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An RNAi Screen for Mitochondrial Proteins Required to Maintain the Morphology of the Organelle in *Caenorhabditis elegans*

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Mitochondria are dynamic organelles that frequently divide and fuse together, resulting in the formation of intracellular tubular networks. In yeast and mammals, several factors including Drp1/Dnm1 and Mfn/Fzo1 are known to regulate mitochondrial morphology by controlling membrane fission or fusion. Here, we report the systematic screening of *Caenorhabditis elegans* mitochondrial proteins required to maintain the morphology of the organelle using an RNA interference feeding library. In *C. elegans* body wall muscle cells, mitochondria usually formed tubular structures and were severely fragmented by the mutation in *fzo-1* gene, indicating that the body wall muscle cells are suitable for monitoring changes in mitochondrial morphology due to gene silencing. Of 719 genes predicted to code for most of mitochondrial proteins, knockdown of >80% of them caused abnormal mitochondrial morphology, including fragmentation and elongation. These findings indicate that most fundamental mitochondrial functions, including metabolism and oxidative phosphorylation, are necessary for maintenance of the tubular networks as well as membrane fission and fusion. This is the first evidence that known mitochondrial activities are prerequisite for regulating the morphology of the organelle. Furthermore, 88 uncharacterized or poorly characterized genes were found in the screening to be implicated in mitochondrial morphology.

Key words: *Caenorhabditis elegans*, elongation, fragmentation, mitochondrial morphology, RNAi screening.

Abbreviations: AAT, aspartate aminotransferase; GFP, green fluorescent protein; MitoP2, Mitochondria Proteome database; mRFP, monomeric red fluorescent protein; RNAi, RNA interference.

In eukaryotic cells, mitochondria are highly motile organelles that undergo continuous division and fusion with other mitochondria, resulting in a network of interconnected tubular shapes (*1–5*). Drp1/Dnm1, Mfn1&2/Fzo1, OPA1/Mgm1 and Fis1, which are conserved in eukaryotes, maintain the morphology of the organelle by regulating membrane fusion and fission (*1*, *6–10*). Recently, several other factors were identified to be implicated in controlling mitochondrial morphology (*11–13*).

To date, genome-wide screening has successfully identified many genes related to mitochondrial morphology and function. For example, systematic screening of yeast deletion mutants led to the identification of the *MDM* genes, which are involved in mitochondrial division and morphology (*14*). However, certain *MDM* genes are conserved only in fungi, suggesting differential regulation of mitochondrial morphology between unicellular and multicellular organisms. Indeed, mitochondria are delivered along microtubules in higher eukaryotes (*15*, *16*), whereas their movement depends on the actin cytoskeleton in yeast (*17*). In *Caenorhabditis elegans*, a large-scale screen for genes whose inactivation increased life span revealed that mitochondrial function affects longevity (*18*). Thus, a relationship between mitochondrial function and aging, but not morphology, has been established in *C. elegans* (*19*, *20*). The mitochondria in nematodes are tubular structures found in the embryo and body wall muscle (*21*, *22*), and similar to other eukaryotes, downregulation of *drp-1* produces elongated mitochondria (*21*).

We developed a screen for genes involved in the maintenance of mitochondrial morphology in *C. elegans*, utilizing the ease of genetic manipulation and superior efficiency of RNA interference (RNAi) offered by this model organism versus higher eukaryotes. In total, 719 genes that code for most of the mitochondrial proteins in *C. elegans* were screened, and knocking down >80% of them resulted in mitochondrial fragmentation and elongation, indicating that most of their functions are related to the maintenance of mitochondrial morphology.

MATERIALS AND METHODS

Strains and Culture Conditions—The *C. elegans* strains used in this study, wild-type Bristol N2, *fzo-1* (tm1133), *gas-1*(fc21), *isp-1*(qm150) and *clk-1*(e2519) mutant worms, were maintained at 20°C on nutrient growth medium (NGM) agar plates seeded with *Escherichia coli* OP50, according to standard techniques (*23*). Mitotracker (Invitrogen, Carlsbad, CA, USA)

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staining was performed essentially as described previously (*24*).

Mitochondrial-Targeted GFP and mRFP Fusion Proteins—To construct the body wall muscle-specific expression plasmid pmyo-3-GFP, the 2.4-kb *Hind*III/*Xba*I fragment of the *myo-3* promoter from pPD96.52 was subcloned into pPD95.77. Next, the region encoding the N-terminal region (*1–54*) of *C. elegans* TOM-20 (F23H12.2) was amplified using EST cDNA clone yk258a6 as a template with the primers (5′catctagaa atgtcggacacaattcttg3′ and 5′ttcctccggatccagcctgg3′), and cloned into the *Xba*I/*Bam*HI site of pmyo-3-GFP to construct pmyo-3-TOM20-GFP. Finally, to construct pmyo-3-TOM20-mRFP, the 2.6 kb *Hind*III/*Kpn*I fragment from pmyo-3-TOM20-GFP was cloned into pST-mRFP2, which is pPD95.77 carrying monomeric RFP (mRFP) (*25*) instead of GFP. The 2.4 kb *Hind*III/*Bam*HI fragment from pPD96.52 was subcloned into pPD96.32 to construct pmyo-3-AAT-GFP.

Generation of Transgenic Worms—A DNA solution (50 μg/ml) containing pmyo-3-TOM20-mRFP and pmyo-3-AAT-GFP was microinjected together with the selectable marker *rol-6* (su1006) (100 μg/ml) into young adult hermaphrodites and transgenic worms with an extrachromosomal array were selected under a fluorescence microscope. Using the resulting transgenic worms, transgenic lines in which the plasmid DNAs were integrated into the chromosome were generated by UV irradiation and outcrossed at least ten times to the wild-type strain. The integrated transgenic lines, which had normal mitochondrial morphology, were selected and used for RNAi screening.

Bioinformatic Analysis of C. elegans Genes Encoding Putative Mitochondrial Proteins—To search for *C. elegans* mitochondrial proteins, we used a comprehensive list of human mitochondrial proteins (score >80) from the Mitochondria Proteome database (MitoP2) (*26*) (http://www.mitop.de:8080/mitop2). The primary sequences of 719 human mitochondrial proteins were retrieved from Swiss-Prot (http://ca.expasy.org/sprot) and a BLAST search for *C. elegans* mitochondrial proteins was performed (http://www.sanger.ac.uk). Initially, 1009 *C. elegans* genes encoding proteins with a *P* value $>1.0 \times e^{-10}$ were selected; however, those genes that were redundant or missing from the RNAi library were omitted, leaving 719 genes for use in RNAi screen.

RNAi Screen for C. elegans Genes Involved in Mitochondrial Morphology—The *C. elegans* RNAi feeding library (*27*) was purchased from MRC Geneservice (Cambridge, UK). A systematic RNAi screen was carried out as described previously (*27*, *28*) with modifications. *Escherichia coli* cells expressing double-stranded RNA specific to each target gene were grown overnight in 2 × YT media containing 25 μg/ml carbenicillin and 12.5 μg/ml tetracycline (both Sigma-Aldrich, St. Louis, MO, USA), diluted 100-fold, grown for an additional 6 h and then seeded onto NGM agar plates containing 25 μg/ml carbenicillin and 5 mM isopropylthiogalactoside (Sigma-Aldrich, St. Louis, MO, USA) for induction of double-stranded RNA. To examine mitochondrial morphology, several young adult transgenic worms were placed onto the NGM agar plates seeded with the *E. coli* cells and incubated for 48 h at 20°C. They were then anesthetized with 10 mM aldcarb (Sigma-Aldrich, St. Louis, MO, USA) in an agar pad on glass slides and the mitochondria were analyzed under a fluorescence or confocal microscope.

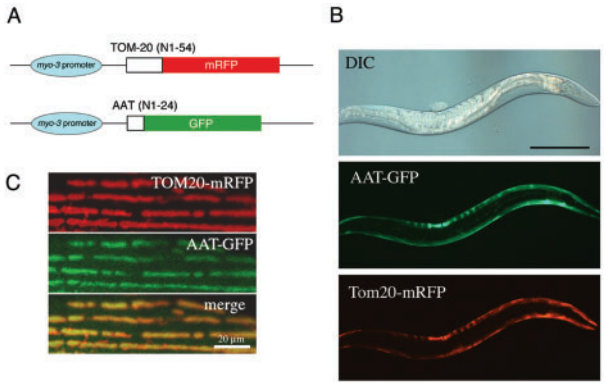

Fig. 1. **Establishment of transgenic worms for visualizing the mitochondria in body wall muscle cells.** (A) mRFP and GFP were fused to the N-terminal regions of *C. elegans* TOM-20 (*1–54*) and chicken aspartate aminotransferase (*1–24*), respectively, and expressed under control of the *myo-3* promoter, which is transcribed preferentially in body wall muscle cells. (B) Isolated transgenic worms stably expressing the mRFP and GFP fusion proteins were anesthetized with 10 mM aldcarb on glass slides and images were collected under a fluorescence microscope. DIC, differential interference contrast. Scale bar, 0.2 mm. (C) Mitochondria in the body muscle cell of a worm were analyzed by confocal microscopy. Scale bar, 20 μm.

RESULTS AND DISCUSSION

Morphological Analysis of C. elegans Mitochondria—To identify genes encoding mitochondrial proteins required to maintain the morphology of the organelle, we used the nematode *C. elegans*, an organism commonly used for microscopic analyses and gene silencing by RNAi. Since mitochondria are easily damaged, two mitochondria-specific fluorescent proteins (TOM20-mRFP and AAT-GFP) were used to visualize the mitochondria without requiring fixation. mRFP was fused to the N-terminal region (*1–54*) of *C. elegans* TOM-20, which is targeted to the mitochondrial outer membrane (*29*), while GFP was fused to the presequence (*1–24*) of chicken aspartate aminotransferase, which is targeted to the matrix (Fig. 1A). By introducing the plasmids, we obtained transgenic worms that constantly expressed both fluorescent proteins in their body wall muscle cells (Fig. 1B). Moreover, the proteins were co-localized in intracellular tubular structures (Fig. 1C), which correspond to mitochondria in *C. elegans* body wall muscle cells (*21*). Since AAT-GFP frequently leaked into the cytoplasm, TOM20-mRFP was used in the subsequent experiments.

In yeast and mammals, Drp1/Dnm1 and Mfn1&2/Fzo1 regulate mitochondrial membrane fusion and fission, respectively (*6–10*). To examine whether the transgenic worms isolated could be used to analyze mitochondrial morphology, *drp-1* and *fzo-1* expression was knocked down by RNAi. Tubular mitochondria were found in the

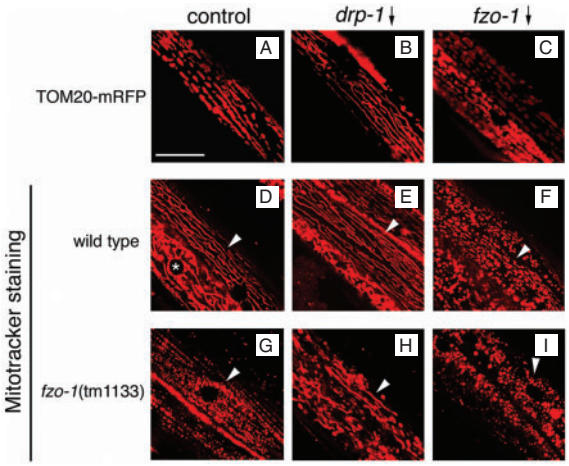

Fig. 2. **Morphological analysis of transgenic worms expressing fluorescent mitochondria-targeted proteins.** Transgenic worms (A–C) were cultured on plates seeded with *E. coli* cells expressing double-strand RNA for *drp-1* or *fzo-1*. The mitochondria in the worms were then analyzed as described in Materials and Methods. After RNAi for *drp-1* or *fzo-1*, wild-type (D–F) and *fzo-1*(tm1133) deletion mutant (G–I) worms were stained with 5 μM Mitotracker for 24 h and images were collected under a confocal microscope. Arrowheads and asterisk represent mitochondria in body wall muscle cells and an intestinal cell (nucleus), respectively. Scale bar, 50 μm.

wild-type worms (Fig. 2A) and in those with RNAi for *vha-7*, which encodes a vacuolar proton pump subunit (data not shown). In contrast, elongated mitochondria were observed in the *drp-1* knocked-down worms (Fig. 2B), consistent with the results of a previous study (*21*). Fragmented mitochondria were detected when *fzo-1* expression was repressed (Fig. 2C). Similar results were obtained by staining of non-transgenic worms with Mitotracker, a membrane potential-dependent mitochondrial vital dye (Fig. 2D–F). Although slightly swollen mitochondria appeared in the transgenic worms in which *fzo-1* expression was knocked down, exogenous expression of the fluorescent proteins had little effect on mitochondrial morphology. We also observed fragmented mitochondria in the body wall muscle cells of worms with an *fzo-1* deletion (Fig. 2G); however, elongated mitochondria were not confirmed in worms with a *drp-1* deletion because the mutation was lethal (*21*). When *drp-1* expression was knocked down in the *fzo-1* deletion mutants, formation of the tubular structures was observed (Fig. 2H; arrowhead), which is consistent with the previous work that loss of both fusion and fission activities restores mitochondrial tubular structures in yeast (*30*). These results indicate that body wall muscle cells are sufficient to monitor mitochondrial morphology and the transgenic worms isolated are suitable for analyzing efficiently the changes due to gene silencing.

Most Mitochondrial Functions are Crucial for Maintaining the Morphology of the Organelle—We used an RNAi feeding library to systematically knock down the expression of genes encoding mitochondrial proteins. The library, which includes 86% of the putative genes

Table 1. **RNAi screening for *C. elegans* genes involved in mitochondrial morphology.**

Procedure	Mitochondrial morphology	Number of genes
human MitoP2*		719
BLAST search		1009
RNAi screen		719 (100%)
	wild type	120 (16.7%)
	elongation	25 (3.5%)
	fragmentation**	574 (79.8%)

*Genes with high scores (>80) in the MitoP2 were selected.
**Includes the aggregation of fragmented mitochondria.

in *C. elegans* (*27*), is used to produce efficiently loss-of-function RNAi phenocopies by feeding the worms with bacteria expressing specific double-stranded RNAs. Unfortunately, no information related to the mitochondrial proteins in nematodes was available; thus, we used the information available for human mitochondrial proteins in the MitoP2 (*26*) to search for *C. elegans* genes encoding mitochondrial proteins. In total, 1009 putative genes were identified by a BLAST search using 719 human proteins. Those genes that were redundant or not included in the library were omitted, meaning that ultimately 719 *C. elegans* genes were examined by RNAi screening. Young adult transgenic worms were cultured for 48 h on plates seeded with bacteria expressing double-stranded RNA corresponding to each of the 719 genes. Changes in mitochondrial morphology were then analyzed under a fluorescence microscope and classified into three groups: normal (wild type), elongation and fragmentation. Surprisingly, 79.8% of the genes caused mitochondrial fragmentation and/or aggregation when they were knocked down, whereas 16.7% of the genes had no influence on morphology (Table 1, Supplementary data). Moreover, loss of function of genes (3.5%) resulted in elongated mitochondria. Note that the mitochondria of the body wall muscle cells were less mobile because of the muscle fibers; consequently, the fragmented mitochondria were not able to disperse and seemed to aggregate. The genes related to mitochondrial fragmentation were subsequently divided into five groups according to the functional category in the MitoP2 (Fig. 3, Supplementary data). No significant bias was detected toward a particular group compared to the human MitoP2 database (data not shown), suggesting that changes in most mitochondrial functions, including oxidative phosphorylation, protein synthesis and metabolism, directly and indirectly influence the morphology of the organelle. To confirm that dysfunction in the basic functions, especially oxidative phosphorylation, induces changes in mitochondrial morphology, three different worms with non-lethal mutations (*gas-1*, *isp-1* and *clk-1*) were stained with Mitotracker. Mutations in *gas-1* (49 kDa subunit of Complex I) and *isp-1* (Rieske Fe/S protein of Complex III) genes are shown to cause low oxygen consumption and/or the deficiency in oxidative phosphorylation (*31*, *32*). *clk-1* mutants are originally isolated as long-lived worms and exhibit low ubiquinone synthesis activity (*33*). All mutants clearly showed abnormal mitochondrial morphologies (Fig. 4).

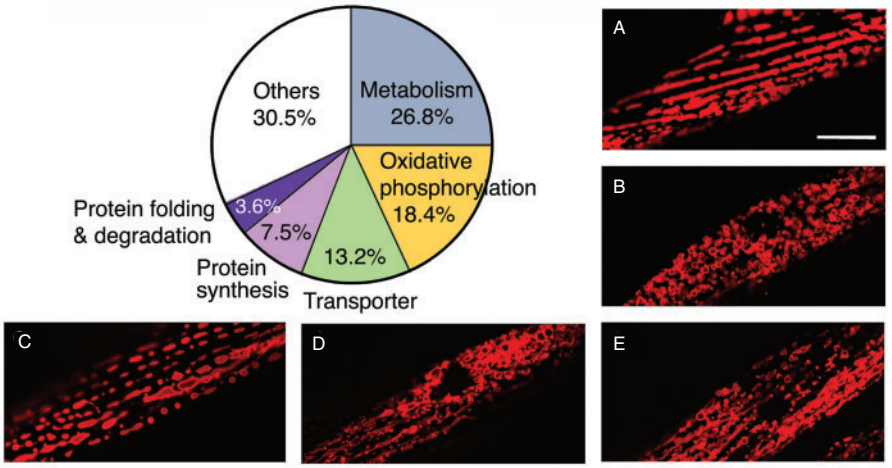

Fig. 3. **Fundamental mitochondrial functions are crucial for maintaining the morphology of the organelle.** Based on the functional categories in the MitoP2, genes related to fragmentation were classified. The circular graph represents the percentage of genes in each of the five categories. The mitochondria of wild-type worms (A) and those with knocked-down expression of (B) DNA polymerase γ (Y57A10A.15), (C) hepatopoietin (F56C11.3), (D) peptidyl-prolyl *cis–trans* isomerase (Y75B12B.2) and (E) 28S ribosomal protein S18c (T14B4.2) were analyzed as described in Materials and methods. Scale bar, 40 μm.

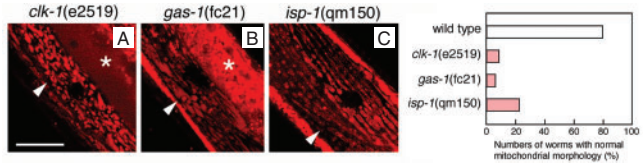

Fig. 4. **The respiratory-deficient mutants exhibit abnormal mitochondrial morphologies.** *clk-1*(e2519) (A), *gas-1* (fc21) (B) and *isp-1*(qm150) mutant worms (C) were stained with 5 μM Mitotracker for 24 h and images were acquired by confocal microscopy. Arrowheads and asterisks represent abnormal mitochondria in body wall muscle cells and intestinal cells, respectively. Scale bar, 50 μm. Data represent percentages of more than 100 individual worms counted.

More than 78% of the mutant worms either lost the normal tubular structures or had expanded mitochondria in body wall muscles. This is the first evidence that fundamental mitochondrial functions are involved in maintaining the organelle's morphology. Interestingly, diverse phenocopies were observed by knocking down the genes related to mitochondrial fragmentation. In particular, the loss of function of putative mitochondrial DNA polymerase γ (Y57A10A.15) caused severe fragmentation (Fig. 3H). Intriguing mitochondrial structures were also observed by knocking down the homologs of hepatopoietin (F56C11.3), which is involved in Fe/S cluster biogenesis (*34*) and peptidyl-prolyl *cis–trans* isomerase (Y75B12B.2), which functions in protein folding and mitochondrial membrane permeabilization (*35*) (Fig. 3C, D). Until now, neither gene had been shown to be directly involved in mitochondrial morphology. In addition, we found 88 uncharacterized or poorly characterized genes whose inactivation caused abnormal mitochondrial morphology (Supplementary data), although further investigation is necessary to determine their individual functions.

The knockdown of >80% of the genes we tested caused abnormal mitochondrial morphology, and especially fragmentation. Why are so many genes involved in maintaining mitochondrial morphology? One simple explanation involves impaired trafficking of mitochondrial membrane components and proteins. In yeast, mitochondrial targeting of Tom20 depends on the outer membrane proteins Tom40 and Mim1 (*36*, *37*). Although *C. elegans* homologues of Mim1 are still unidentified, mitochondrial fragmentation was observed when fed with the bacteria expressing double-strand RNA for C18E9.6, a *C. elegans* homologue of Tom40 (Supplementary data), suggesting that preexisting TOM-20-mRFP is sufficient to monitor changes in mitochondrial morphology even after 48 h RNAi feeding. However, it is possible that normal tubular mitochondria pretend to be fragmented as a consequence of abnormal distribution of TOM-20-mRFP existing on the outer membranes, that is induced by RNAi of certain genes. Second, a reduction in the ATP content caused by defects in oxidative phosphorylation would produce morphological changes because a close correlation exists between mitochondrial swelling and ATP depletion (*38*). Indeed, RNAi of *C. elegans* mitochondrial HSP70 led to a decrease in ATP and changes in mitochondrial morphology, although HSP70 is involved indirectly in oxidative phosphorylation (*24*). We also demonstrated that the mutants with the deficiency in activities of the respiratory chains exhibited abnormal mitochondrial morphology. Interestingly, malfunctions in mitochondrial DNA replication in *C. elegans* caused severe fragmentation (Fig. 3B), while ρ° HeLa cells, which lack mitochondrial DNA, still maintained tubular networks (*39*). To further elucidate the regulation of mitochondrial morphology, additional studies of individual genes, especially uncharacterized genes, are required. Moreover, our morphological assay can be used to investigate the contribution of individual genes to mitochondrial morphology in higher eukaryotes.

Supplementary Data are available at *JB* online.

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