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). The continuity of the mitochondrial reticulum is maintained by continuous membrane fusion and fission events (Nunnari et al., 1997; Bleazard et al., 1999; Sesaki and Jensen, 1999). Two proteins of the outer membrane, Fzo1 and Ugo1, were found to be involved in the fusion of mitochondria. Yeast mutants lacking either one of these proteins exhibit fragmented mitochondria because fusion is blocked in the presence of ongoing fission. As a secondary
consequence of aberrant mitochondrial morphology, \textit{fzo1} and \textit{ugo1} mutants fail to inherit mitochondrial DNA (mtDNA),\footnote{Abbreviations used in this paper: cRFP, cytosolic red fluorescent protein; mtDNA, mitochondrial DNA; mtGFP, mitochondrial matrix–targeted GFP.} 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; Tieu and Nunnari, 2000; Cerveny et al., 2001; Sesaki and Jensen, 2001).

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, \textit{MDM}. One of the mutants isolated in this screen, \textit{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**

\textit{MDM33} encodes a novel 54-kD protein

The \textit{MDM33} gene (systematic name \textit{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 \textit{Candida albicans} (Andaluz et al., 2001) and 18\% with SPAC823.13Cp of \textit{Schizosaccharomyces pombe} (GenBank/EMBL/DDBJ 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).
Mdm33 is located in the mitochondrial inner membrane

The NH$_2$ 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. 1 A). Possible cleavage sites for the matrix processing peptidase (Hartl et al., 1989) are after residues 24 and 30. To examine whether the NH$_2$ 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. 2 A), 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 ~60 kD was observed (Fig. 2 B, 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. 2 B, lane 2) and translocated to a protease-protected location (Fig. 2 B, lane 3) in a membrane potential–dependent manner (Fig. 2 B, 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. 2 B, 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. 2 B, lane 7), indicating that it was a derivative of imported Mdm33. As the imported protein was resistant to alkaline extraction (Fig. 2 C), it is an integral part of the membrane. We conclude that Mdm33 is located in the mitochondrial inner membrane with its NH$_2$-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 Δmdm33 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 (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 Dld1, an integral protein of the inner membrane, and Mge1, p, precursor; m, mature form of Mdm33. Mdm33 controls mitochondrial morphology | Messerschmidt et al. 555

space, 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 (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 mitochondrial rings that were often interconnected (Fig. 3 C). The remainder of the cells displayed either single elongated structures or aggregated or fragmented mitochondria. Branched tubular networks resembling wild-type cells (Fig. 3 A) could never be observed in Δmdm33 cells. A quantification of mitochondrial morphology of Δmdm33 cells and its isogenic wild type is presented in Table I. Confocal fluorescence microscopy of Δmdm33 cells coexpressing mtGFP and cytosolic red fluorescent protein (cRFP) suggested that the interior of mitochondrial rings contained cytosolic proteins (Fig. 3 D). The distribution of mtGFP and mtRFP in a representative zygote containing a medial bud (asterisk) is shown. (E 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 intermixed in zygotes, indicating that Mdm33 is not required for mitochondrial 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 Δmdm33 mitochondria form hollow spheres, interconnected sheets, and rings**
To examine the three-dimensional organization of Δmdm33 mitochondria, cells expressing mtGFP were analyzed by confocal fluorescence microscopy. A representative Δmdm33 cell containing a giant spherical organelle is depicted in Fig. 4. Large mitochondrial rings were observed in several consecutive 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 depicted in Fig. 4 O. Similar structures were observed in many other Δmdm33 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 concomitant 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 Δmdm33 yeast strains. Induction of the GAL1 promoter by change of the carbon source of medium (YPD→YPGal→YPG) strongly altered the mitochondrial morphology (Table I).

**Table I. Quantification of mitochondrial morphology in Δmdm33 cells**

| Strain/medium | Mitochondrial morphology (% of cells) | No. of cells scored |
|---------------|---------------------------------------|---------------------|
|               | Wild type–like | Large rings | Small rings | Elongated/tubules | Aggregated/fragmented | |
| MDM33/YPD     | 97            | –          | –          | 1                | 2                     | 394 |
| MDM33/YPGal   | 99            | –          | –          | –                | 1                     | 281 |
| MDM33/YPG     | 89            | –          | –          | –                | 11                    | 109 |
| Δmdm33/YPD    | –            | 41         | 39         | 13               | 7                     | 433 |
| Δmdm33/YPGal  | –            | 40         | 41         | 10               | 9                     | 242 |
| Δmgm33/YPG    | –            | 52         | 17         | 5                | 26                    | 104 |

**Figure 4. Mitochondrial sphere in a Δmdm33 mutant cell.** Δmdm33 cells expressing mtGFP were grown to log phase in YPGal medium. A representative cell was analyzed by confocal fluorescence microscopy. (A–K) Single optical planes (x/y); distance between serial planes is 285 nm. (L) Section corresponding to a z/y plane. (M) Section corresponding to a z/x plane. (N) Bright field image taken in the transmission mode. (O) Surface-rendered three-dimensional representation. This image has been turned 90° counter clockwise when compared with panels A–K.
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 $\Delta mdm33$ strains carrying the empty vector. It increased to $\sim 10$ 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), $\Delta mdm33$ 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 $\Delta mdm33$ 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 $\Delta mdm33$ 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 $\Delta mdm33$ 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). 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 seption 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-
onstrate an important role of Mdm33 in maintenance of the structure of the mitochondrial inner membrane.

Epistatic relationships of Δmdm33, Δfis1, and Δfzo1 mutations

Establishment and maintenance of a tubular mitochondrial network depends on antagonistic fission and fusion events (Nunnari et al., 1997). Block of mitochondrial fission by deletion of any one of the DNMI, MDV1, or FIS1 genes leads to the formation of long tubular mitochondria or closed mitochondrial nets (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999; Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001), whereas block of mitochondrial fusion by deletion of either one of the FZO1 or UGO1 genes leads to highly fragmented mitochondria (Hermann et al., 1998; Rapaport et al., 1998; Sesaki and Jensen, 2001). To investigate whether Mdm33 is involved in these processes, we constructed Δmdm33/Δfis1 and Δmdm33/Δfzo1 double mutants by genetic crosses and examined their phenotypes.

The Δmdm33/Δfis1 double mutant was viable on nonfermentable carbon sources (unpublished data). Mitochondrial morphology was very similar to the Δmdm33 parent, and clearly different from Δfis1. Most of the Δmdm33/Δfis1 cells displayed mitochondrial ring structures, whereas the elongated net-like mitochondria of Δfis1 cells (Mozdy et al., 2000) could never be found in the double mutant (Table III). Thus, the Δmdm33 mitochondrial morphology defect is epistatic to the Δfis1 mitochondrial morphology defect. Similarly, overexpression of Mdm33 in Δfis1 cells induced the same growth arrest and mitochondrial aggregation phenotypes that were observed in a FIS1 wild-type background (unpublished data). These results demonstrate that Mdm33 acts upstream of Fis1.

The Δmdm33/Δfzo1 double mutant is inviable on nonfermentable carbon sources (unpublished data), as is the Δfzo1 single mutant (Hermann et al., 1998; Rapaport et al., 1998). Mitochondrial morphology was indiscernible from the Δfzo1 parent. Most of the Δmdm33/Δfzo1 cells displayed highly fragmented mitochondria, whereas the characteristic ring-shaped mitochondria of Δmdm33 cells could not be found in the double mutant (Table IV). Thus, the Δfzo1 mitochondrial morphology defect is epistatic to the Δmdm33 mitochondrial morphology defect, implying that Fzo1 is required to form the extended mitochondrial structures seen in the Δmdm33 mutant. This genetic finding is consistent with our morphological observations, which suggest the formation of mitochondrial rings and spheres by self-fusion of the organelles in Δmdm33 cells.

Mdm33 is part of a high molecular weight complex with homo-oligomeric interactions

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 Mdm33, 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

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

| Strain                  | Mitochondrial morphology (%) of cells | No. of cells scored |
|-------------------------|---------------------------------------|---------------------|
|                         | Wild type–like | Rings | Elongated/tubules | Aggregated/fragmented |                  |
| MDM33                  | 98               | –     | –                 | 2                   | 104             |
| Δmdm33                 | –                 | 84    | 10                | 6                   | 101             |
| MDM33[GAL–MDM33]       | 16               | –     | 5                 | 79                  | 109             |
| Δmdm33[GAL–MDM33]      | 2                | 11    | 5                 | 82                  | 102             |

### Table III. Quantification of mitochondrial morphology in Δmdm33/Δfis1 cells

| Strain            | Mitochondrial morphology (%) of cells | No. of cells scored |
|-------------------|---------------------------------------|---------------------|
|                   | Wild type–like | Large rings | Small rings | Elongated/tubules | Elongated net-like | Aggregated/fragmented |                  |
| MDM33/FIS1        | 95               | –          | –          | –                 | –                 | –                 | 5                 | 114             |
| MDM33/Δfis1       | –                 | –          | 1          | 9                 | 84                | 6                 | 108               |
| Δmdm33/ΔFIS1      | –                 | 29         | 41         | 17                | –                 | 13                | 115               |
| Δmdm33/Δfis1      | –                 | 17         | 44         | 22                | –                 | 17                | 115               |
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...
Mdm33 controls mitochondrial morphology | Messerschmitt et al. 561

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

| Strain       | Mitochondrial morphology (% of cells) | No. of cells scored |
|--------------|---------------------------------------|--------------------|
|              | Wild type-like | Large rings | Small rings | Elongated/tubules | Fragmented | Aggregated |                |
| MDM33/FZO1   | 98           | –           | –           | –               | 2          | –         | 146             |
| MDM33Δfzo1   | –            | –           | –           | –               | 83         | 17        | 103             |
| Δmdm33/FZO1  | –            | 43          | 43          | 13              | 1          | –         | 126             |
| Δmdm33Δfzo1  | –            | –           | <1          | –               | 78         | 22        | 218             |

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.

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 this is indeed observed by electron microscopy. The enclosed spheres seen by confocal microscopy are probably generated when two-dimensional rings subsequently close also in the third dimension.
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 apposing inner membranes would interact with each other from the matrix side, possibly by forming α-helical bundles via Mdm33 coiled coils. Formation of α-helical bundles would then mediate constriction and/or fission of the inner membrane from the matrix side.

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 Δfis1. 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; Mozdy 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 Δmdm33Δfzo1 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 oncocytes 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 (Kataoka 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.
yeast DNA using the primers 5’-GGG AAG CTT ATG TGG AGA TAC TAT GGG GGC-3’ and 5’-GGG GGA TCC ACC GTT CAT TAG AGA ATG TGA 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 CCT ATG TAC TGC AGC C-3’ and 3’-TTT GGA TCC AGA ATA TGA ACT ATA ATG AAA TGG-3’, cloned with SacI/BamHI; (2) yeast enhanced GFP encoding region amplified from pUG34 (GenBank/EMBL/DDBJ accession no. AF298784) using primers 5’-AAA CTT TTT GTACAA TTC ATC CAT ACC AT-3’ and 5’-AAA AAG CTT TCA AGC ACC AAC 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, pKS321.1, was generated by PCR amplification of the Mdm32 coding region from genomic yeast DNA using the primers 5’-AAA GAG TCC ATG TCT AAA GGT GAA GAA TTA TTC-3’ and 5’-AAA CCA ATG TTG AGA TAC TAT CCA AAG TAC TCT C-3’, cloned with HindIII/HindIII; and (3) Mdm33 mature part encoding region and Mdm33 terminator amplified from genomic yeast DNA using primers 5’-AAA AAG CTT TCA ACT ATG AAA TGG-3’, cloned with BamHI/HindIII; and (4) Mdm33 mature part encoding region and Mdm33 terminator amplified from genomic yeast DNA using primers 5’-AAA CCT ATG TAC TGC AGC C-3’ and 3’-TTT GGA TCC AGA ATA TGA ACT ATA ATG AAA TGG-3’, cloned with SacI/BamHI; (2) yeast enhanced GFP encoding region amplified from pUG34 (GenBank/EMBL/DDBJ accession no. AF298784) using primers 5’-AAA GAG TCC ATG TCT AAA GGT GAA GAA TTA TTC-3’ and 5’-AAA AAG CTT TCA AGC ACC AAC CCA CC-3’ and ligation into the pGEM-T vector (Promega) 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 TCT CTC AAT TTC ATG ATG GTC GAT C-3’ and 5’-TTT GGA TCA ATG TTA GGA GAA GAA TTA TTC-3’, cloned with SacI/BamHI; (2) yeast enhanced GFP encoding region amplified from pUG34 (GenBank/EMBL/DDBJ accession no. AF298784) using primers 5’-AAA GAG TCC ATG TCT AAA GGT GAA GAA TTA TTC-3’ and 5’-AAA AAG CTT TCA AGC ACC AAC CCA CC-3’ and ligation into the pGEM-T vector (Promega) according to the manufacturer’s instructions. Yeast strain constructions Standard methods were used for growth and manipulation of yeast strains (Sherman et al., 1986; Gietz et al., 1992). Growth of yeast was always at 30°C. All strains used for microscopy and genetic studies were isogenic to BY4741, BY4742, and BY4743 (Brachmann et al., 1998). Haploid deletion strains Δmdm33, Δfis1, and Δzro1 were obtained from EUROSCARF (Frankfurt, Germany). Double mutants Δmdm33/Δfis1 and Δmdm33/Δzro1 were generated by mating of haploid strains, sporulation, and tetrad dissection. Genotypes of haploid progeny were determined by PCR. For overexpression of Mdm33, yeast strains were transformed with pYX223-Mdm33 and grown on galactose-containing medium lacking histidine to select for the overexpressing plasmid. For expression of GFP–Mdm33 in yeast, pMM112 was transformed into Δmdm33. For visualization of mitochondria, yeast strains were transformed with plasmid pVT10U-mtGFP, pYX113-mtGFP, pYX122-mtGFP, pYX142-mtGFP (Westermann and Neupert, 2000), or pRS416-GAL1-p+PrG-ATP9-RFP (Mozydly et al., 2000) encoding the red fluorescent protein, DsRed, into vector pVT10U-2 (Vernet et al., 1987). The plasmid for expression of cRFP, pVT102U-cRFP, was generated by cloning a BamHI/Xhol fragment of pRS424-ADH1-pF-ATP9-RFP (Mozydly et al., 2000) encoding the red fluorescent protein, DsRed, into vector pVT102U (Vernet et al., 1987). Yeast strains transformed with plasmid pVT100U-mtGFP, pYX113-mtGFP, pYX122-mtGFP, pYX142-mtGFP (Westermann and Neupert, 2000), or pRS416-GAL1-p1F-ATP9-RFP (Mozydly et al., 2000) for visualization of ER, yeast strains were transformed with pVT1055 (Prinz et al., 2000), and transformants were grown in the absence of methionine to induce the MET25 promoter for expression of ER-targeted GFP. For visualization of the cytosol, yeast strains were transformed with pVT102U-cRFP. Microscopy Yeast cultures were grown in liquid YPD medium (yeast extract, peptone, dextrose) to mid-logarithmic growth phase, if not indicated otherwise. Epifluorescence microscopy was according to standard procedures (Westermann and Neupert, 2000). Quantification of mitochondrial morphology defects was performed without prior reference to strain identity. Confocal images were taken with a Leica TCS SP2 beam scanning confocal microscope equipped with a 1.4 NA oil immersion lens (Leica 100X; Planapo). For imaging, living cells were embedded in 1% low melting point agarose. Electric microscopy and immunocytochemistry were performed as previously described (Kargel et al., 1996). In brief, yeast cells were harvested by centrifugation and fixed for 1 h with 4% formaldehyde and 0.5% glutaraldehyde under culture conditions (pH and temperature were kept constant), cryoprotected by a mixture of 25% polyvinylpyrrolidone (PVP K15; M 10,000; Fluka) and 1.6 M sucrose for 3 h, and frozen in liquid nitrogen. Ultrathin cryosections were prepared with glass knives and transferred to formvar-carbon–coated copper grids using the cryoprotectant mixture. Labeling with anti-GFP IgG primary antibodies and secondary antibody–gold complexes (10 nm; Dianova) was performed as described (Kargel et al., 1996). Finally, the sections were stained and stabilized by a freshly prepared mixture of 3% turgosticic acid hydrate (Fluka) and 2.5% polyvinyl alcohol (M 10,000; Sigma-Aldrich).

Assay of mitochondrial fusion in vivo Mitochondrial fusion was examined in vivo essentially as previously described (Nunnari et al., 1997; Mozydly et al., 2000) with some minor modifications. Cells of opposite mating types harboring plasmids encoding mtGFP or mtRFP under control of the GAL1 promoter were precultured in synthetic raffinose-containing medium under selection for the plasmids. Then, cells were grown to log phase in synthetic medium containing raffinose and galactose to induce expression of the fluorescent proteins. Then, yeast cells were incubated for 2 h in YPD medium (pH adjusted to 3.5) to shut off expression of the fluorescent proteins and to synchronize the cell cycle. Cultures were mixed, cells were transferred to nitrocellulose, placed for 3 h on YPD plates (pH 4.5) to allow mating, resuspended in water, and analyzed by fluorescence microscopy.

Miscellaneous Gel filtration (Rapaport et al., 1998) and coimmunoprecipitation (Herrmann et al., 2001) were performed according to published procedures. Staining of the actin cytoskeleton with rhodamine-phalloidin (Molecular Probes) was performed as previously described (Amberg, 1998). Staining of the vacuole with 7-amino-4-chloromethylcoumarin, 1-arginine amide (Molecular Probes) was performed according to the manufacturer’s instructions. Online supplemental material The supplemental figures (Figs. S1–S6) are available online at http://wlcb.org/cgi/content/full/wlcb.200211113/DC1. Mitochondrial morphology of wild-type and Δmdm33 cells expressing mtGFP was analyzed by confocal fluorescence microscopy as in Fig. 4.

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