Fzo1p Is a Mitochondrial Outer Membrane Protein Essential for the Biogenesis of Functional Mitochondria in *Saccharomyces cerevisiae*

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Mitochondria exist in a particular cell type in a characteristic copy number, size, and position, often reflecting the energy needs of the cell. The inheritance of mitochondria, the maintenance of their characteristic shape, and their positioning is mediated by active transport along cytoskeletal elements and depends on continuous fusion and fission of the organelles (1, 2). Only little is known about the molecular components mediating these processes.

The budding yeast *Saccharomyces cerevisiae* is an excellent model organism to study these processes because genetic and biochemical techniques can be readily combined. In *S. cerevisiae*, mitochondria form a giant branched network below the cell cortex (3). During vegetative growth, the continuity of this network is maintained by a balanced frequency of fission and fusion events (4). During mitotic cell division early in the cell cycle, a portion of the paternal mitochondrial network is actively transported into the developing bud, where mitochondria continue to accumulate until cytokinesis is completed (2). Upon fusion of two mating cells, parental mitochondria immediately fuse, and their contents mix (4). Several proteins are known to be important for mitochondrial morphology and inheritance in yeast. These proteins include the mitochondrial outer membrane proteins Mdm10p, Mmm1p, and Mdm12p, the fatty acid desaturase Mdm2p/Ole1p, the dynamin-like protein Mgm1p, the intermediate filament-like protein Mdm1p, yeast actin, Act1p, and a component important for the organization of the actin cytoskeleton, Mdm20p (5–12). Disruption or mutation of the respective genes leads to the formation of mitochondria with an abnormal morphology and/or to a defect in the partitioning of mitochondria to the daughter cell. None of these proteins appears to play a direct role in mitochondrial fusion.

Because the integrity of the three-dimensional structure of the cell depends on fusion of intracellular membranes, the identification and characterization of the molecular components responsible for this process is subject to intense investigation. The best characterized system of intracellular membrane fusion is that of the organelles of the secretory pathway, which are interconnected by a complex network of transport vesicles (13). Both the transport vesicles and the target membranes carry a specific set of integral membrane receptors on their surface, the SNARE proteins which determine the identity of organelles and control the specificity of docking between intracellular membranes (14, 15). At the same time, SNAREs constitute the minimal machinery for membrane fusion (16). The ATPase NSF and SNAP proteins play an essential role in SNARE-dependent fusion processes in vivo and are thought to be a general machinery for the recycling of SNARE proteins after fusion (16, 17). Mitochondria, however, appear to be independent of the action of NSF (4), and no SNARE-like proteins are known in mitochondria. Thus, it seems likely that mitochondria employ a different mechanism for fusion.

Recently, the first known protein mediator of mitochondrial fusion has been identified in *Drosophila* (18). The fuzzy onions (fzo) gene encodes a large predicted transmembrane GTPase that is expressed during spermatogenesis late in meiosis II. In male fzo mutants, mitochondria aggregate and are defective in postmeiotic fusion. They develop structures that look like “fuzzy onions.” This deficient organellar development results in defective sperm production and male sterility. Similar proteins of unknown function exist in mammals, nematodes, and yeast (18).

Here, we report the characterization of the yeast homolog of Fzo, Fzo1p. Disruption of the FZO1 gene in yeast results in a petite phenotype and in the loss of mitochondrial DNA, indicating an important function of Fzo1p in mitochondrial biogenesis. Cells lacking Fzo1p show a fragmented mitochondrial morphology. Fzo1p is located in the mitochondrial outer membrane exposing the major part of the protein to the cytosol, and can be imported into isolated mitochondria in a receptor-dependent manner. Fzo1p is part of a high molecular weight

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1 The abbreviations used are: SNARE, SNAP receptor; NSF, N-ethylmaleimide–sensitive fusion protein; SNAP, soluble NSF attachment protein; GFP, green fluorescent protein; mt-GFP, mitochondrial GFP; MOPS, 4-morpholinopropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; Hsp60, heat shock protein of 60 kDa; KLH, keyhole limpet hemocyanin; COXII, cytochrome oxidase subunit II; DiOC6, 3’,5’-dihexyloxacarbocyanine iodide; Y1, carboxyl cyanide cyanohydrin YPP, yeast extract-peptone-dextrose; YPG, yeast extract-peptone-glycerol; YPGal, yeast extract-peptone-galactose; DIC, differential interference contrast; PCR, polymerase chain reaction.
complex, and presumably is the first identified component of a yeast mitochondrial fusion machinery.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Disruption of the FZO1 Gene—**Standard genetic techniques were used for growth and manipulation of yeast strains (19).

Transformation of yeast was carried out as described (20).

To obtain disruption mutants of FZO1, most of the open reading frame was first replaced with the Ty903 kanamycin resistance gene in the diploid strain YPH501 (21) by a PCR-based approach as described (22) using primers used to generate the disrupting DNA fragment were

179KA-5 (5′-GTT GAT GTA AAT ACT GGT GTC CCT TTC AAC TCT C) and 179KA-3 (5′-GCA AAG AGC GAG ACC AGT ATT TAC ATC ACC). Haploid deletants were obtained after sporulation and tetrad dissection. The wild type strains D273–10B (ATCC 24657) and W303A were used for the preparation of subcellular and sub mitochondrial fractions.

To obtain the construct for in vitro transcription of FZO1, the FZO1 open reading frame was amplified from genomic DNA by PCR using the primers Z36L (5′-CTG TAT ATG AGT GCC TCC ACT CGT GTC C) and Z36U (5′-GAG CTT ATG GCC TCC ACC ATG GTC C) and cloned into the pGEM4 (Promega).

**Recombinant DNA Techniques and Plasmids Constructions—**Standard methods were used for the manipulation of DNA (23). To obtain the construct for in vitro transcription of FZO1, the FZO1 open reading frame was amplified from genomic DNA by PCR using the primers Z36L (5′-CCC GGA TCC ACC ATG TCT GAA GGA AAA CAA C) and Z36U (5′-CCC GTT AAC GTG GTA TCA ATG GAT GA CAA CTA A) and cloned into the BamHI and SalI sites of the in vitro expression vector pGEM4 (Promega).

To obtain the construct for expression of the green fluorescent protein (GFP) in mitochondria, the presequence of the subunit 9 of the F0 ATPase of Neurospora crassa was amplified by PCR using the primers SU9N (5′-GGG AGA CTT ATG GCC TCC ACC ATG GTC C) and SU9C (5′-GGG GGA TCC GGA AGA GTA GGC GCG CTT) and cloned into the HindIII and BamHI sites of the vector pGEM3 (Promega) yielding plasmid pGEM3-Su9(1–69). The open reading frame coding for GFP from Aequorea victoria containing the 657 mutation (24) was amplified by PCR from plasmid pFP20 (kind gift of Dr. F. Parlati, New York) using the primers GFP-N (5′-CGG AAT TCT TAT TTG TAT) and GFP-C (5′-CCG AAT TCT TAT TTG TAT AGT TCA TCC) and cloned into the KpnI and EcoRI sites of pGEM3-Su9(1–69) yielding plasmid pGEM3-Su9-GFP2. The HindIII/EcoRI fragment of pGEM3-Su9-GFP2 was subcloned into the yeast expression vector pYES2.0 (Invitrogen) yielding plasmid pYES-GFP2.

**Import of Precursor Proteins into Mitochondria—**The in vitro import of Fzo1p was carried out essentially as described (25). Precursor protein was synthesized in the presence of [35S]methionine in reticulocyte lysate (Promega). Import mixtures (100 μl) usually contained 1–3% reticulocyte lysate (v/v) in 3% bovine serum albumin (w/v), 0.6 M sorbitol, 10 mM MOPS-KOH, 80 mM KCl, pH 7.2. Protease treatment was performed to generate the disrupting DNA fragment was amplified by PCR from plasmid pFP20 (kind gift of Dr. F. Parlati, New York) using the primers GFP-N (5′-CGG AAT TCT TAT TTG TAT) and GFP-C (5′-CCG AAT TCT TAT TTG TAT AGT TCA TCC) and cloned into the KpnI and EcoRI sites of pGEM3-Su9(1–69) yielding plasmid pGEM3-Su9-GFP2. The HindIII/EcoRI fragment of pGEM3-Su9-GFP2 was subcloned into the yeast expression vector pYES2.0 (Invitrogen) yielding plasmid pYES-GFP2.

**RESULTS**

**FZO1 Is Essential for the Formation of Respiratory-competent Mitochondria—**The FZO1 gene in yeast (systematic name YBR179c) encodes a protein that is 20% identical to Drosophila Fzo (18). Both proteins possess a similar domain structure, namely an N-terminal predicted coiled-coil, followed by a highly conserved GTPase domain, a second coiled-coil region, a predicted transmembrane domain in the C-terminal third of the protein, and a third coiled-coil close to the C terminus (18).

Based on these similarities, we reasoned that like Fzo in Drosophila, Fzo1p might play an important role for the biogenesis of mitochondria in yeast. To test whether the FZO1 gene is essential for the viability of yeast cells, we disrupted one of the two copies of FZO1 in diploid cells. The disruption was done by replacing almost the entire open reading frame of FZO1 by the kanamycin resistance gene (see “Experimental Procedures”). After sporulation and tetrad dissection, we found that all four spores in each tetrad were viable on glucose-containing medium, but that two spores in each tetrad showed a slow growth phenotype (Fig. 1A). Cells from the small colonies carried the disrupted gene (Δfzo1), whereas cells from normal-sized colonies carried the wild type gene. The deletion mutant failed to grow on nonfermentable carbon sources (Fig. 1B), suggesting an important role of Fzo1p for mitochondrial function.

The cells containing the disruption in FZO1 had lost a functional mitochondrial genome. This was concluded from two independent observations. First, mating the Δfzo1 strain with a rho+ tester strain lacking mitochondrial DNA, Δmdj1 (26), did

**FIG. 1. Disruption of the FZO1 gene results in a petite phenotype and a fragmented mitochondrial morphology. A, dissection of tetrads after sporulation of a FZO1 heterozygous diploid strain. Dissected tetrads were incubated for 3 days on YPD medium (2%) glucose) at 30 °C. B, replica-plate analysis of dissected tetrads from a FZO1/fzo1 ΔTn903 heterozygous diploid strain. Yeast cells were grown on a fresh YPD plate and were replica-plated onto a YPD plate, a YPG plate (3% glycerol), and a YPD plate containing 500 μg/ml Geneticin (G418). Plates were incubated for 2 days at 30 °C. The latter plate allows growth of only those cells that carry the Δfzo1 allele disrupted by the Tn903 kanamycin resistance marker. C, fluorescence microscopy of wild type cells and the Δfzo1 mutant expressing mt-GFP. Cells were grown in liquid YPGal medium (2% galactose) at 25 °C, washed in water, and immobilized in 0.5% low melting point agarose. For each strain, one representative cell is shown. The upper part shows DIC images, and the lower part shows mitochondrial staining.
not result in diploid cells able to grow on glycerol. Thus, the Δfzo1 strain did not contain a functional mitochondrial genome that would be able to restore growth on nonfermentable carbon sources in the presence of the wild type copy of the FZO1 gene in the diploid strain (not shown). Second, we performed an in organello translation assay for mitochondrial-encoded proteins (28). Mitochondria were incubated in the presence of 35S-labeled methionine, which resulted in the labeling of mitochondrial-synthesized proteins in wild type mitochondria. In mitochondria isolated from the Δfzo1 strain, this labeling was completely absent (not shown). Moreover, COXII, a protein encoded by the mitochondrial DNA, was absent in immunoblots of Δfzo1 mitochondria. Fzo1p (Fig. 1C). An identical staining was obtained with mitochondria-specific dyes such as Mitotracker or DiOC6 (not shown). In the Δfzo1 strain expressing mt-GFP, the mitochondrial morphology was completely altered. Mitochondria were highly fragmented, and only in a few cells could some short tubular structures be seen (Fig. 1C). Because it is known that the morphology of yeast mitochondria is changed in the absence of an intact mitochondrial genome (31), we expressed mt-GFP in a cytoplasmic petite strain lacking mitochondrial DNA that is otherwise wild type (W303 rho0). In this strain, a mitochondrial morphology similar to that in the Δfzo1 mutant was observed. Most of the rho0 cells harbored fragmented mitochondria, and only a few well developed tubular structures were seen (data not shown). We conclude that Fzo1p is required for a normal mitochondrial morphology. It is, however, unclear as to whether the fragmented mitochondrial morphology is a direct consequence of deletion of FZO1, or an indirect effect of the rho0 state of the deletion mutant.

Fzo1p Is a Mitochondrial Protein Located at the Outer Membrane—To determine the location and topology of Fzo1p, antibodies against an N-terminal and a C-terminal peptide were prepared (see “Experimental Procedures”). First, we investigated the subcellular location of Fzo1p. Cells were harvested from a liquid culture at mid-logarithmic growth phase, and subcellular fractions were prepared and used for immunoblotting. Fzo1p was detected in the mitochondrial fraction and cofractionated with Tom70, a mitochondrial protein. Fzo1p could not be detected in the cytosolic or nonmitochondrial membrane fractions (Fig. 2A). Thus, Fzo1p is a mitochondrial protein. Next, the sub mitochondrial location of Fzo1p was determined. Hydropathy analysis suggests that Fzo1p may contain one or two putative membrane-spanning segments (Fig. 2B). Indeed, Fzo1p could not be extracted from mitochondrial membranes following treatment with 0.1 M sodium carbonate, indicating that it is an integral membrane protein (Fig. 2C). To determine in which of the two mitochondrial membranes Fzo1p resides, we prepared outer membrane vesicles. Fzo1p is enriched in these vesicles together with other outer membrane proteins such as Tom70 (Fig. 2C). In contrast, inner membrane proteins such as COXII were hardly detectable in these vesicles. Furthermore, Fzo1p was accessible to externally added proteases in intact mitochondria under conditions where proteins exposed to the intermembrane space were protected (Fig. 2C) indicating that at least a part of the protein is exposed to the cytosol. We conclude that Fzo1p is an integral protein of the mitochondrial outer membrane.

To understand the role of Fzo1p in the biogenesis of normal mitochondria, it is important to know its topology in the outer membrane. We noticed that after treatment of mitochondria
with very low amounts of protease, fragments were generated that were slightly smaller than the full-length protein. Because these fragments could be detected in a Western blot using the antibody against a C-terminal peptide of Fzo1p (Fig. 2D), the protease must have cleaved the N terminus of the protein, indicating that this part is exposed to the outside. Thus, the major part of Fzo1p including the predicted GTPase domain faces the cytosol.

In Vitro Translated Fzo1p Can Be Imported into Isolated Mitochondria—Next, we tested the in vitro import of Fzo1p into isolated mitochondria. This is an independent approach to show its mitochondrial localization, and at the same time, Fzo1p is a novel model protein to study protein import into the mitochondrial outer membrane. Radiolabeled protein was synthesized in vitro and incubated with isolated wild type mitochondria. After the import reaction, carbonate extraction was performed. Most of the imported protein was recovered in the pellet, indicating insertion of the protein into the membrane (Fig. 3A). As a control, carbonate extraction was performed on lysate in the absence of mitochondria. Here, the protein was found in the supernatant, excluding the possibility that it was aggregated under these conditions. The inserted protein like the endogenous one was sensitive to proteinase K (Fig. 3A). As expected from the lack of a typical cleavable presequence, no change in the molecular mass of Fzo1p was observed upon import. We investigated the kinetics of the import process and found that, within 5 min, most of the protein was inserted into the outer membrane (Fig. 3B). Similar to other outer membrane proteins, the import of Fzo1p was independent of a membrane potential ΔΨ across the inner membrane as the addition of the uncoupler CCCP did not change the import efficiency (Fig. 3C). Pretreatment of mitochondria with trypsin to cleave import receptors on the mitochondrial surface significantly reduced the amount of inserted protein, suggesting that Fzo1p uses protease-sensitive import receptors for its insertion into the outer membrane (Fig. 3C). The import and protease sensitivity of Fzo1p was not affected by the presence or absence of nucleotides such as AMP, ADP, ATP, or GTP (Fig. 3C and data not shown). These observations are consistent with a localization of Fzo1p in the mitochondrial outer membrane. It appears that its import is receptor-dependent and does not require ATP-dependent cytosolic chaperones.

Fzo1p Is Part of a High Molecular Weight Complex—It is conceivable that Fzo1p interacts with other proteins to fulfill its role in mitochondrial biogenesis. In particular, the predicted coiled-coil domains may be responsible for formation of hetero and/or homo oligomers. Thus, we investigated whether Fzo1p is part of a high molecular weight complex. Mitochondria were solubilized with detergent, and the proteins were separated on a gel filtration column. Fzo1p was eluted from the column in a relatively sharp peak, corresponding to a molecular weight of about 800 kDa (Fig. 4). The protein was eluted in a fraction corresponding to a similar size when GTP was present during lysis and elution (not shown). These results suggest that Fzo1p is part of a complex of high molecular weight, which might represent the mitochondrial fusion machinery in yeast, or a part thereof.

DISCUSSION

Fzo in Drosophila is the first known protein mediator of mitochondrial fusion (18, 32). Several lines of evidence suggest that Fzo1p might play a similar role in yeast. First, Fzo1p shares a significant sequence homology with the Drosophila protein and possesses a similar predicted domain structure (18). Second, Fzo1p in yeast cells and Fzo in sperm cells have the same subcellular location in mitochondria. Third, Fzo1p is an integral protein of the mitochondrial outer membrane with the major part facing the cytosol, a topology which would be expected for a fusion protein that has to interact with proteins on the opposite membrane or in the cytosol.

The assumption that Fzo1p plays a role in mitochondrial fusion is further strengthened by the observed phenotype of the deletion mutant. Deletion of FZO1 leads to the loss of mitochondrial DNA. The inheritance of mitochondrial DNA in yeast is an ordered event that is thought to depend on the integrity of

![Fig. 3.](image-url)
that proteins of different molecular weights are present in the same complex. Thus, Fzo1p is likely to be only the first identified component of a much more complex mitochondrial fusion machinery.

The precise role of Fzo1p in mitochondrial fusion is still unknown. Our data indicate that it is an integral protein of the mitochondrial outer membrane with the major part of the protein exposed to the cytosol. The exposed part includes the GTPase domain and two predicted coiled-coil regions. Such a topology seems to be ideally suited for a fusion protein that is expected to interact with other proteins on the opposite membrane and/or in the cytosol. It is still unclear whether the C-terminal end of the protein, which carries an additional putative coiled-coil region, may contribute to such interactions. It was not possible to detect a protected C-terminal fragment in Western blots of protease-treated mitochondria or outer membrane vesicles, which would be expected if the C terminus would be in the intermembrane space, or even in the matrix (not shown).

One possibility is that Fzo1p itself plays a central role in the recognition of the partner organelle and/or fusion of lipid bilayers. The predicted coiled-coil regions might be the domains mediating such interactions, similar to pairing of cognate SNARE proteins on opposite membranes via coiled-coils. An intact GTPase domain has been shown to be essential for the function of the protein in Drosophila (18). Similar to dynamin GTPases, GTP hydrolysis could provide biomechanical energy that could be used for membrane fusion. Alternatively, Fzo1p could be a key regulator for mitochondrial fusion, as many GTPases play regulatory roles in diverse biological processes. Rab GTPases, for example, are important regulators for fusion events in the secretory pathway (34). The challenge for the future is to identify the components interacting with Fzo1p, and to determine the precise role of Fzo1p and each of its yet unknown partner proteins in mitochondrial membrane fusion.

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