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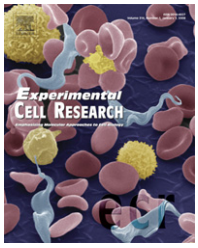

Research Article

Caenorhabditis elegans par2.1/mtssb-1 is essential for mitochondrial DNA replication and its defect causes comprehensive transcriptional alterations including a hypoxia response

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ABSTRACT

DNA polymerase γ and mtSSB are key components of the mtDNA replication machinery. To study the biological influences of defects in mtDNA replication, we used RNAi to deplete the gene for a putative mtSSB, *par2.1*, in *Caenorhabditis elegans*. In previous systematic RNAi screens, downregulation of this gene has not caused any clearly defective phenotypes. Here, we continuously fed a dsRNA targeting *par2.1* to *C. elegans* over generations. Seventy-nine percent of F1 progeny produced 60–72 h after feeding grew to adulthood but were completely sterile, with an arrest of germline cell proliferation. Analyses of mtDNA copy number and cell cytology indicated that the sterile hermaphrodites had fewer mitochondria. These results indicated that *par2.1* essentially functions for germline cell proliferation through mtDNA replication; we therefore termed it *mtssb-1*. Comprehensive transcriptional alterations including hypoxia response induction dependent on and independent of *hif-1* function, occurred by RNAi depletion of *mtssb-1*. Treatment with ethidium bromide, which impairs mtDNA replication and transcription, caused similar transcriptional alterations. In addition, the frequency of apoptosis in the germline cells was reduced in fertile progeny with a partial RNAi effect. These suggest that RNAi depletion of *C. elegans mtssb-1* is useful as a model system of mitochondrial dysfunction.

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Introduction

Without mitochondria, present-day eukaryotes would be dependent on the relatively inefficient process of glycolysis for all of their ATP production. Whereas unicellular eukaryotes like yeast could be supported by this inefficient process, complex multicellular organisms could not grow and proliferate without mitochondria. Thus, mitochondrial biogenesis and the accompanying mitochondrial DNA (mtDNA) replication are essential for the growth and proliferation of complex multicellular organisms. DNA polymerase γ (*POLG*) and mitochondrial single-stranded DNA-binding protein (mtSSB) are key components of the mtDNA replication machinery and are encoded by nuclear DNA [1–6]. Mutations in *POLG* cause large-scale mtDNA deletions to accumulate and are associated with progressive external ophthalmoplegia, a rare human disease [7]. In addition, clinical assessments have shown significant cosegregation of parkinsonism and an ataxia syndrome with *POLG* mutations [8–10]. mtSSB greatly enhances the overall activity of *POLG* by increasing primer recognition, stimulating the initiation rate of DNA strands, and increasing the processivity of *POLG* in DNA strand elongation in vitro [1]. Despite these observations, little is known about how mtSSB dysfunction influences human diseases. The *lopo* (*low power*) gene mutant in *Drosophila melanogaster* was the first mtSSB mutant to be isolated in a higher eukaryote, and its characterization showed that mtSSB is required for mtDNA replication, and cell proliferation and development [5]. In this mutant, a drastic decrease in mtDNA copy number is coupled with a loss of respiration.

In addition to being the site of respiration and oxidative phosphorylation, mitochondria also function in heme, lipid, and amino acid biosynthesis and fatty acid oxidation, among other important functions [11]. Moreover, mitochondria contribute to aging, cancer, and programmed cell death (apoptosis). The reactive oxygen species (ROS) produced by mitochondria are involved in apoptosis signaling and the hypoxia response [12–15].

The nematode *Caenorhabditis elegans* is a convenient model system for physiological experiments at the molecular level because it is easy to handle, its development and life cycle are well characterized, and abundant genetic information about it is available. The roles of mitochondria and mtDNA copy number in each developmental stage have been reported for wild-type animals and sperm-deficient or oocyte-deficient mutants [16,17]. In addition, the transcriptional profiles of cells, tissues, and developmental stages in normal conditions [18] and under several stress conditions, including hypoxia [19], have been reported. Many proteins, including the hypoxia-inducible transcription factor HIF-1 and the von Hippel–Lindau tumor suppressor protein VHL, are evolutionarily conserved between *C. elegans* and mammals [19,20]. In contrast, neither *C. elegans POLG* nor the *mtSSB* gene has been well characterized. Based on sequence similarities, *Y57A10A.15* and *par2.1* have, respectively, been annotated as *POLG* and *mtSSB*, but RNAi-based genome-wide screening studies have not revealed any defective phenotypes caused by RNAi of either of these genes [21–25].

Ethidium bromide treatment preferentially impairs mtDNA replication and transcription, creating cells with no mitochondria (ρ^0 cells) in mammalian cells [26–28] or diminished numbers of mitochondria in *C. elegans* [16]. In this study, we characterized *C. elegans par2.1* (termed *mtssb-1* in this study) gene functions by its RNAi. mtDNA-diminished animals were produced by continuous feeding of a dsRNA targeting the *mtssb-1* gene to the worms, as well as using the general method of ethidium bromide treatment. This produced several defects, including arrest of germline cell proliferation and decrease in apoptotic activity. In addition, comprehensive transcriptional alterations, including the induction of the hypoxia response, were identified in the mtDNA replication-defective animals.

Materials and methods

C. elegans strains and RNAi methods

C. elegans N2 wild-type hermaphrodites and the strains MD701: bcIs39[P(*lim-7*) *ced-1*::*GFP*+*lin-15*(+)] [29], and ET65: *cul-2* [ek1]/*unc-64* [e246] [30] were used. General methods of culturing and handling *C. elegans* were described previously [31]. All nematode experiments were performed at 20 °C.

To construct RNAi feeding bacteria, a cDNA fragment derived from *mtssb-1* (nucleotides 31–484) was amplified with an RT–PCR kit (Qiagen GmbH) using total RNA from N2 adult hermaphrodites as a template and the primer set *mtssb-1* FW and *mtssb-1* RV (Supplementary material 1). The cDNA fragment was cloned into the plasmid LITMUS 28 (New England BioLabs, Inc.). The resulting plasmid was transformed into *Escherichia coli* strain HT115 (DE3), and the transformants were termed HT115 (DE3)/pdT7-*mtssb-1* and HT115 (DE3)/pLITMUS28 (control). The optimal RNAi feeding method described by Kamath et al. [32] was slightly modified, in that the RNAi feeding plate was prepared once a day during the I0 generation, and the following F1 progeny fed on the RNAi bacteria at the L4 larval stage again as a method of continuous feeding over generations.

Ethidium bromide was added at a final concentration of 125 μg/ml to NGM plates seeded with bacteria [16], and synchronized L3 larvae were placed directly onto the plates. The adult hermaphrodites grown on the plates 40 h after transfer (24 h from the L4 larval stage) were pooled and used for analyses of transcriptional alterations and mtDNA copy number.

Expression analyses using RT–PCR and DNA microarrays

Total RNA was isolated with TRIzol Reagent (Gibco BRL Co., Ltd.) from synchronized adult hermaphrodites (24 h after the L4 larval stage) of the wild-type N2 strain with or without continuous feeding of dsRNA targeting *mtssb-1*. To measure the expression differences in each gene, real-time quantitative RT–PCR was performed using MiniOpticon (Bio-Rad Laboratories Inc.) with a SYBER ExScript™ RT–PCR Kit (TaKaRa Bio Inc.). Each primer set used is listed in Supplementary material 1. The expression level of an elongation factor *eft-2* was used as an internal standard and relative ratio of each gene expression was calculated.