, T., Fortunato, A., and Torre, R. (2015). An ultra-rapid cryo-technique for complex organisms. Cryobiology *71*, 391-397. 10.1016/j.cryobiol.2015.10.144.

47.   McDonald, K.L., and Webb, R.I. (2011). Freeze substitution in 3 hours or less. J

Microsc *243*, 227-233. 10.1111/j.1365-2818.2011.03526.x.

Figure legends

Figure 1 Identification of *slc-25A46* mutant

(A) Schematic of PHA neuron morphology (upper panel) and a representative image showing

the morphology and distribution of mitochondria in PHA neuron (lower photo). The arrowhead

indicates the cell body. TOMM-20(1-54) was fused with GFP and expressed using the *flp-15*

promoter. Bar, 10 µm.

(B) Alleles of *slc-25A46* and their impact on mitochondrial morphology. The *jpn15* allele,

isolated through EMS mutagenesis, has a nonsense mutation in the *slc-25A46* gene and

exhibits fragmented mitochondria phenotype in the PHA neuron. Using CRISPR/cas9

technology, the nonsense mutation in the *slc-25A46(jpn15)* allele was corrected. The resultant

allele *slc-25A46(jpn33)* has normal mitochondria. Bars, 10 µm.

(C) Dot plots showing the number of mitochondria in the PHA dendrite. Each dot shows the

number of mitochondria in a single PHA dendrite. Green bars represent median values. n = 20

dendrites from 20 worms. Kruskal-Wallis test followed by Dunn's multiple comparison test.

***, p < 0.001. ****, p < 0.0001. ns, p > 0.05 and statistically not significant.

(D) Dot plots showing the size distribution of mitochondria in the PHA dendrite. Each dot

represents the size of an individual mitochondrion in the PHA dendrite. Green bars represent

median values. n = 85, 83 and 85 mitochondria. Kruskal-Wallis test followed by Dunn's

multiple comparison test. ***, p < 0.001. ****, p < 0.0001. ns, p > 0.05 and statistically not

significant.

Figure 2 SLC-25A46 and DRP1 have opposite functions in mitochondrial morphogenesis

(A) Identification of *jpn73*, a suppressor mutant of the *slc-25A46* mutant. Representative

images showing mitochondria in the cell body of *wild type*, *slc-25A46*, *slc-25A46*; *jpn73*

mutants. Note that fragmented mitochondria phenotype in *slc-25A46* is suppressed in

slc-25A46; jpn73 double mutant. Bars, 2 µm.

(B) Schematic drawing showing the genome structure of *drp-1* gene and the *jpn73* mutation.

Deleted regions in *jpn73* and *tm1108* alleles are shown by bars.

(C) Representative images showing the mitochondrial morphology in the dendrite of *wild type*,

slc-25A46(jpn15), *drp-1(jpn73)* and *drp-1(tm1108)*. Bars, 10 μm.

Figure 3

(A) Schematic drawing showing the mitochondrial dynamics and functions of

FZO-1(Mitofusin), EAT-3(OPA1) and DRP-1(DRP1).

(B) Schematic drawing showing the category of mitochondrial morphology used in this study.

(C and D) Mitochondrial morphology in the cell body of PHA neuron. Representative images

showing the mitochondrial morphology in PHA cell body and their categories (C). In panel (D),

a stacked bar graph illustrates the ratio of categories in each mutant. N = 40 worms. Bars, 2 μm.

(E) Representative images showing mitochondrial morphology in the PHA neuron. Bars, 10

μm.

(F) Dot plots showing the number of mitochondria in the PHA dendrite. Each dot shows the

number of mitochondria in a single PHA dendrite. Green bars represent median values. n = 20

dendrites from 20 worms. Kruskal-Wallis test followed by Dunn's multiple comparison test. *,

p < 0.05. ****, p < 0.0001. ns, p > 0.05 and statistically not significant.

(G) Dot plots showing the size distribution of mitochondria in the PHA dendrite. Each dot

represents the size of an individual mitochondrion in the PHA dendrite. Green bars represent

median values. n = 85, 83, 53 and 60 mitochondria for each genotype. Kruskal-Wallis test

followed by Dunn's multiple comparison test. ****, p < 0.0001. ns, p > 0.05 and statistically

not significant.

Figure 4 Ectopic expression of FZO-1 suppresses the mitochondrial morphology in the

slc-25A46 mutant

(A) Representative images showing the mitochondrial morphology in the PHA neuron of *wild*

type, *slc-25A46(jpn15)* and *fzo-1* mutants, as well as strains overexpressing FZO-1 in each

mutant background. Note that overexpression of FZO-1 rescue mitochondrial defects in both

slc-25A46(jpn15) and *fzo-1* mutants. Bars, 5 μm.

(B) Dot plots showing the number of mitochondria in the PHA dendrite. Each dot shows the

number of mitochondria in a single PHA dendrite. Green bars represent median values. n = 20

dendrites from 20 worms. Kruskal-Wallis test followed by Dunn's multiple comparison test.

****, p < 0.0001. ns, p > 0.05 and statistically not significant.

(C) Dot plots showing the size distribution of mitochondria in the PHA dendrite. Each dot

represents the size of an individual mitochondrion in the PHA dendrite. Green bars represent

median values. n = 85, 70, 83, 57, 53 and 60 mitochondria, respectively. Kruskal-Wallis test

followed by Dunn's multiple comparison test. ****, p < 0.0001. ns, p > 0.05 and statistically

not significant.

Figure 5 Ectopic expression of SLC-25A46 induces fragmentation of mitochondria

(A) Representative images showing the mitochondrial morphology in the PHA neuron of *wild*

type, *slc-25A46(jpn15)* and *fzo-1* mutants, as well as strains overexpressing SLC-25A46 in

each mutant background. Note that overexpression of SLC-25A46 caused fragmentation of

mitochondria in the wild type but overexpression of SLC-25A46 could not rescue

mitochondrial defects in *slc-25A46(jpn15)* and *fzo-1* mutants. Bars, 5 µm.

(B) Dot plots showing the number of mitochondria in the PHA dendrite. Each dot shows the

number of mitochondria in a single PHA dendrite. Green bars represent median values. n = 20

dendrites from 20 worms. Kruskal-Wallis test followed by Dunn's multiple comparison test. ns,

p > 0.05 and statistically not significant.

(C) Dot plots showing the size distribution of mitochondria in the PHA dendrite. Each dot

represents the size of an individual mitochondrion in the PHA dendrite. Green bars represent

median values. n = 85, 81, 83, 82, 53 and 64 mitochondria, respectively. Kruskal-Wallis test

followed by Dunn's multiple comparison test. ****, p < 0.0001. ns, p > 0.05 and statistically

not significant.

Figure 6 Establishment of SLC25A46-associated disease models

(A) A sequence comparison between human SLC25A46 and *C. elegans* SLC-25A46. Three

amino acid changes associated with SLC25A46-related diseases (P333L, E335D and R340C)

and the corresponding amino acid changes in *C. elegans*, analyzed in this study, are illustrated.

(B) Schematic drawing showing the predicted structure of *C. elegans* SLC-25A46 and

mutations analyzed in this study.

(C and D) Mitochondrial morphology in the cell body of PHA neuron. Representative images

showing the mitochondrial morphology in PHA cell body in *slc-25A46* alleles and their

categories (C). In panel (D), a stacked bar graph illustrates the ratio of categories in each

mutant. Mitochondrial morphology was categorized using the criteria shown in Figure 3C. N =

40 worms. Bars, 2 µm.

(E) Representative images showing the mitochondrial morphology in the PHA neuron of *wild*

type, *slc-25A46* mutant alleles. Arrow heads indicate the cell body. Bars, 5 µm.

(F) Dot plots showing the number of mitochondria in the PHA dendrite. Each dot shows the

number of mitochondria in a single PHA dendrite. Green bars represent median values. n = 20

dendrites from 20 worms for each genotype. Kruskal-Wallis test followed by Dunn's multiple

comparison test. ***, $p < 0.001$. ns, $p > 0.05$ and statistically not significant.

(G) Dot plots showing the size distribution of mitochondria in the PHA dendrite. Each dot

represents the size of an individual mitochondrion in the PHA dendrite. Green bars represent

median values. n = 85, 83, 83, 80, 80 and 80 mitochondria for each genotype, respectively.

Kruskal-Wallis test followed by Dunn's multiple comparison test. *, $p < 0.05$. ***, $p < 0.001$.

****, $p < 0.0001$. Actual p value is shown for *slc-25A46(E301D)* mutant.

Figure 7 Neuronal morphology in slc-25A46 mutant worms

(A) Schematic drawing of the morphology of PVD neuron. The drawing also illustrates

examples of ectopic branches. Measured area in panels C and D are shown by a gray box.

(B) Representative images showing the morphology of PVD neurons in *wild type* and

slc-25A46 mutant alleles at Day 1 and 5.

(C) A graph showing the number of bead-like structures along the dendrite of PVD neuron at

days 1, 3, 5 and 7. Measured area is indicated in panel A. Mean ± standard errors of the mean

(SEM) is shown in the graph. n = 20 worms for each genotype and each day. Kruskal-Wallis

test followed by Dunn's multiple comparison test was performed using wild type as a control.

**, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$. ns, $p > 0.05$ and statistically not significant.

(D) Dot plots showing the percentage of area occupied by PVD neurons within the region

illustrated in panel A. An elevated value is considered to represent an increase in ectopic

branches. n = 40 PVD neurons from 40 worms for each genotype and each day. Kruskal-Wallis

test followed by Dunn's multiple comparison test. ***, $p < 0.001$. ****, $p < 0.0001$. ns, $p >$

0.05 and statistically not significant.

A

Tail region

PHA neuron

mitochondria

cell body

axon

dendrite

wild type

B

slc-25A46 (jpn15)

Rescue *(jpn33)*

slc-25A46 (40B1B.8) genome

ATG

1 kb

	123							130
	I	Q	T	F	W	K	G	A
wild type	ATT	CAG	ACA	TTC	TGG	AAA	GGA	GCA
jpn15 [W127*]	ATT	CAG	ACA	TTC	TaG	AAA	GGA	GCA
jpn33 (Rescue)	ATc	CAa	ACc	TTt	TGG	AAg	GGc	GCc

C

ns

*** ****

Number of mitochondria per dendrite

wild type   slc-25A46 (jpn15)   Rescue (jpn33)

D

ns

**** ****

Size of mitochondria (µm²)

wild type   slc-25A46 (jpn15)   Rescue (jpn33)

Figure 1

A

TOMM-20::GFP

wild type   *slc-25A46*   *slc-25A46; jpn73*

B

ATG

drp-1 genome

jpn73

tm1108

1kb

C

wild type   *slc-25A46(jpn15)*

drp-1(jpn73)   *drp-1(tm1108)*

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B

C **D**

Figure 7