The inner membrane protein Mdm33 controls mitochondrial morphology in yeast

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Mitochondrial distribution and morphology depend on MDM33, a Saccharomyces cerevisiae gene encoding a novel protein of the mitochondrial inner membrane. Cells lacking Mdm33 contain ring-shaped, mostly interconnected mitochondria, which are able to form large hollow spheres. On the ultrastructural level, these aberrant organelles display extremely elongated stretches of outer and inner membranes enclosing a very narrow matrix space. Dilated parts of Δmdm33 mitochondria contain well-developed cristae. Overexpression of Mdm33 leads to growth arrest, aggregation of mitochondria, and generation of aberrant inner membrane structures, including septa, inner membrane fragments, and loss of inner membrane cristae. The MDM33 gene is required for the formation of net-like mitochondria in mutants lacking components of the outer membrane fission machinery, and mitochondrial fusion is required for the formation of extended ring-like mitochondria in cells lacking the MDM33 gene. The Mdm33 protein assembles into an oligomeric complex in the inner membrane where it performs homotypic protein–protein interactions. Our results indicate that Mdm33 plays a distinct role in the mitochondrial inner membrane to control mitochondrial morphology. We propose that Mdm33 is involved in fission of the mitochondrial inner membrane.

Introduction

Mitochondria perform essential metabolic functions in virtually every eukaryotic cell (Scheffler, 2001). Each cell type contains mitochondria in characteristic copy number, distribution, and shape, often reflecting the energy requirements of the cell. As mitochondria cannot be synthesized de novo, they must be inherited upon cell division. Mitochondrial inheritance and maintenance of normal mitochondrial morphology depend on active transport of the organelles along cytoskeletal tracks and balanced membrane fusion and fission events (Bereiter-Hahn, 1990; Warren and Wickner, 1996; Yaffe, 1999; Griparic and van der Bliek, 2001; Westermann, 2002). Recent work has shown that mitochondrial dynamics plays an important role in cellular function and cell differentiation processes. For example, mitochondrial fusion is required for sperm development in Drosophila (Hales and Fuller, 1997). Mitochondrial fission is crucial for embryonic development in nematodes (Labrousse et al., 1999) and is involved in apoptosis in mammalian cells (Frank et al., 2001). Moreover, exchange of mitochondrial content by frequent fusion and fission is thought to be a defense mechanism against cellular aging (Ono et al., 2001).

Baker’s yeast, Saccharomyces cerevisiae, turned out to be a powerful model organism to study the molecular machinery mediating mitochondrial distribution and morphology (Hermann and Shaw, 1998; Jensen et al., 2000; Boldogh et al., 2001; Shaw and Nunnari, 2002). Yeast mitochondria form a branched tubular network located immediately below the cell cortex (Hoffmann and Avers, 1973; Egner et al., 2002). Recent work has shown that mitochondrial dynamics plays an important role in cellular function and cell differentiation processes. For example, mitochondrial fusion is required for sperm development in Drosophila (Hales and Fuller, 1997). Mitochondrial fission is crucial for embryonic development in nematodes (Labrousse et al., 1999) and is involved in apoptosis in mammalian cells (Frank et al., 2001). Moreover, exchange of mitochondrial content by frequent fusion and fission is thought to be a defense mechanism against cellular aging (Ono et al., 2001).

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consequence of aberrant mitochondrial morphology, fzo1 and ugo1 mutants fail to inherit mitochondrial DNA (mtDNA), resulting in a respiratory-deficient growth phenotype (Hermann et al., 1998; Rapaport et al., 1998; Sesaki and Jensen, 2001).

Division of the mitochondrial outer membrane in yeast depends on the dynamin-related GTPase Dnm1, which assembles on the surface of mitochondrial tubules at sites of organelle constriction and fission (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999). Dnm1 cooperates with the soluble protein Mdv1 (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001; Tieu et al., 2002) and the integral outer membrane protein Fis1 (Mozdy et al., 2000). Mutants lacking either one of these proteins exhibit long tubular mitochondria or closed planar nets of interconnected mitochondria, a phenotype indicative of a strong defect in mitochondrial division. Inheritance of mtDNA is not affected in these mutants. Mitochondrial fragmentation and the respiratory growth defect of mutants defective in fusion can be suppressed by deletion of components of the outer membrane division machinery (Bleazard et al., 1999; Sesaki and Jensen, 1999; Mozdy et al., 2000; Cerveny et al., 2002). Mitochondria are complex organelles bounded by two membranes. Thus, mechanisms must exist that coordinate fusion and fission events of the outer and inner membranes. The fusion machinery of the outer membrane is in contact with as yet unknown factors of the inner membrane, and this connection appears to be critical to coordinate fusion of four mitochondrial membranes (Fritz et al., 2001). A second mitochondrial dynamin-related protein, Mgm1, is located in the intermembrane space. In contrast to Dnm1, it is not clear whether Mgm1 is involved in membrane fission events. However, it has been suggested that Mgm1 is involved in remodeling events of the mitochondrial inner membrane (Wong et al., 2000). Other proteins that might be involved in fusion and fission of the inner membrane have not been described.

We recently conducted a systematic genome-wide screen to identify novel yeast genes important for mitochondrial distribution and morphology, MDM. One of the mutants isolated in this screen, mdm33, exhibits giant ring-like mitochondrial structures, a phenotype that has never been described before for any other yeast mutant (Dimmer et al., 2002). Here, we report on the functional characterization of Mdm33 and discuss a possible role of this protein in fission of the mitochondrial inner membrane.

Results

MDM33 encodes a novel 54-kD protein

The MDM33 gene (systematic name YDR393W) encodes a protein of 456 amino acid residues (~54 kD). The Mdm33 protein shares 19% amino acid identity with the predicted protein CaShe9 of Candida albicans (Andaluz et al., 2001) and 18% with SPAC823.13Cp of Schizosaccharomyces pombe (GenBank/EMBL/DDJB accession no. CAB90158) (for an alignment see Fig. 1 A). These three proteins have a similar domain structure. Hydropathy analysis (Hofmann and Stoffel, 1993) predicts two transmembrane segments, one after about two thirds of the protein and another one at the extreme COOH terminus (Fig. 1 A). The COILS algorithm (Lupas et al., 1991) predicts a high propensity of the protein to form coiled coils (Fig. 1 B).

Abbreviations used in this paper: cRFP, cytosolic red fluorescent protein; mtDNA, mitochondrial DNA; mtGFP, mitochondrial matrix–targeted GFP.

Figure 1. Mdm33 is a novel protein with predicted transmembrane segments and coiled-coil structures. (A) Comparison of the predicted amino acid sequences of Mdm33-related proteins. The amino acid sequences of Mdm33 of Saccharomyces cerevisiae (S.c.), CaShe9 of Candida albicans (C.a.), and SPAC823.13C of Schizosaccharomyces pombe (S.p.) were aligned using the DNAMAN software (Lynnon BioSoft). Amino acids that are identical in all three proteins are in black boxes, and gaps introduced to maximize the alignment are indicated by dashes. The positions of two predicted transmembrane segments (Hofmann and Stoffel, 1993), which are present in all three homologues, are indicated by gray boxes. (B) Plots of coiled-coil probabilities (Lupas et al., 1991) for the protein sequences shown in A. The window size used was 21. The positions of predicted transmembrane segments (TM1 and TM2) are indicated by arrows.

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Mdm33 is located in the mitochondrial inner membrane

The NH₂ terminus of Mdm33 resembles classical mitochondrial presequences (Hartl et al., 1989); it is rich in positively charged residues, lacks acidic amino acid residues, and has a high content of hydroxylated residues (Fig. 1A). Possible cleavage sites for the matrix processing peptidase (Hartl et al., 1989) are after residues 24 and 30. To examine whether the NH₂ terminus of Mdm33 functions as a presequence in vivo, we fused the first 41 amino acid residues to GFP and expressed the chimeric protein in yeast. Specific mitochondrial staining was observed by fluorescence microscopy (Fig. 2A), suggesting that Mdm33 is targeted to mitochondria.

We tested the import of Mdm33 into isolated mitochondria. Upon translation in vitro in the presence of [³⁵S]methionine, SDS-PAGE, and autoradiography, a prominent band with an apparent molecular mass of \( \approx 60 \) kD was observed (Fig. 2B, lane 1). The relatively slow running behavior of Mdm33 in SDS-PAGE might be explained by its rather basic nature (the isoelectric point is 9.36). Upon incubation with isolated mitochondria, Mdm33 was processed to a slightly smaller form (Fig. 2B, lane 2) and translocated to a protease-protected location (Fig. 2B, lane 3) in a membrane potential–dependent manner (Fig. 2B, lanes 6 and 7).

When the outer membrane was opened by osmotic swelling of mitochondria after import, a specific fragment was generated by protease treatment of mitoplasts (Fig. 2B, lane 5). The apparent molecular mass of this fragment corresponds well to the predicted size of a segment ranging from the processing site to the end of the first predicted transmembrane domain (34 kD), suggesting that this part is protected by the inner membrane in mitoplasts. The fragment was not generated in the absence of a membrane potential (Fig. 2B, lane 7), indicating that it was a derivative of imported Mdm33. As the imported protein was resistant to alkaline extraction (Fig. 2C), it is an integral part of the membrane. We conclude that Mdm33 is located in the mitochondrial inner membrane with its NH₂-terminal part in the matrix and its COOH-terminal part exposed to the intermembrane space.

To further investigate the intracellular localization, we expressed epitope-tagged versions of Mdm33 in a \( \Delta \text{mdm33} \) background, and Mge1, a soluble matrix protein. p, precursor; m, mature form of Mdm33. Mdm33 was imported into mitochondria as in B. Aliquots were either left untreated on ice (lane 2) or treated with trypsin (lane 3). Then, protease treatment was stopped, and trypsin-treated samples were extracted with carbonate \((\text{CO}_3^-)\) and separated into pellet (P; lane 4) and supernatant (SN; lane 5) fractions. All samples were precipitated with TCA and analyzed by SDS-PAGE and autoradiography. The fractionation into soluble and membrane proteins was controlled by immunoblotting using antisera against the ADP/ATP carrier (AAC), an integral protein of the inner membrane, and Mge1, p, precursor; m, mature form of Mdm33. (D) Immunocytochemistry of GFP–Mdm33. Yeast cells expressing a chimeric protein consisting of Mdm33 and GFP, inserted between the presequence and the mature part of Mdm33, were analyzed by immunoelectron microscopy using antibodies against GFP. Arrowheads point to inner membrane regions containing GFP–Mdm33. Bar, 100 nm.
background. A tagged version carrying 13 copies of the myc epitope fused to the COOH terminus of Mdm33 was exclusively detected in mitochondria upon subfractionation of cells (unpublished data). However, this construct was unable to rescue the mitochondrial morphology defect of the deletion mutant, suggesting that the COOH-terminal end of Mdm33 is important for function of the protein. Next, we constructed a chimeric protein carrying a GFP moiety inserted between the mitochondrial presequence and the NH₂-terminal end of mature Mdm33. This construct, GFP–Mdm33, rescued the mitochondrial defect of the deletion mutant (unpublished data). Immunoelectron microscopy demonstrated an association of GFP–Mdm33 with the inner membrane, both at the cristae and the inner boundary membrane, the part of the inner membrane that faces the outer membrane (Fig. 2 D). The fact that a fraction of GFP–Mdm33 was found in cristae suggests that Mdm33 does not form stable contacts to the outer membrane.

**Δmdm33 cells harbor aberrant mitochondria**

We examined the phenotype of the Δmdm33 mutant by various methods. Cells harboring mitochondrial matrix–targeted GFP (mtGFP) were grown to mid-logarithmic growth phase in liquid cultures containing different carbon sources and examined by fluorescence microscopy. Under all conditions, the majority of Δmdm33 cells harbored either large mitochondrial ring-like structures (Fig. 3 B) or two to four smaller interconnected ring-like mitochondria. (D) Double staining of mitochondria and cytosol. Δmdm33 cells coexpressing mtGFP and cRFP were grown to log phase in YPD medium and examined by confocal fluorescence microscopy. Left, bright field image taken in the transmission mode; right, merged confocal green and red fluorescence images. (E) Mitochondrial fusion. Δmdm33 cells of opposite mating types containing mitochondria preloaded with mtGFP or mtRFP were mated and analyzed by fluorescence microscopy. The distribution of mtGFP and mtRFP in a representative zygote containing a medial bud (asterisk) is shown. (F and I) Morphology of the ER. Wild-type and Δmdm33 cells expressing ER-targeted GFP were grown to log phase in glucose-containing synthetic minimal medium lacking methionine and examined by phase contrast and fluorescence microscopy. (G and J) Vacuolar morphology. Wild-type and Δmdm33 cells were grown to log phase in YPD medium, stained with 7-amino-4-chloromethylcoumarin, l-arginine amide to label vacuoles and examined by phase contrast and fluorescence microscopy. (H and K) Structure of the actin cytoskeleton. Wild-type and Δmdm33 cells were grown to log phase in YPD medium, fixed, stained with rhodamine-phalloidin to visualize filamentous actin, and examined by phase contrast and fluorescence microscopy.
ing different fluorescent matrix markers (Nunnari et al., 1997), the fluorescent labels immediately intermix in zy-
gotes, indicating that Mdm33 is not required for mitochon-
drial fusion (Fig. 3 E). Deletion of the MDM33 gene did
not affect the structure of other organelles, such as the ER
(Fig. 3 I), the vacuole (Fig. 3 J), or the actin cytoskeleton
(Fig. 3 K). We conclude that Mdm33 is specifically required
for maintenance of mitochondrial shape.

Aberrant ∆mdm33 mitochondria form hollow spheres,
interconnected sheets, and rings
To examine the three-dimensional organization of ∆mdm33
mitochondria, cells expressing mtGFP were analyzed by
confocal fluorescence microscopy. A representative ∆mdm33
cell containing a giant spherical organelle is depicted in Fig.
4. Large mitochondrial rings were observed in several con-
secutive focal planes (Fig. 4, A–K). Ring-shaped structures
similar to those seen in the x/y planes were observed also in
sections corresponding to z/y (Fig. 4 L) and z/x planes (Fig.
4 M). Apparently, this mitochondrion formed a large hollow
sphere of ~4 μm diameter. A view onto a surface-rendered
three-dimensional representation of this organelle is de-
picted in Fig. 4 O. Similar structures were observed in many
other ∆mdm33 cells. Often these organelles appeared to be
closed, sometimes they contained openings of varying sizes,
or they formed sheets typically located at one side of the cell
(see supplemental figures, Figs. S1–S6, available at http://
www.jcb.org/cgi/content/full/jcb.200211113/DC1).

Overexpression of Mdm33 leads to growth arrest
and aggregation of mitochondria
The MDM33 gene (alternate name SHE9; sensitive to high
expression) was previously found in a screen for genes that
cause growth arrest when highly overexpressed (Espinet et
al., 1995). We asked whether this growth arrest is concomi-
tant with changes of mitochondrial morphology. The
MDM33 coding sequence was placed under control of the
strong inducible GAL1 promoter in a multicopy plasmid
and transformed into wild-type and ∆mdm33 yeast strains.
Induction of the GAL1 promoter by change of the carbon

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<td></td>
<td>Wild type–like</td>
<td>Large rings</td>
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<td>MDM33/YPD</td>
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<td>–</td>
</tr>
<tr>
<td>MDM33/YPGal</td>
<td>99</td>
<td>–</td>
</tr>
<tr>
<td>MDM33/YPG</td>
<td>89</td>
<td>–</td>
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<td>52</td>
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source from glucose to galactose resulted in a severe reduction of growth in both strains (Fig. 5 A). The generation time in minimal medium containing galactose as carbon source was 3 h for both the wild-type and Δmdm33 strains carrying the empty vector. It increased to ~10 h for the strains overexpressing Mdm33 from the GAL1 promoter. Mitochondria were highly aggregated and/or fragmented in these cells (Fig. 5 B; Table II). A similar aggregation behavior was observed upon GAL1-induced overexpression of an Mdm33 fusion protein carrying a GFP moiety fused to its COOH terminus, Mdm33–GFP. Fluorescence microscopy indicated that mitochondrial targeting was not affected under these conditions (unpublished data). From the comparison of fluorescent signals of GAL1-overexpressed Mdm33–GFP and nonoverexpressed GFP–Mdm33, we estimate that GAL1-driven overexpression was at least 100-fold (unpublished data). We conclude that overexpression of Mdm33 severely affects mitochondrial morphology.

Mdm33 affects the internal structure of mitochondria

To investigate the role of Mdm33 in the organization of the mitochondrial membranes, wild-type cells (Fig. 6, A–C), Δmdm33 mutant cells (Fig. 6, D–F), and Mdm33-overexpressing cells (Fig. 6, G–J) were examined by electron microscopy. In some experiments, mtGFP was labeled with immunogold to unequivocally identify the mitochondrial matrix compartment. Sections of Δmdm33 cells often showed extremely elongated mitochondria that extended through half of the cell (Fig. 6 F). These organelles displayed very long stretches of mitochondrial double membranes that enclosed a very narrow matrix space (see immunogold label in Fig. 6, D and F). Typically, these structures were dilated at the ends where the inner membrane formed cristae (Fig. 6 F). It is conceivable that these mitochondria are similarly extended in the third dimension perpendicular to the cross section, and that they correspond to the large rings or elongated structures seen in the fluorescence microscope (see above).

Some images showed extended mitochondrial structures in close proximity originating from the same organelle. As Δmdm33 mitochondria are not defective in membrane fusion, these structures are able to fuse with each other (the arrow in Fig. 6 F points to a putative fusion site). Such fusion events would form mitochondrial rings (Fig. 6, D and E) that eventually might close also in the third dimension and form the hollow structures that were observed by confocal fluorescence microscopy (see above). We propose that Δmdm33 cells contain abnormally extended mitochondria that may form hollow spheres by fusion of mitochondrial membranes of the same organelle.

We examined the internal structure of mitochondria in cells overexpressing Mdm33 (Fig. 6, G–J). The most striking phenotype was the formation of inner membrane septa, membranous partitions separating the inner compartment into distinct chambers. Apparently, these partitions consisted of two parallel membranes in direct continuity with the inner membrane (Figs. 6, G and I, arrows). Furthermore, vesicular structures accumulated within the organelles, some of which appeared to be disconnected from the inner boundary membrane (Fig. 6, H and J). Some organelles were seen that were largely devoid of cristae (Fig. 6 H). All mitochondria were still bounded by a double membrane. We consider it likely that overexpression of Mdm33 initially results in the formation of inner membrane septa. With ongoing septation of the mitochondrial interior, smaller inner membrane fragments are generated that eventually become disconnected from the inner boundary membrane, leaving a largely cristae-free space. Our results dem-
The function of Mdm33 in mitochondrial morphogenesis may involve protein–protein interactions. In particular, the predicted coiled-coil domains could be responsible for formation of homo- and/or hetero-oligomers. We investigated whether Mdm33 assembles into a high molecular weight complex. In vitro–translated Mdm33 protein was imported into mitochondria. After completion of import, mitochondria were solubilized with detergent, and protein complexes were analyzed by gel filtration chromatography. Mdm33 was eluted from the column in fractions corresponding to a molecular weight of ~300 kD (Fig. 7 A), indicating assembly into an oligomeric complex.

To investigate protein–protein interactions involving Mdm33, we performed coimmunoprecipitation experiments. In vitro–translated precursor proteins were imported into wild-type mitochondria or mitochondria harboring GFP–Mdm33. After completion of import, mitochondria were lysed and subjected to coimmunoprecipitation with antibodies against GFP. We tested the interactions of GFP–Mdm33 with imported Mdm31, Mdm32, and Oxa1. Mdm31 and Mdm32 are two predicted inner membrane proteins that were shown to be essential for mitochondrial distribution and morphology. Mutants lacking these proteins, however, do not exhibit the mitochondrial ring structures seen in Δmdm33 mutants (Dimmer et al., 2002). Oxa1 is an inner membrane protein not related to mitochondrial morphogenesis and served as a negative control. Imported Mdm33 was

Table II. Quantification of mitochondrial morphology in Mdm33-overexpressing cells

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<tr>
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<th>Rings</th>
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<th>Aggregated/fragmented</th>
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Table III. Quantification of mitochondrial morphology in Δmdm33/Δfis1 cells

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specifically coprecipitated with GFP–Mdm33, indicating that Mdm33 performs homotypic protein–protein interactions (Fig. 7 B, lane 3). Mdm33 was not precipitated from mitochondria lacking the GFP tag (Fig. 7 B, lane 5), and no interactions of GFP–Mdm33 with Mdm31, Mdm32, and Oxa1 were found (Fig. 7 B).

Stable interactions of Mdm33 with outer membrane proteins known to be involved in mitochondrial morphogenesis

Figure 6. **Ultrastructure of mitochondria in Mdm33-deficient and Mdm33-overexpressing cells.** (A–C) Cross sections of mitochondria of wild-type cells. (D–F) Cross sections of mitochondria of Δmdm33 cells. N, nucleus. Arrowheads point to extended interconnected mitochondrial structures; the arrow points to a putative intramitochondrial fusion site. (G–J) Cross sections of mitochondria of Mdm33-overexpressing cells. Induction of GAL–MDM33 was as described for Fig. 5 B. Arrows point to inner membrane septa. (B, C, D, F, G, H and J) Immunogold labeling of mtGFP to identify the mitochondrial matrix compartment. It should be noted that the mitochondrial outer membrane appears relatively faint due to its low protein content. Bar, 500 nm.
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Discussion

Our studies establish an important function of Mdm33 in the regulation of mitochondrial morphology. Several lines of evidence point to a possible role of Mdm33 in fission of the inner membrane. First, the formation of ring-like and spherical mitochondria is unique for the Δmdm33 mutant. However, the phenotype of the Δmdm33 mutant bears some similarities to other yeast mutants known to be affected in mitochondrial fission, such as Δdnm1, Δmdv1, and Δfis1 (Otsuga et al., 1998; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Mutant cells contain only one or a few individual mitochondria per cell; the aberrant mitochondrial structures often remain interconnected and are located at only one side of the cell; and, in contrast to many other mitochondrial morphology mutants, mutants defective in mitochondrial fission do not lose mtDNA and do not acquire a respiratory-deficient growth phenotype.

Second, deletion of the MDM33 gene leads to the formation of large organelles that typically contain long stretches of outer and inner membranes enclosing a very narrow matrix space. These extremely extended structures can form only in the absence of frequent organellar division events. As mitochondrial fusion is not compromised in the Δmdm33 strain, elongated organelles likely undergo self-fusion when two ends of the same organelle approach one another. Such a self-fusion event initially would form a ring structure, and then a self-fusion event initially would form a ring structure. If Mdm33 is present both in the inner boundary membrane and in cristae (Fig. 2 D), it would be expected to be located exclusively in parts of the inner membrane that face the outer membrane. Moreover, genetic evidence suggests that Mdm33 function is not coupled to that of the outer membrane proteins Fis1 or Fzo1 (see above). Yet, we cannot exclude that Mdm33 interacts with other known or unknown proteins. However, the observed homotypic interactions of Mdm33 might explain why mdm33 was the only mutant displaying giant ring-shaped mitochondria in a comprehensive genome-wide screen for mitochondrial morphology mutants (Dimmer et al., 2002). Thus, no other known MDM component would be a likely interaction partner of Mdm33 based on its location, function, or phenotype. We suggest that Mdm33 performs homo-oligomeric interactions.

Table IV. Quantification of mitochondrial morphology in Δmdm33/Δfzo1 cells

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<td>Δmdm33/Δfzo1</td>
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Figure 7. Mdm33 assembles into higher molecular complexes. (A) Gel filtration of Mdm33. Mdm33 protein synthesized in vitro was imported into wild-type mitochondria as in Fig. 2 B. After completion of import, mitochondria (1 mg) were washed, reisolated, and solubilized in 300 μl buffer containing 0.5% Triton X-100. Nonsolubilized and aggregated material was separated by centrifugation, and the supernatant was loaded on a superose-6 column. After chromatography, the collected fractions were analyzed by SDS-PAGE, blotting to nitrocellulose, and autoradiography. Recovered Mdm33 was quantified by densitometry. Marker proteins thyroglobulin, apoferritin, alcohol dehydrogenase, and bovine serum albumin were used to calibrate the column. (B) Coimmunoprecipitation of Mdm33. Mdm33 translated in vitro was imported into mitochondria harboring GFP-Mdm33 or nontagged Mdm33 (WT). Mitochondria were washed, reisolated, lysed with Triton X-100, and subjected to coimmunoprecipitation with antibodies directed against GFP. In control reactions, two other putative inner membrane proteins involved in mitochondrial morphogenesis, Mdm31 and Mdm32, and a nonrelated inner membrane protein, Oxa1, were imported. Lane 1, [35S]methionine-labeled precursor proteins, corresponding to 10% of the amounts added to the import reactions. Lane 2, import reactions using GFP–Mdm33 mitochondria, corresponding to 10% of the amounts used for the coimmunoprecipitations. Lane 3, coimmunoprecipitations from GFP–Mdm33 mitochondria using anti-GFP antiserum. Lane 4, import reactions using wild-type mitochondria. Lane 5, coimmunoprecipitations from wild-type mitochondria using anti-GFP antiserum. p, precursor forms; m, mature forms of imported proteins.
Third, overexpression of Mdm33 results in septation and vesiculation of the inner membrane and disappearance of inner membrane cristae. This phenotype can be best explained by greatly enhanced inner membrane fission activity in the presence of excess Mdm33. In particular, the appearance of septated mitochondria induced by Mdm33 overexpression bears striking similarity to dividing mitochondria in various tissues described in the literature (e.g., Tandler et al., 1969; Larsen, 1970; Tandler and Hoppel, 1972; Wakabayashi et al., 1974; Duncan and Greenaway, 1981; for review see Griparic and van der Bliek, 2001). Partitioning of mitochondria is likely due to enhanced fission activity of the inner membrane in the absence of division of the outer membrane.

Fourth, Mdm33 is an integral protein of the inner membrane and has extensive coiled-coil domains exposed to the matrix side. This topology and the observed homotypic protein–protein interactions are in agreement with the following possible mode of action. Mdm33 protein complexes on the mitochondrial outer and inner membranes persisted in the absence of division of the outer membrane.

Maintenance of normal mitochondrial morphology by balanced membrane fission and fusion depends on several distinct, yet interdependent, processes. Based on the results reported herein and in previous studies by others, we propose a hypothetical cycle of sequential reactions of inner membrane fission, outer membrane fission, and mitochondrial fusion (Fig. 8). The first step of the cycle is inner membrane fission. Division of the mitochondrial outer and inner membranes can be uncoupled. Mitochondria of worms expressing dominant interfering mutants of DRP-1, the Caenorhabditis elegans homologue of yeast Dnm1, had swollen mitochondria with irregular shapes and sizes. Intriguingly, these mitochondria contained separated matrix compartments that were still connected by thin tubules of outer membrane. Thus, inner membrane fission persists, even when outer membrane fission is blocked by loss of DRP-1 function (Labrousse et al., 1999). An independent machinery appears to mediate fission of the mitochondrial inner membrane. Mdm33 is likely to play an important role to control this process in yeast.

The next step of the cycle is fission of the outer membrane by Dnm1 and its cofactors, Fis1 and Mdv1. Membrane pinching by dynamin-like proteins requires a considerable constriction of the organelle before a dynamin ring can assemble (Labrousse et al., 1999). In the case of mitochondria, constriction of the outer membrane may be facilitated by severing of the inner membrane. Consistent with this idea, the Δmdm33 mutation was found to be epistatic to Δfis. Thus, the outer membrane fission machinery is not required for the formation of extended mitochondria in Δmdm33 cells. Conversely, Mdm33 is required to form the net structures in Δfis1 mutants. Thus, Mdm33 acts upstream of the outer membrane fission machinery.

The cycle is completed by mitochondrial fusion. In yeast, mutations leading to deficiency in outer membrane fission are epistatic to mutations leading to deficiency in fusion, i.e., mitochondrial fragmentation and loss of mtDNA in fusion mutants is blocked by mutation of components of the outer membrane fission machinery (Bleazard et al., 1999; Sesaki and Jensen, 1999, 2001; Fekkes et al., 2000; Mozdzy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). On the other hand, mitochondrial fusion is a prerequisite for the formation of extended Δmdm33 mitochondria, as we found that Δfzo1 is epistatic to Δmdm33. Why does deletion of the MDM33 gene not prevent fragmentation of mitochondria in Δfzo1 cells, similar to deletion of genes encoding components of the outer membrane fission machinery? Interestingly, Sesaki and Jensen (1999) observed that mitochondrial fragments persisted in ~40% of the cells when cells were first disrupted for FZO1 and subsequently for DNM1. This suggests that once the equilibrium of fission and fusion has been shifted in the direction of fission, it is difficult to rebuild larger mitochondrial structures without fusion activity. In case of the Δmdm33/Δfzo1 strain, the action of Dnm1 and its cofactors might well be sufficient to induce and maintain fragmentation of mitochondria, even in the absence of Mdm33.

Abnormal ring-shaped or cup-shaped mitochondria in various animal and human tissues have been described. These include, for example, parathyroid gland cells of senile dogs (Setoguti, 1977), renal oncocyes in rats (Krech et al., 1981), avian retinal pigment epithelium cells under continuous light (Lauber, 1982), Purkinje cells of the rat cerebellar cortex after prolonged alcohol consumption (Tavares and Paula-Barbosa, 1983), and human salivary tumor cells (Katoaka et al., 1991). It will be interesting to see in the future whether there is a link between defects of mitochondrial division and various pathological conditions, and whether proteins similar to Mdm33 are involved.

**Materials and methods**

**Plasmid constructions**

Standard methods were used for cloning procedures (Sambrook et al., 1989). PCR was performed using Pfu polymerase (Stratagene) according to the manufacturer’s instructions. The plasmid for expression of GFP fused to the presequence of Mdm33 in yeast, pYES-Mdm33(1–41)–GFP, was generated by PCR amplification of the first 123 bp of the MDM33 gene from genomic DNA.
yeast DNA using the primers 5′-GGG AAG CTT ATG TGG AGA TAT CAC GGC TGG-3′ and 5′-GGG GGA TCC ACC GCT TAT CTT TAG AGA ATG AGC AC-3′ and cloning into the HindIII/BamHI sites of vector pYES-mtGFP (Westermann and Neupert, 2000). The plasmid for in vitro transcription of Mdm33, pMM106-1, was generated by PCR amplification of the Mdm33 coding region from genomic yeast DNA using the primers 5′-AAA GAG CTC ACC ATG TCT TTT ACC ACG C-3′ and 5′-AAA AAG CTT TCA GCC ACC AAA CCA CC-3′ and ligation into the pGEM-T vector (Promega) according to the manufacturer’s instructions. The plasmid for in vitro transcription of Mdm32, pKD531.1, was generated by PCR amplification of the Mdm32 coding region from genomic yeast DNA using the primers 5′-AAA GAG CTC ACC ATG TCT TTT ACC ACG C-3′ and 5′-TTT GGA TCA TTA AAC GGT AGT AGC AG-3′, cloned with SacI/BamHI; (2) yeast enhanced GFP encoding region amplified from pUG14 (GenBank/EMBL/DDBJ accession no. AF298784); (3) yeast 5′-AAA AAG CTC ACC ATG TAT TCT TTA TTT ACC ACG C-3′ and 5′-TT TTT AAG CTT TTA GTA ATG AGC AGC ACC CCC GGA TAC C-3′ and ligation into the pPCR-script Amp SK(+)-vector (Stratagene) according to the manufacturer’s instructions. For construction of the plasmid expressing GFP–Mdm33 under control of the Mdm33 regulatory sequences, pMM112, the following three DNA fragments were PCR amplified and successively cloned into vector pRS316 (Sikorski and Hieter, 1989): (1) Mdm33 promoter and Mdm33 presequence encoding region amplified from genomic yeast DNA using the primers 5′-AAA GAG CTC CAA TTC TTT ATG GTG GAT GTG C-3′ and 5′-TTT GGA TCA TTA AAC GGT AGT AGC AG-3′, cloned with SacI/BamHI; (2) yeast enhanced GFP encoding region amplified from pUG14 (GenBank/EMBL/DDBJ accession no. 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