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Investigation of yeast genes possibly involved in mtDNA stability using the nematode *Caenorhabditis elegans*

Addo M. G.[1,2*], Cossard R.[1], Pichard D.[1], Obiri-Danso K.[2], Rötig A.[3] and Delahodde A.[1]

[1]Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette cedex, France.
[2]Department of Theoretical and Applied Biology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[3]INSERM UMR 1163, Laboratory of Genetics of Mitochondrial Disorders, Paris Descartes – Sorbonne Paris Cité University, Imagine Institute, 24 Boulevard du Montparnasse, Paris 75015, France.

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Screening of *Caenorhabditis elegans* genes possibly involved in the mitochondrial genome maintenance was performed using our previous validated method of RNAi combined with ethidium bromide. This was to knock down *C. elegans* genes homologous to yeast genes known to be involved in mtDNA stability but of unknown molecular function or to identify transient components that could play important role on the stability of mtDNA in a temporal and/or spatial manner. *C. elegans* homologs for 11 genes among 27 yeast genes for which deletion leads to a rho0 state were found, however, only 5 genes were present in the RNAi library. Out of these 5 genes, 1 gene (homolog of *GEM1*) gave a clear L3 arrest on RNAi and ethidium bromide indicating its involvement on mtDNA stability. Four other genes homologs of *MTG2, YER087W, AVL9* and *RRG3* did not lead to L3 arrest even though their deletion in *Saccharomyces cerevisiae* leads to rho0 state. Although *MTG2* has been reported to be important in the function and structure on mtDNA stability in yeast, our results did not support those findings in *C. elegans*. The human homolog of this gene (MIRO1) can be considered as a candidate gene involved in mtDNA stability and sequenced in patients with mtDNA depletion diseases.

Key words: mtDNA, *Caenorhabditis elegans*, nucleoid, RNAi, candidate genes, homolog, MIRO1.

INTRODUCTION

The clinical and biochemical heterogeneity of mitochondrial diseases is one of the major problems for identifying the disease genes. Genetic defects of oxidative phosphorylation (OXPHOS) account for a large variety of clinical symptoms in childhood. The mitochondrial respiratory chain (RC) is made up of about 100 different

*Corresponding author. E-mail: maddosci@yahoo.co.uk.

proteins (Hatefi, 1985). It is hypothesized that several hundreds of nuclear genes are also needed for various functions of the RC. Mutations in any of these genes will result in mitochondrial RC disorders. Since the discovery of mutations in mitochondrial DN (mtDNA), the organization and segregation of mtDNA and nucleoid proteins has become a topic of active investigation and significant advances have been made in understanding the composition and dynamics of human mitochondrial nucleoids (Gerhold et al., 2015). Various approaches have been developed by different groups which resulted in the identification of disease genes. Yet, owing to the large number of possible candidate genes, these approaches most often remains laborious (De Lonlay et al., 2002). Again, considering the large number of genes involved in mitochondrial functions, using a systematic study to identify candidate genes for mitochondrial disorders is unrealistic.

The extensive conservation of mitochondrial structure, composition, and function across evolution offers a unique opportunity to expand the understanding of human mitochondrial biology and disease. By investigating the biology of much simpler model organisms, it is often possible to answer questions that are unreachable at the clinical level. Model organisms have an essential role in this process and might help to make these genes and their products amenable to pharmacological intervention. They are invaluable for understanding and elucidating the molecular bases of the pathophysiologies resulting from mitochondrial dysfunction. The discovery in the late 1980s of the first association of mtDNA mutations with human disorders (Holt et al., 1988; Wallace et al., 1988) has given an enormous boost to mitochondrial genetic research.

Qualitative or quantitative anomalies of this genome result in various types of mitochondrial diseases. Mutations in nuclear genes encoding proteins involved in mtDNA maintenance can result in large-scale mtDNA rearrangements and abnormal copy number of the mitochondrial genome.

The yeast *Saccharomyces cerevisiae* is probably the most studied model organism for acquisition of basic knowledge on mitochondrial function and biogenesis. Its suitability as a model for human mitochondrial disease studies has been well demonstrated (Bolotin-Fukuhara et al., 2010; Rinaldi et al., 2010). About 40% of human genes whose mutations lead to diseases have an ortholog in yeast (Bassett et al., 1996) and genomic screens have been extended to mitochondrial diseases (Steinmetz et al., 2002). The study of mitochondrial functions and dysfunctions is of special interest in the yeast *S. cerevisiae* because it can survive without its mtDNA (also called rho factor) if it is supplied with fermentative substrate. Thus, all mutations of the mitochondrial genome can be studied without cell lethality. Also, a clear advantage in the use of *S. cerevisiae* is the ease with which to introduce nuclear- gene mutations, mitochondrial gene mutations and screens for complementation or synthetic lethality. It is therefore an ideal organism for dissecting the molecular processes required for maintenance of respiratory competent mitochondria (Dimmer et al*.,* 2002; Steinmetz et al*.*, 2002).

However, notable differences in mtDNA structure and dynamics between yeast and human did not make the use of *S. cerevisiae* a perfect tool to study the mtDNA maintenance. Indeed, human cells contain 10^2–10^4 mtDNA copies, whereas yeast cells contain only 20 to 100 copies. The mitochondrial genome, 16.6 kb in human and 85.8 kb in yeast, is predominantly linear in yeast but is circular in human (Burger et al., 2003; Legros et al., 2004; Williamson, 2002). Finally, heteroplasmy is very frequently observed for mtDNA mutations in human, whereas yeast cannot normally maintain stably heteroplasmy (Shoubridge, 1998; Zeng et al., 2007). Furthermore, because this yeast can grow robustly by fermentation in the absence of mtDNA, it loses its mitochondrial genome very rapidly upon inactivation of a large class of genes encoding mitochondrial proteins involved in almost all the mitochondrial biogenesis pathways (mitochondrial translation, adenosine triphosphate (ATP) synthesis, iron homeostasis, mitochondrial import, and morphology). As such, it cannot be used easily to address the question of mtDNA transmission control (Contamine and Picard, 2000).

The characteristics of *Caenorhabditis elegans* make it a perfect complement to the yeast system. The success of *C. elegans* as a model organism in biological research is attributed to a number of biological and easy handling properties of the worm, such as short generation time, fixed cell lineage, transparent body, ease of maintenance and cryopreservation, sequenced genome, life synchronization, power of its genetics and a wide spectrum of tools for genome manipulation.

Previous studies have shown that, down expression by RNA interference of genes involved in mtDNA replication such as polg-1, encoding the mitochondrial DNA polymerase, results in reduced mtDNA copy number but in a normal phenotype of the F1 worms. By combining RNAi of genes involved in mtDNA maintenance and EtBr exposure, Addo et al. (2010) were able to reveal a strong and specific phenotype (L3-developmental larval arrest) associated to a severe decrease of mtDNA copy number.

Genome-scale approaches and various computational predictions of mitochondrial proteins (Small et al., 2004) have catalyzed the identification of a large number of mitochondrial proteins. From all these data, a human mitochondrial proteome has been proposed as MitoP2 (Andreoli et al., 2004). Ryohei et al. (2008) used the information available for human mitochondrial proteins in the MitoP2 to search for *C. elegans* genes encoding mitochondrial proteins. In total, 1009 putative genes were identified by a BLAST search using 719 human proteins (Ryohei et al., 2008). The availability of RNAi library, which is used to produce efficiently loss-of-function RNAi

phenocopies by feeding the worms with bacteria expressing specific double-stranded RNAs, includes 86% of the putative genes in *C. elegans* (Kamath and Arhinger, 2003).

In a recent genome-wide study in *S. cerevisiae*, Merz and Westermann (2009) found that out of 319 respiratory deficient deletion strains, 51% (162 strains) displayed the irreversible phenotype corresponding to the loss of intact wild-type mitochondrial genome. It is therefore important to know if these genes are also involved in mtDNA stability in *C. elegans.* To address this question, this study aims at investigating in *C. elegans* the role of the homologs of yeast genes of unknown or poorly assigned function, by knocking-down by RNAi combined with ethidium bromide (EtBr), the *C. elegans* genes homologous to the yeast genes, which upon deletion lead to a rho0 state.

MATERIALS AND METHODS

Media and cultivation of *E. coli*

Luria-Bertani-Media (LB) (Sambrook et al., 2001): 1% (w/v) Tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl pH 7.5. For solid media, 1.5% (w/v) agar was added before sterilization. For plasmid selection, the antibiotic Ampicillin was used at a concentration of 0.1 mg/ml. *E. coli* was cultivated in LB-medium over night at 37°C. Liquid cultures were incubated with shaking (150 rpm). To select transformants, the antibiotic Ampicillin (100 mg/L) was added to the media.

Yeasts strain, media and cultivation

Yeast deletion mutant strain used in this study was BY4741 (Openbiosystem). Cells were grown in complete liquid medium at 28°C overnight or for selection in minimal medium complemented with the necessary requirements at 28°C. Liquid cultures were incubated with shaking (150 rpm). Complete medium: 1% (w/v) yeast extract; 1% (w/v) bactopeptone; 2% (w/v) of carbon source Glucose (YPD) and Glycerol (YPG). For solid medium, 2% agar (Difco) was added before sterilization.

C. elegans strain and growth conditions

The *C. elegans* wild type worm N2 Bristol and *unc*-119 (ed3)III were used in this work and these were provided by the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota. The worms were maintained at 20°C on NGM plates seeded with *Escherichia coli* strains OP50 or HT115(DE3) following standard protocols (Brenner, 1974).

Bacterial preparation and induction

Nematode Growth Medium (NGM) agar (Epstein and Shakes, 1995) was prepared including 25 µg/ml carbenicillin (carb) and 1 mM IPTG (Kamath and Ahringer, 2003). 4.0 ml of agar was dispensed into 5 cm plates (Nunc). Plates were allowed to dry inverted at room temperature before use. Bacteria (HT115 strain) expressing the gene of interest were streaked onto LB–agar plates including 50 µg/ml Ampicillin (LBA) and 15 µg/ml Tetracycline and incubated over-night. Large innocula of bacteria were picked, inoculated into LB broth with 50 µg/ml Ampicillin and grown for 6 to 8 h with shaking at 37°C. Twenty (20) microliters of 0.1 M IPTG was added to 2 ml of the incubated LBA (final concentration of 1 mM) to induce expression of dsRNA gene of interest. About 200 µl of this culture was seeded/spread unto each of the aforementioned NGM derivative plates. The plates were dried thoroughly before being incubated overnight (~12 to 24 h) at room temperature in the dark to allow the bacteria to grow and to continue induction of the dsRNA gene of interest. *E. coli* transformation was performed with the $CaCl_2$ method.

RNA interference and EtBr assay

RNAi experiments were performed using the feeding procedure described by Kamath and Ahringer (2003) with slight modifications (Addo et al., 2010). For ethidium bromide (EtBr) plates, stock solution of 10 mg/ml was used to prepare different concentrations of 0, 50 and 125 µg/ml as required and added before dispensing into the 5 cm plates. Plates were then incubated at room temperature in a dark container for 48 h to allow the expression of the double-stranded RNA (dsRNA). Worms feeding on HT115 bacteria carrying the empty vector (L4440) were used as controls in all the experiments. Synchronized L1-stage N2 worms were placed onto NGM (RNAi) plates seeded with bacteria expressing the dsRNA gene of interest and were incubated for 72 h at 20°C. Four adult worms were independently picked up and transferred to fresh RNAi plates with or without different concentrations of ethidium bromide (EtBr). Worms were allowed to lay between 80 and 100 eggs before being removed. Eggs were immediately counted and the F1 progeny produced was analyzed after 3 and 4 days. At day 4, evaluation of the F1 progeny arrested at the L3 stage was compared to the number of adults on the same plate. The phenotype was scored as sensitive to RNAi and EtBr if more than 80% of worms were arrested at the L3-stage on plates containing 50 µg/ml of EtBr. A gene was considered as positive for a given phenotype if the same result was observed in at least two independent feeding experiments.

Bioinformatics

BLAST information available in the *Saccharomyces* Genome Database was used to explore the *C. elegans* homologs for yeast genes at: http://www.yeastgenome.org/cgi-bin/blast-sgd.pl. MITOP2 was used to find the *C. elegans* homologs to yeast genes that are completely devoid of mtDNA (rho0) upon their inactivation: http://ihg.gsf.de/mitop2.

Staining

MtDNA presence was also estimated by DAPI staining and confocal fluorescence microscopy.

RESULTS AND DISCUSSION

A systematic functional screen using the pre-existing whole genome pool of yeast deletion mutants (BY4741, *Openbiosystem*) was performed to expand the list of genes involved in respiratory competence at different temperatures. Using the Blast algorithm available in the SGD database (*Saccharomyces* Genome Database), potential human homologs for 108 yeast genes for which no function has yet firmly been assigned were found.

Among them, 75 encode proteins present in the yeast mitochondrial proteome (Sickmann et al., 2003; Pflieger et al. 2002). Twenty seven mutant strains completely devoid of mtDNA (rho0) were found by using DAPI staining and deconvolution fluorescence microscopy (Merz and Westermann, 2009). *C. elegans* homologs for 11 genes among 27 yeast genes for which deletion lead to a rho0 state were also identified. The genes identified as required for mtDNA stability in *C. elegans* as reported in earlier work (Addo et al., 2010) do not only mirror those already known in human to be implicated in human diseases but also support predictions of the importance of the nucleoid dynamics for mtDNA stability. Out of these 11 genes, only 5 were present in our RNAi library (Kamath and Ahringer, 2003). Screening of these 5 genes by combining RNAi and EtBr was performed and found that RNAi of 4 genes (*M01E5.2, T27F6.5, T26A5.6* and *C45G3.3* with the *S. cerevisiae* ortholog as MTG2*, YER087W (AIM10), AVL9* and *AIM22 respectively*) did not lead to L3 arrest whereas 1 of these genes *K08F11.5* (*GEM1*) gave a clear L3 arrest on EtBr (Table 1 and Plate 1, respectively).

RNAi EtBr non-sensitive genes (MTG2 orthologous gene GTPB5_HUMAN)

Mtg2 is a GTPase, member of the Obg family that comprises a group of GTPases acting as translation factors. The Obg subfamily of GTPases has been identified in all organisms sequenced to date. It is a mitochondrial inner membrane protein that is essential for mitochondrial ribosome function in yeast. It associates with the large ribosomal subunit; required for mitochondrial translation, possibly via a role in ribosome assembly. It has been shown that elevated levels of Mtg2p partially suppress the thermosensitive loss of mtDNA in a 21S rRNA methyltransferase mutant, *mrm2* (Datta et al., 2005). It is therefore important to check the plasmid sequence used for the RNAi expressing the dsRNA-*MTG2* in subsequent work, as some errors may have occurred during the construction of the library. The efficiency of the RNAi by RT-q-PCR should also be controlled as well as the measurement of the mtDNA content after RNAi in subsequent work. Inactivation of the three other genes did not seem to affect the mtDNA content in *C. elegans*. *YER087W (AIM10)* is a protein with similarity to tRNA synthetases. This gene encodes a mitochondrial protein with similarity to tRNA synthetases. *AVL9* encodes a conserved protein of unknown function involved in exocytic transport from the Golgi whilst *AIM22* encodes a putative lipoate-protein ligase.

RNAi EtBr sensitive genes (*GEM1* (MIRO-1 or RHOT1))

In yeast, *GEM1* encodes an evolutionary-conserved tail-anchored outer mitochondrial membrane GTPase which regulates mitochondrial morphology. Cells lacking Gem1p contain collapsed, globular, or grape-like mitochondria that are not caused by defects in mitochondrial fission and/or fusion. It has been shown that Gem1p functions to maintain mitochondrial morphology, retain mitochondrial DNA nucleoids, and promote mitochondrial inheritance in yeast (Frederick et al., 2004). Vance (2014) reports that the contact sites between mitochondria and the ER are hubs for lipid translocation and Ca^{2+} traffic between the ER and mitochondria. The endoplasmic reticulum-mitochondria encounter structure (ERMES) forms a junction between mitochondria and the endoplasmic reticulum (ER). Four ERMES proteins are known in yeast, the ER-anchored protein Mmm1 and three mitochondria-associated proteins, Mdm10, Mdm12 and Mdm34, with functions related to mitochondrial morphology and protein biogenesis (Stroud et al., 2011). There is evidence that the metazoan Gem1 ortholog Miro-1 localizes to sites of ER–mitochondrial contact, suggesting that some of the features ascribed to Gem1 may be evolutionarily conserved and that ERMES-mediated ER–mitochondria connections lie at a cross-roads of several biosynthetic pathways (Kornmann et al., 2011). Their study again identified the Ca^{2+}-binding Miro GTPase Gem1 asan ERMES subunit. Michel and Kornmann (2012) also report that the protein complex is physiologically involved in a plethora of mitochondrial processes, suggesting that ER-mitochondria connections play a central co-ordinating role in the regulation of mitochondrial biology. Defects of mitochondrial inheritance were also observed after inactivation of the Miro GTPase (homolog to *GEM1*) in fly and human suggesting a conserved role in mitochondrial distribution (Guo et al., 2005). More recent work suggests that the Miro GTPases form a link between the mitochondria and the trafficking apparatus of the microtubules (Cox and Spradling, 2006; Fransson et al., 2006; Guo et al., 2005; Glater et al., 2006) whereas in yeast Gem1 interacts with actin cables to anchor mitochondria to the cytoskeleton. Our work supports the predictions that the gene homologous to human MIRO-1 (RHOT-1) is involved in the attachment of mitochondria to the cytoskeleton that are required for mtDNA maintenance in *C. elegans* post-mitotic cells. Involvement of RHOT1 in mtDNA stability has never been described for MIRO-1. Mitochondrial RHOT1 (MIRO-1) gene encodes a tail-anchored outer mitochondrial membrane GTPase which has been shown to bind directly to the microtubule-dependent motor KIF5 proteins (Macaskill et al., 2009). These proteins that link Miro to the cytoskeleton, are required for normal distribution of mitochondria and have been shown to often locate in the vicinity of nucleoids (Iborra et al., 2004). Thus, microtubule-mediated mitochondrial transport seems to play an important role on the stability of the mitochondrial genome in *C. elegans*. Recent biochemical characterization of this protein complex has led to the

Table 1. Yeast deletion strains (genes) that were either negative or positive to ethidium bromide and RNAi treatment and may or may not be involved in mtDNA stability.

SGD	Strain with human homolog	SGD comment	RNAi EtBr 50 µg/ml screen 30	Similarity (%)	Worm ortholog	Human ortholog	Similarity (%)
AIM10	YER087W	Protein with similarity to tRNA synthetases; non-tagged protein is detected in purified mitochondria; null mutant is viable and displays elevated frequency of mitochondrial genome loss	Not sensitive 30%	1.50E-40 (78)	T27F6.5	SYPM_HUMAN, Probable prolyl-tRNA	1.9e-68 (78)
MTG2	YHR168W	Putative GTPase, member of the Obg family; peripheral protein of the mitochondrial inner membrane that associate with the large ribosomal subunit; required for mitochondrial translation, possibly via a role in ribosome assembly	Not sensitive 60%	1.50E-48 (78)	M01E5.2	GTPBP5, GTP binding protein 5 (putative)	3.8e-77 (99.2)
AVL9	YLR114C	ORF, uncharacterized, defective in late secretory pathway. Conserved protein involved in exocytic transport from the Golgi; mutation is synthetically lethal with apl2 vps1 double mutation; member of a protein superfamily with orthologs in diverse organisms	Not sensitive 28%	1.20E-26 (78)	T26A5.6	NP_055875.1,	3.4e-36 (93)
AIM22	YJL046W	Putative lipoate-protein ligase, required along with Lip2 and Lip5 for lipoylation of Lat1p and Kgd2p; similar to E. coli LplA; null mutant displays reduced frequency of mitochondrial genome loss	Not sensitive 71%	6.90E-31 (78)	C45G3.3	LIPT1, Lipoyltransferase 1, mitochondrial	3.0e-45 (96.9)
GEM1	YAL048C	Outer mitochondrial membrane GTPase, subunit of the ERMES complex; potential regulatory subunit of the ERMES complex that links the ER to mitochondria and may promote inter-organellar calcium and phospholipid exchange as well as coordinating mitochondrial DNA replication and growth; cells lacking Gem1p contain collapsed, globular, or grape-like mitochondria; ortholog of metazoan Miro GTPases	Yes sensitive 95%	1.50E-77 (78)	K08F11.5, RNAi no abnormalities	RHOT1, calcium ion binding; GTP binding	6.7e-144 (98.6)

SGD: Saccharomyces genome database; *C. elegans* wild type worm N2 Bristol was used as controls.

discovery that GTPases of the Miro family are part of ER-mitochondria connections.

The yeast Miro GTPase Gem1 localizes to ER-mitochondria interface and influences the size and distribution of mitochondria. Thus Miro GTPases may serve as regulators of the ER-mitochondria connection (Michel and Kornmann, 2012).

Apart from yeast, a relationship between MIRO-like proteins and mtDNA copy number has never

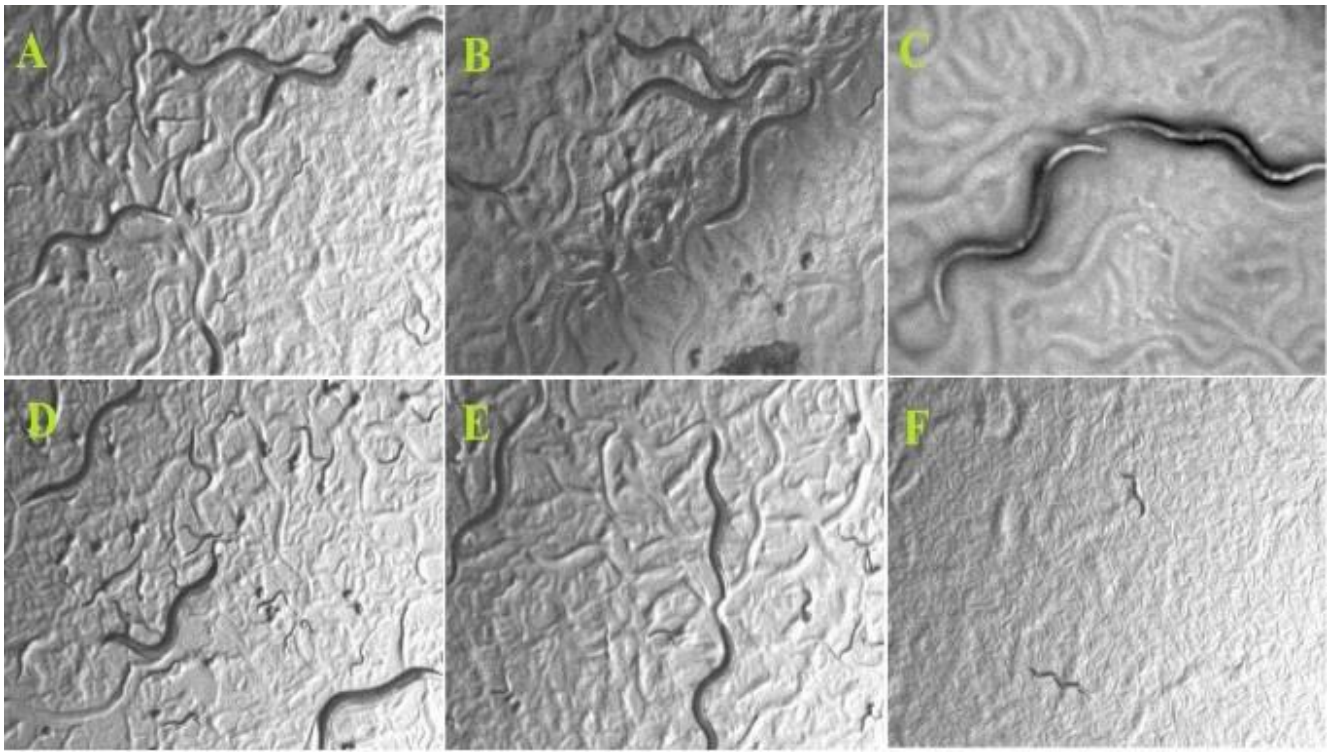

Plate 1. Microscopic images of L3 developmental arrest after RNAi of (A) N2 wild-type control worms, (B) *MTG2* gene, (C) *AIM10* gene, (D) *AVLA9* gene, (E) *AIM22* gene and (F) *GEM1* gene. Inactivation of A – E with 50 µg/ml ethidium bromide did not affect mtDNA stability. *GEM1* mutants however were sensitive to the RNAi knockdown.

been observed presumably because it has not been addressed. Nevertheless, our results do suggest that there is an unexplored interplay between mitochondrial network dynamics and nucleoid dynamics/segregation at least in the yeast *S. cerevisiae* and *C. elegans*.

This supports the assumption made by Spelbrink (2010) of a potential conservation of function between yeast and mammalian nucleoid organization and dynamics (Spelbrink, 2010). In yeast, replicating nucleoids have been observed in close vicinity of Mdm10/Mdm34/Mdm12/Mmm1 (ERMES) complex that is at least responsible for ER-mitochondrial tethering (Kornmann et al., 2009; Michel and Kornmann, 2012) and that associates with the actin cytoskeleton involving the Arp2/3 and Puf3 proteins (Boldogh et al*.,* 2003). In Spelbrink's (2010) scenario, mammalian MIRO-1 would anchor the mtDNA nucleoids to the cytoskeleton and potentially also to the ER-mito junctions, thus playing a very important role in mtDNA nucleoid dynamics. Our results therefore give credence to Spelbrink's assumption.

To conclude, our result supports the existence of a membrane scaffold structure, as suggested earlier (Hobbs et al., 2001), that is at least functionally conserved in all respiring eukaryotes and would coordinate mtDNA maintenance with mitochondrial replication, transcription, translation, protein assembly and mitochondrial dynamics. The human homolog (MIRO-1) of the *GEM1* gene can be considered as a candidate gene to be sequenced in patients suffering of mtDNA depletion syndrome.

Conflict of Interests

The authors have not declared any conflict of interests.

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