Mitochondria constantly fuse and divide to adapt organellar morphology to the cell’s ever-changing physiological conditions. Little is known about the molecular mechanisms regulating mitochondrial dynamics. F-box proteins are subunits of both Skp1-Cullin-F-box (SCF) ubiquitin ligases and non-SCF complexes that regulate a large number of cellular processes. Here, we analyzed the roles of two yeast F-box proteins, Mfb1 and Mdm30, in mitochondrial dynamics. Mfb1 is a novel mitochondria-associated F-box protein. Mitochondria in mutants lacking Mfb1 are fusion competent, but they form aberrant aggregates of interconnected tubules. In contrast, mitochondria in mutants lacking Mdm30 are highly fragmented due to a defect in mitochondrial fusion. Fragmented mitochondria are docked but nonfused in an mdm30 cells. Mitochondrial fusion is also blocked during sporulation of homoygous diploid mutants lacking Mdm30, leading to a mitochondrial inheritance defect in ascospores. Mfb1 and Mdm30 exert nonredundant functions and likely have different target proteins. Because defects in F-box protein mutants could not be mimicked by depletion of SCF complex and proteasome core subunits, additional yet unknown factors are likely involved in regulating mitochondrial dynamics. We propose that mitochondria-associated F-box proteins Mfb1 and Mdm30 are key components of a complex machinery that regulates mitochondrial dynamics throughout yeast’s entire life cycle.

INTRODUCTION

Mitochondria carry out a variety of different functions in eukaryotic cells. They supply the cell with ATP generated by oxidative phosphorylation, they are the site of many anaerobic and catabolic pathways, they play an essential role in the assembly of Fe/S clusters, and they are key regulators of programmed cell death (Scheffler, 2000; Lill and Mullenhoff, 2005; Youle and Karowski, 2005). Depending on the cell’s physiological conditions, mitochondria frequently change their shape and intracellular position (Bereiter-Hahn, 1990; Yaffe, 1999; Griparic and van der Bliek, 2001; Westermann et al., 2002; Mozdy and Shaw, 2003; Scott et al., 2003; Chen and Chan, 2004). This dynamic behavior is important for a number of cellular processes. For example, mitochondrial fusion is required for sperm development in Drosophila (Hales and Fuller, 1997), for embryonic development in mice (Chen et al., 2003), and for maintenance of mitochondrial DNA in yeast (Chen and Butow, 2005). Conversely, mitochondrial division plays a crucial and evolutionarily highly conserved role in the apoptotic pathway (Jagasia et al., 2005; Youle and Karowski, 2005). Although several proteins mediating mitochondrial motility, fusion and fission have been identified during the past several years (Okamoto and Shaw, 2005), little is known about regulatory components of mitochondrial dynamics.

The core machinery mediating mitochondrial membrane fusion in yeast consists of three proteins, namely, Fzo1, a conserved GTPase in the outer membrane (Hermann et al., 1998; Rapaport et al., 1998); the outer membrane protein Ugo1 (Sesaki and Jensen, 2001); and Mgm1, a conserved GTPase located in the intermembrane space (Wong et al., 2000). Ugo1 physically interacts with Fzo1 and Mgm1 and presumably coordinates their function (Wong et al., 2003; Sesaki and Jensen, 2004). Genetic and morphological evidence suggests that the mitochondrial fusion and fission machineries act antagonistically and operate in a perfectly balanced manner. Defects in cells lacking components of the fusion machinery can be relieved by deletion of genes encoding proteins of the fission machinery (Bleazard et al., 1999; Sesaki and Jensen, 1999). Wild-type cells growing logarithmically on glucose-containing medium show tightly balanced mitochondrial fusion and fission activities at a rate of 1.7 fusion and fission events per minute and cell (Jakobs et al., 2003). Thus, fusion and fission must be strictly coordinated to maintain a reticular mitochondrial network. A regulatory role in mitochondrial fusion has been proposed for Pcp1, a rhomboid-like protease in the mitochondrial inner membrane. Pcp1 generates two isoforms of Mgm1 by site-specific processing (Herlan et al., 2003; McQuibban et al., 2003; Sesaki et al., 2003). It has been suggested that Pcp1...
dependent processing of Mgm1 adapts mitochondrial fusion activity to the ATP requirements of the cell (Herlan et al., 2004). F-box proteins are particularly versatile regulators of a variety of cellular functions (Willems et al., 2004; Petroski and Deshaies, 2005). Typically, they serve as substrate adaptors in Skp1-Cullin-F-box (SCF) E3 ubiquitin ligases, where they are required to target proteins to ubiquitylation and degradation by the 26S proteasome. However, some F-box proteins may also function independently of the SCF complex and the 26S proteasome (Galan et al., 2001). In budding yeast, at least 21 proteins contain a discernible F-box motif (Willems et al., 2004). One of these proteins, Mdm30, is required for maintenance of fusion-competent mitochondria (Fritz et al., 2003). A subfraction of Mdm30 is associated with mitochondria (Fritz et al., 2003; Sickmann et al., 2003), and cells lacking Mdm30 contain aggregated or fragmented mitochondria, tend to lose their mitochondrial DNA, and fail to fuse mitochondria in vivo. The steady-state level of Fzo1 is dependent on the cellular level of Mdm30, suggesting that Mdm30 regulates mitochondrial fusion by directly or indirectly stimulating degradation of Fzo1 (Fritz et al., 2003). However, recent results by Neutzner and Youle (2005) suggest that the situation may be more complex. These authors showed that treatment of yeast cells with the mating pheromone alpha factor evokes a fragmentation of the mitochondrial network into small pieces. Mitochondrial fragmentation is accompanied by proteasome-dependent degradation of Fzo1 but does not require Mdm30 (Neutzner and Youle, 2005; Escobar-Henriques et al., 2006). These results point to the existence of multiple pathways for the regulation of the mitochondrial fusion machinery. To gain more insights into the role of F-box proteins in the regulation of mitochondrial behavior, we analyzed the functions of Mdm30 and Mfb1, a novel mitochondria-associated F-box protein, in vegetatively growing cells and during mating and sporulation.

**MATERIALS AND METHODS**

**Plasmid Constructions**

Standard methods were used for cloning procedures. The plasmid for expression of Mfb1 from the GALI promoter in yeast, pYES-MFB1, was generated by
been described previously (Fritz et al., 2000). Plasmid pYES-BamHI sites of vector pYES2.0 (Invitrogen, Groningen, The Netherlands).

ATG ACA TTA TTT TCT TGT AGT GTA C-3
/H11032

TAT ATT AAA ATC GGT ATT AGC G-3

ATG ACA TTA TTT TCT TGT AGT GTA C-3
/H11032

MFB1

MTD30

MFB1

MTD30

MFB1

GFP

N-terminal amino acids (pJDCEX2-MFB1-ΔF-box) was constructed the same way using forward primer 5'-AAA AAG ATC TGT TAT CGG CTC AAA GGA TAT G-3'.

PCR amplification of the mitochondria-associated F-box protein 1 (MFB1) coding region from genomic yeast DNA using primers 5'-AAA AAG ATC TTA TTT TCT TGT AGT GTA C-3' and 5'-AAA GGA TAT G-3' targeted GFP have been described previously (Dimmer et al., 2003). Plasmids pVT100U-mtGFP, pYX113-mtGFP (Westermann and Neupert, 2000), pRS416-Plasmid pYES-MFB1

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Strain/medium & Wild-type-like & Aggregated & Fragmented & No. of cells scored \\
\hline
YPD & WT & 91.1 & 5.1 & 3.8 & 315 \\
& \Delta mfb1 & 9.4 & 84.7 & 5.9 & 374 \\
& \Delta mfb1/\Delta mdm30 & 10.4 & 88.4 & 1.2 & 346 \\
& \Delta mfb1/\Delta mdm30 & 1.8 & 83.7 & 14.5 & 339 \\
YPG & WT & 84.6 & 0.9 & 14.5 & 331 \\
& \Delta mfb1 & 39.2 & 2.9 & 57.9 & 339 \\
& \Delta mdm30 & 3.7 & 0.6 & 95.7 & 350 \\
& \Delta mfb1/\Delta mdm30 & 1.3 & 1.3 & 97.4 & 312 \\
YPGal & WT/pYES & 90.8 & 3.2 & 6.0 & 532 \\
& WT/pYES-MFB1 & 97.5 & 2.5 & 0.0 & 305 \\
& WT/pYES-MDM30 & 85.6 & 6.6 & 7.8 & 408 \\
& \Delta mfb1/pYES-MFB1 & 82.8 & 10.4 & 7.7 & 412 \\
& \Delta mdm30/pYES-MDM30 & 84.8 & 7.6 & 7.6 & 422 \\
& \Delta mfb1/pYES-MDM30 & 39.3 & 39.3 & 5.1 & 553 \\
& \Delta mdm30/pYES & 6.8 & 86.4 & 6.8 & 308 \\
& \Delta mdm30/pYES-MFB1 & 3.7 & 80.5 & 10.4 & 307 \\
YPD + Cu²⁺ & WT & 14.7 & 80.3 & 5.0 & 300 \\
& \Delta mfb1 & 69.5 & 27.2 & 3.3 & 305 \\
& \Delta mfb1/MFB1 & 78.5 & 20.5 & 1.0 & 302 \\
& \Delta mfb1/MFB1ΔFbox & 14.3 & 80.0 & 5.7 & 300 \\
& \Delta mdm30 & 48.0 & 48.7 & 3.3 & 304 \\
& \Delta mdm30/MDM30 & 59.3 & 38.7 & 2.0 & 300 \\
YPD & WT & 99.0 & 0 & 1.0 & 300 \\
& cdc53 & 2.5 & 0.5 & 97 & 200 \\
& cdc34 & 100.0 & 0 & 0 & 100 \\
& pre1 & 97.5 & 0 & 2.5 & 200 \\
YPG & WT & 91.5 & 0 & 8.5 & 200 \\
& cdc53 & 2.5 & 0 & 97.5 & 200 \\
& cdc34 & 78.0 & 0 & 22.0 & 100 \\
& pre1 & 90.0 & 0 & 10.0 & 200 \\
YPD + Dox & WT & 99.7 & 0.3 & 0 & 300 \\
& cdc53 & 4.5 & 3.0 & 92.5 & 200 \\
& cdc34 & 14.0 & 0 & 86.0 & 100 \\
& pre1 & 24.7 & 0 & 75.3 & 300 \\
YPG + Dox & WT & 90.0 & 0 & 10.0 & 200 \\
& cdc53 & 3.0 & 2.0 & 95.0 & 100 \\
& cdc34 & 24.0 & 0 & 76.0 & 100 \\
& pre1 & 31.5 & 0 & 68.5 & 200 \\
\hline
\end{tabular}
\caption{Mitochondrial phenotypes during vegetative growth}
\end{table}

Construction and Manipulation of Yeast Strains

Growth and manipulation of yeast strains was according to published procedures (Sherman, 1991; Sherman and Hicks, 1991; Gietz et al., 1992). All strains used in this study are isogenic to BY4741, BY4742, and BY4743 (Brachmann et al., 1998). Haploid deletion strains (Giaever et al., 2002) were obtained from EUROSCARF (Frankfurt, Germany), and strains expressing essential genes under control of a titratable promoter (Mnaemneh et al., 2004) were obtained from BioCat (Heidelberg, Germany). A strain expressing MFB1-GFP (Huh et al., 2003) was obtained from Invitrogen. Mitochondria have a wild-type-like appearance in this strain, indicating that the MFB1-GFP fusion protein is functional (Figure 1B; our unpublished observations). Homozygous diploid wild-type and \Delta mfb1 and \Delta mdm30 mutants expressing mitochondria-targeted GFP have been described previously (Dimmer et al., 2002). A haploid double mutant, \Delta mfb1/\Delta mdm30, was generated by mating of haploid deletion
strains, sporulation, and tetrad dissection. Genotypes of haploid progeny were determined by PCR. Promoter shutoff in cdc3, cdc34, cdc53, etf1, met30, and prb1 mutants carrying titratable promoter alleles (Miaimeeh et al., 2004) was as described previously (Altman and Westermann, 2005). Expression of full-length tagged Mfb1 and Mdm30 proteins and F-box variants thereof was induced by addition of 100 μM CuSO4 to the medium. Assay of mitochondrial fusion in vivo was according to published procedures (Nunnari et al., 1997; Fritz et al., 2003). Mitochondrial behavior during sporulation was observed essentially as described previously (Gorsch and Shaw, 2004) with minor modifications. Before sporulation, strains were incubated for 3 d on yeast extract-peptone-dextrose (YPD) plates at 30°C. Cells were allowed to form tetrads for 12 d at 30°C in liquid sporulation medium (1% potassium acetate; 20 μg/ml each of adenine, arginine, histidine, methionine, and tryptophan; and 30 μg/ml each of isoleucine, leucine, lysine, phenylalanine, threonine, and valine).

Microscopy

Yeast cells were prepared for electron microscopy according to published methods (Neuher and Youle, 2005), early log phase cells grown on YPD medium were treated with 20 mM sodium citrate and 10 μM Cd. At the indicated time points, cells corresponding to 3 optical density units were collected, lysed at alkaline pH, and trichloroacetic acid (TCA)-precipitated (Tatsuta and Langer, 2006), and protein extracts were analyzed by SDS-PAGE and immunoblotting using antisera against the GTPase domain of Fzo1 (kind gift from Jodi Nunnari, University of California, Davis, CA).

Assay of Fzo1 Turnover after a Factor-induced Cell Cycle Arrest

To examine Fzo1 degradation upon a factor-induced cell cycle arrest (Joseph and Youle, 2005), early log phase cells grown on YPD medium were treated with 20 mM sodium citrate and 10 μM Cd. At the indicated time points, cells corresponding to 3 optical density units were collected, lysed at alkaline pH, and trichloroacetic acid (TCA)-precipitated (Tatsuta and Langer, 2006), and protein extracts were analyzed by SDS-PAGE and immunoblotting using antisera against the GTPase domain of Fzo1 (kind gift from Jodi Nunnari, University of California, Davis, CA).

Molecular Biology of the Cell
Mitochondria-associated F-Box Proteins

Table 2. Role of yeast F-box proteins in mitochondrial morphology

<table>
<thead>
<tr>
<th>Strain/medium</th>
<th>Mitochondrial morphology (% of cells)</th>
<th>No. of cells scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPD</td>
<td>Wild-type-like</td>
<td>Aggregated</td>
</tr>
<tr>
<td>WT</td>
<td>91.1</td>
<td>5.1</td>
</tr>
<tr>
<td>Δmfb1 (Δf-box)</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>Δcig1 (ΔCgc1)</td>
<td>95.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Δmet30 (Δf-box)</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>Δmfb1 (Δf-box)</td>
<td>10.4</td>
<td>88.4</td>
</tr>
<tr>
<td>Δcig1 (ΔCgc1)</td>
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</tr>
<tr>
<td>Δskp2 (ΔSkp2)</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Δmet30 (Δf-box)</td>
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<td>5.0</td>
</tr>
<tr>
<td>Δmfb1 (Δf-box)</td>
<td>97.0</td>
<td>2.0</td>
</tr>
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</tr>
<tr>
<td>Δmfb1 (Δf-box)</td>
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<td>0</td>
</tr>
<tr>
<td>YPD + Dox</td>
<td>Wild-type-like</td>
<td>Aggregated</td>
</tr>
<tr>
<td>WT</td>
<td>99.0</td>
<td>0</td>
</tr>
<tr>
<td>Δcig1 (ΔCgc1)</td>
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</tr>
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<td>Δcig1 (ΔCgc1)</td>
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<td>Δcig1 (ΔCgc1)</td>
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</tr>
<tr>
<td>Δcig1 (ΔCgc1)</td>
<td>91.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

wild-type–like mitochondrial reticulum and that they have nonredundant functions. The F-box comprises a conserved binding site for Skp1 (Willems et al., 2004; Petroski and Deshaies, 2005). To test whether the presence of an intact F-box is essential for the proteins’ functions, we overexpressed truncated Mfb1 and Mdm30 proteins lacking their F-boxes from a CUP1 promoter (MFB1ΔFbox and MDM30ΔFbox). Surprisingly, these constructs rescued the mitochondrial morphology defects of the null mutants to a large extent (Table 1), suggesting that at least some of the proteins’ functions in mitochondrial dynamics do not require assembly into SCF complexes. It should be noted, however, that complementation of the mitochondrial phenotypes was seen under conditions that involved overexpression of the ΔF-box constructs from a strong promoter. Under these conditions, truncated, non-functional F-box proteins might be still able to bind to accumulated substrate proteins, such as Fzo1. Even though we consider it unlikely, we cannot exclude the possibility that this binding might inactivate excess Fzo1, or other substrate proteins, and thereby restore normal mitochondrial morphology in an indirect manner.

Several lines of evidence assign an important role to the ubiquitin/26S proteasome system in mitochondrial morphogenesis. For example, overexpression of a mutant ubiquitin variant unable to form polyubiquitin chains results in aberrant mitochondrial morphology (Fisk and Yaffe, 1999), and cells lacking functional proteasome subunits have severe mitochondrial defects (Rinaldi et al., 1998; Altman and Westermann, 2005). However, the exact role of the proteasome in mitochondrial biogenesis is still unclear. We asked whether the mitochondrial phenotypes seen in F-box protein mutants resemble mitochondria in cells after depletion of Cdc53/Cullin and Cdc34, essential core components of SCF ubiquitin ligases, or Pre1, an essential subunit of the 20S proteasome core particle. Yeast mutants containing the CDC53, CDC34, or PRE1 gene under control of a promoter that can be shut off by the addition of doxycycline to the medium (Mnaimneh et al., 2004) were grown under inducing and repressing conditions on fermentable and nonfermentable carbon sources, and mitochondrial morphology was analyzed. Mitochondria were fragmented in cdc53 cells under all conditions and in pre1 and cdc34 cells under repressing conditions on either carbon source (Figure 2C). Surprisingly, aggregated mitochondria, the major phenotypic class seen in mutants lacking mitochondria-associated F-box proteins grown on YPD medium, could only very rarely be observed in cdc53, cdc34, and pre1 mutants (Figure 2C and Table 1). Thus, mitochondrial defects are clearly distinguishable in cdc53, cdc34, and pre1 mutants on the one hand and mfb1 and mdm30 mutants on the other hand. Together, our observations point to the involvement of other, yet unidentified factors, in F-box protein-dependent regulation of mitochondrial dynamics.

Mfb1 and Mdm30 Are the Only F-box Proteins Required for Maintenance of Mitochondrial Morphology

The yeast genome contains 21 genes potentially encoding F-box proteins (Willems et al., 2004). We asked whether in addition to MFB1 and MDM30 any of the remaining 19 genes is required for normal mitochondrial distribution and morphology. Mitochondria were stained with mitochondria-
targeted GFP and examined by fluorescence microscopy in the following deletion mutants lacking nonessential genes: ∆mtn1, ∆coi11, ∆swh2, ∆sde1, ∆grr1, ∆grr3, ∆ycr1, ∆skp2, ∆fzo1, ∆fbr280c, ∆gdr131c, ∆gdr306c, ∆gdr149w, ∆gfr224w, ∆gfr352w, and ∆mtime285c. Three genes encoding F-box proteins, CDC4, CFT13, and MDM30, are essential for viability in yeast. Mitochondrion morphology was analyzed in strains carrying these genes under control of a doxycycline-repressible promoter (Mnaimneh et al., 2004). All of these mutant strains lacking F-box proteins showed wild-type-like mitochondria (Table 2 and Supplemental Figure 1). We conclude that Mfb1 and Mdm30 are the only members of the F-box protein family that are essential for maintenance of normal mitochondrial morphology in yeast.

To examine whether deletion of the MFB1 gene has pleiotropic effects on the morphology of other intracellular structures we analyzed vacuoles, ER, and filamentous actin by fluorescence microscopy of wild-type and ∆mfb1 cells. Cause these cellular structures seemed normal in ∆mfb1 (Supplemental Figure 2) and ∆mdm30 cells (Fritz et al., 2003), we conclude that organellar morphology defects in ∆mfb1 and ∆mdm30 mutants primarily affect mitochondria.

**Mfb1 Is Not Required for Mitochondrial Fusion and Turnover of Fzo1**

Mitochondrial fusion is blocked in ∆mdm30 cells, presumably because the turnover of Fzo1 is inhibited and surplus Fzo1 protein accumulates in the cell (Fritz et al., 2003). To test whether Mfb1 is required for mitochondrial fusion, we assayed fusion by mating haploid cells of opposite mating types preloaded with different fluorescent mitochondrial matrix markers. At least 20 zygotes were analyzed per strain. Fluorescent labels immediately intermixed in 100% of the zygotes, indicating efficient mitochondrial fusion and content mixing both in wild-type and ∆mfb1 cells (Figure 3A). Consistent with this finding, the steady-state level of Fzo1 in vegetatively growing cells is not elevated in ∆mfb1 cells in comparison with the wild type, and deletion of the MFB1 gene in the ∆mdm30 mutant does not elevate Fzo1 levels further in comparison with the ∆mdm30 single mutant (Figure 3B). We conclude that Mfb1 is not required for mitochondrial fusion or turnover of Fzo1 during vegetative growth.

Interestingly, Neutzner and Youle (2005) reported that Mdm30 is not required for proteasome-dependent degradation of Fzo1 after G1 cell cycle arrest evoked by treatment of yeast wild-type cells with the mating pheromone α factor. These findings were genetically confirmed using yeast strains carrying mutations in genes encoding proteasome subunits (Escarob-Henriques et al., 2006). Intriguingly, deletion of the MDM30 gene in a ∆fzo1 background produces a synthetic growth defect, suggesting that Mdm30 may have other yet unknown target proteins in

**Recruitment of Mdm30 to Mitochondria Is Independent of Fzo1**

A major fraction of Mdm30 is associated with mitochondria (Fritz et al., 2003), and Mdm30 physically interacts with Fzo1 in an F-box–independent manner (Escarob-Henriques et al., 2006). To examine whether deletion of the MFB1 gene has pleiotropic effects on the morphology of other intracellular structures we analyzed vacuoles, ER, and filamentous actin by fluorescence microscopy of wild-type and ∆mfb1 cells. Cause these cellular structures seemed normal in ∆mfb1 (Supplemental Figure 2) and ∆mdm30 cells (Fritz et al., 2003), we conclude that organellar morphology defects in ∆mfb1 and ∆mdm30 mutants primarily affect mitochondria.

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addition to Fzo1 (Fritz et al., 2003). To examine whether Mdm30 binds to mitochondria in the absence of its known binding partner, Fzo1, we fractionated Δfzo1 cells expressing FLAG-tagged Mdm30 (Mdm30FLAG) and FLAG-tagged Mdm30 lacking the F-box (ΔF-boxFLAG). On fractionation of yeast cells, we observed that Mdm30 cofractionated with mitochondria, even in the absence of Fzo1 (Figure 4). This suggests that Mdm30 has one or more mitochondrial binding partners in addition to Fzo1 and thus may have multiple target proteins on mitochondria.

Ultrastructure of Cells Lacking Mfb1 and Mdm30

Next, we examined the ultrastructure of wild-type, Δmfb1, Δmdm30, and Δmfb1/Δmdm30 cells by electron microscopy. To obtain high-resolution images of the three-dimensional organization of mitochondria, we prepared serial ultrathin

Figure 5. Ultrastructure of cells lacking Mfb1 or Mdm30. (A) WT, Δmfb1, Δmdm30, and Δmfb1/Δmdm30 cells were grown to log phase in YPD medium, fixed with glutaraldehyde, treated with zymolyase to remove the cell wall, postfixed with osmium tetroxide, and en bloc stained with uranyl acetate. After embedding in Spurr’s resin, serial ultrathin sections (70 nm) were prepared and analyzed by transmission electron microscopy. The three-dimensional structure of mitochondria was reconstructed using IMOD software (Kremer et al., 1996). Morphologically separate organelles are displayed in different colors. The contour of the plasma membrane is shown by a white line for each section. The model of the wild-type cell was reconstructed from 31 consecutive sections, the Δmfb1 cell from 27 sections, the Δmdm30 cell from 30 sections, and the Δmfb1/Δmdm30 cell from 18 sections. (B) Three representative consecutive sections that were used for reconstruction of the three-dimensional models displayed in A. (C) Enlarged detail of a transmission electron micrograph of a Δmfb1/Δmdm30 cell (taken from the third section displayed in B). White arrows point to close contacts between morphologically separate nonfused mitochondria. It should be noted that yeast strains related to BY4743 generally contain only few cristae on glucose-containing medium. However, strains lacking Mfb1 or Mdm30 are able to form normal cristae (not visible here).
Mitochondria undergo extensive remodeling during sporulation. Mdm30 is required for remodeling of mitochondria. In Figure 5, A and B, we found that all mitochondria within this volume were interconnected and formed one closed continuum consisting of clumped tubules (Figure 5A). In contrast, three-dimensional reconstruction of 20 sections (corresponding to ∼2170 nm) of a wild-type cell, three morphologically distinct organelles were observed within this volume (Figure 5A). After three-dimensional reconstruction of 27 sections (corresponding to ∼1890 nm) of a ∆mfb1 cell, we found that all mitochondria within this volume were interconnected and formed one closed continuum consisting of clumped tubules (Figure 5A). In contrast, three-dimensional reconstruction of 30 sections (corresponding to ∼2100 nm) of a ∆mdm30 cell revealed an aggregate of seven distinct organelles, and 18 sections (corresponding to ∼1260 nm) of a ∆mfb1/∆mdm30 cell contained 16 small and morphologically separable organelles (Figure 5A). These analyses were repeated with at least one additional cell for each strain, yielding very similar results (our unpublished observations). All cells were harvested from logarithmically growing cultures, and for each strain at least one cell that clearly carried a bud was analyzed.

Interestingly, mitochondria in ∆mdm30 (our unpublished data) and ∆mfb1/∆mdm30 cells (Figure 5C) were sometimes observed to be in close contact without fusion. This phenotype resembles clustered mitochondria seen in mammalian cells overexpressing Fzo1 homologues Mfn2 (Rojo et al., 2002) or Mfn1 (Santel et al., 2003). It is conceivable that surplus Fzo1 forms nonfunctional fusion complexes in strains lacking Mdm30. These complexes might tether neighboring organelles, as it has been observed for mammalian cells expressing mutant forms of Mfn2 (Eura et al., 2003) or truncated forms of Mfn1 (Koshiba et al., 2004). Interestingly, some electron-dense material is seen in the contact areas (Figure 5C, arrows), which might consist of accumulated Fzo1. A block of mitochondrial fusion in cells lacking Mdm30 leads to fragmentation of mitochondria by ongoing fission (Fritz et al., 2003). Because fusion is not blocked in ∆mfb1 cells, mitochondria may fuse and form a continuum in cells lacking Mfb1 as long as Mdm30 is present. We conclude that mitochondrial aggregates are formed by different mechanisms in cells lacking Mfb1 and Mdm30.

Figure 6. Mdm30 is required for mitochondrial remodeling and inheritance during sporulation. (A) Homozygous diploid WT, ∆mfb1 and ∆mdm30 cells expressing mitochondria-targeted GFP were grown to log phase (top) in YPD medium. To induce sporulation, strains were grown to stationary phase on YPD plates, transferred to sporulation medium, and incubated at 30°C for 12 d. At each stage of sporulation, representative cells were analyzed by fluorescence and DIC microscopy, as in Figure 2B. Right is a merged image of DIC and fluorescence micrographs. Mitochondrial inheritance defects were observed in early and late tetrads. Ascospores devoid of mitochondria are marked with an asterisk. Bar, 2 μm. (B) Percentage of asci is indicated that contained at least one spore devoid of mitochondria at the early or late tetrad stage (number of asci scored: WT, 413; ∆mfb1, 422; and ∆mdm30, 264). (C) Percentage of cells is indicated that had formed ascii with two to four spores at the late tetrad stage (number of cells scored: 400 for each strain).
Late tetrads (Miyakawa et al., 1984; Gorsich and Shaw, 2004; Egner et al., 2002; Jakobs et al., 2003). During mitotic growth, mating, and sporulation, mitochondria are partitioned to newly formed cells by highly ordered inheritance mechanisms and dynamic remodeling processes (Miyakawa et al., 1984; Nunnari et al., 1997; Simon et al., 1997; Okamoto et al., 1998; Yang et al., 1999; Gorsich and Shaw, 2004). How are mitochondrial fusion and fission orchestrated during these events?

The complement of F-box proteins that is present in every eukaryotic cell is thought to have sweeping regulatory powers by virtue of its ability to directly link SCF substrates to signaling pathways (Willems et al., 2004). Consistently, our results assign a key role to F-box proteins in regulating mitochondrial dynamics. Mfb1 and Mdm30 are both required for proper mitochondrial distribution and morphology during mitotic growth. A comprehensive analysis of mutants of all known yeast F-box protein-encoding genes suggests that the remaining 19 members of this protein family are dispensable for maintenance of wild-type–like mitochondria. Consistent with this finding, large-scale analysis of protein localization in yeast (Huh et al., 2003) and analysis of the mitochondrial proteome (Sickmann et al., 2003) did not reveal any other mitochondria-associated F-box protein. Genetic and morphological evidence suggests that Mfb1 and Mdm30 function independently of each other and may have different target proteins. A Δmfbl/Δmdm30 double mutant does not show a more severe phenotype than a Δmdm30 single mutant; overexpression of one F-box protein does not compensate for the loss of the other; and deletion of the MDM30 gene leads to a block in fusion, whereas deletion of the MFB1 gene leads to clumped interconnected mitochondria. In addition to their role during mitotic growth, mitochondria-associated F-box proteins are important for mitochondrial dynamics during the execution of cell developmental programs. Consistent with a role in mitochondrial dynamics during development, both genes are several-fold induced during sporulation (Chu et al., 1998). Although a role of Mfb1 in regulating mitochondrial behavior during meiosis is still unknown, we consider it likely that Mdm30 modulates mitochondrial fusion activity because fusion of fragmented mitochondria to a tubular network is blocked in pretetrads lacking Mdm30 (Figure 6A). Because Mdm30 is also required for fusion of mitochondria during mating (Fritz et al., 2003), F-box proteins play an

### Table 3. Mitochondrial phenotypes during sporulation

<table>
<thead>
<tr>
<th>Stage/strain</th>
<th>Mitochondrial morphology (% of cells)</th>
<th>No. of cells or asci scored</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tubular</td>
<td>Aggregated</td>
</tr>
<tr>
<td>Stationary phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Δmfb1/Δmfb1</td>
<td>5.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Δmdm30/Δmdm30</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>Pretetrad</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>75.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Δmfb1/Δmfb1</td>
<td>62.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Δmdm30/Δmdm30</td>
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<td>5.3</td>
</tr>
<tr>
<td>Early tetrad</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>91.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Δmfb1/Δmfb1</td>
<td>85.3</td>
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</tr>
<tr>
<td>Δmdm30/Δmdm30</td>
<td>4.1</td>
<td>14.2</td>
</tr>
<tr>
<td>Late tetrads</td>
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<td></td>
</tr>
<tr>
<td>WT</td>
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</tr>
<tr>
<td>Δmdm30/Δmdm30</td>
<td>0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

posibility that mitochondria-associated F-box proteins are required for progression through the mitochondrial remodeling program during meiosis and spore formation. To test this, we observed mitochondrial behavior during sporulation in diploid wild-type cells and homozygous diploid cells lacking Mfb1 or Mdm30. The tubular mitochondrial network of logarithmically growing wild-type cells fragments upon entry into stationary phase. Mitochondrial fragments fuse again in pretetrads to build a complex tubular network. Tubular mitochondria are then partitioned to the newly formed cells by entry into stationary phase. Mitochondrial fragments fuse again in pretetrads to build a complex tubular network. Tubular mitochondria show a complex behavior. Volume, number, and morphology of mitochondria are readily adapted to the carbon source of the growth medium and oxygen supply (Stevens, 1981; Visser et al., 1995; Egner et al., 2002; Jakobs et al., 2003). During mitotic growth, mating, and sporulation, mitochondria are partitioned to newly formed cells by highly ordered inheritance mechanisms and dynamic remodeling processes (Miyakawa et al., 1984; Nunnari et al., 1997; Simon et al., 1997; Okamoto et al., 1998; Yang et al., 1999; Gorsich and Shaw, 2004). How are mitochondrial fusion and fission orchestrated during these events?

The complement of F-box proteins that is present in every eukaryotic cell is thought to have sweeping regulatory powers by virtue of its ability to directly link SCF substrates to signaling pathways (Willems et al., 2004). Consistently, our results assign a key role to F-box proteins in regulating mitochondrial dynamics. Mfb1 and Mdm30 are both required for proper mitochondrial distribution and morphology during mitotic growth. A comprehensive analysis of mutants of all known yeast F-box protein-encoding genes suggests that the remaining 19 members of this protein family are dispensable for maintenance of wild-type–like mitochondria. Consistent with this finding, large-scale analysis of protein localization in yeast (Huh et al., 2003) and analysis of the mitochondrial proteome (Sickmann et al., 2003) did not reveal any other mitochondria-associated F-box protein. Genetic and morphological evidence suggests that Mfb1 and Mdm30 function independently of each other and may have different target proteins. A Δmfbl/Δmdm30 double mutant does not show a more severe phenotype than a Δmdm30 single mutant; overexpression of one F-box protein does not compensate for the loss of the other; and deletion of the MDM30 gene leads to a block in fusion, whereas deletion of the MFB1 gene leads to clumped interconnected mitochondria. In addition to their role during mitotic growth, mitochondria-associated F-box proteins are important for mitochondrial dynamics during the execution of cell developmental programs. Consistent with a role in mitochondrial dynamics during development, both genes are several-fold induced during sporulation (Chu et al., 1998). Although a role of Mfb1 in regulating mitochondrial behavior during meiosis is still unknown, we consider it likely that Mdm30 modulates mitochondrial fusion activity because fusion of fragmented mitochondria to a tubular network is blocked in pretetrads lacking Mdm30 (Figure 6A). Because Mdm30 is also required for fusion of mitochondria during mating (Fritz et al., 2003), F-box proteins play an
important role in mitochondrial dynamics throughout the entire life cycle of budding yeast. A synthetic growth defect in Δfzo1/Δmdm30 double mutants (Fritz et al., 2003) and Fzo1-independent binding of Mdm30 to mitochondria (Figure 4) suggest that Mdm30 may have other yet unknown mitochondrial target proteins in addition to Fzo1. Interestingly, it has recently been reported that Mdm30 is a transcriptional coactivator of Gal4. This activity has been proposed to be required for activation of Gal4 target genes and growth on synthetic galactose medium (Muratani et al., 2005). However, we and others observed that Δmdm30 mutants are able to grow on galactose-containing media (Okamoto and Shaw, 2005), and mitochondria-targeted fluorescent proteins are efficiently expressed from the GAL1 promoter in Δmdm30 strains (Fritz et al., 2003). Thus, it is not clear whether Mdm30 is of general importance for expression of Gal4-dependent genes, and possible additional target proteins of mitochondria-associated F-box proteins remain to be identified.

Numerous lines of evidence point to an important role of the ubiquitin/26S proteasome system in mitochondrial biogenesis. Yeast mutants lacking functional subunits of ubiquitin ligases or the proteasome harbor aberrant mitochondria (Rinaldi et al., 1998; Fisk and Yaffe, 1999; Altmann and Westermann, 2005), and proteasome inhibitors (Neuzner and Youle, 2005) and mutations in proteasome subunit-encoding genes (Escobar-Henriques et al., 2006) reduce the rate of degradation of Fzo1 in a factor-arrested cells. Consistent with a specific role of ubiquitylation of mitochondrial proteins, the mitochondrial outer membrane harbors a deubiquitinating enzyme, Ubp16. However, neither deletion nor overexpression of the UBP16 gene produces any obvious phenotype, and mitochondrial morphology and inheritance are not affected in ubp16 mutants (Kimmer and Kolling, 2003). What might be the role of Mfb1 and Mdm30 in mediating turnover of Fzo1 and other mitochondrial target proteins? Mdm30 has been shown to interact with Skp1 and Cdc53 in the yeast two-hybrid system (Uetz et al., 2000), recombinant Mdm30 assemblies with Skp1, Cdc53, and Rbx1 into an SCF complex in vitro (Kus et al., 2004), and Mfb1 has been found in a complex with Skp1 in a large-scale identification of yeast protein complexes by mass spectrometry (Ho et al., 2002) and in communoprecipitation experiments (Kondo-Okamoto et al., 2006). However, Mfb1–Skp1 complexes apparently do not contain Cdc53 (Kondo-Okamoto et al., 2006). Intriguingly, we observed that the mitochondrial phenotype of yeast strains lacking Mfb1 and Mdm30 cannot be mimicked by deletion of SCF core complex or proteasome subunits and that the F-box motif of Mfb1 and Mdm30 is not essential for rescue of the mitochondrial morphology defect of the null mutants. Furthermore, proteasome-dependent degradation of Fzo1 in a factor-arrested cells does not require Mdm30 (Neuzner and Youle, 2005) or Mfb1 (Figure 3C), and Mdm30-mediated turnover of Fzo1 in vegetatively growing cells is independent of the proteasome (Escobar-Henriques et al., 2006). Thus, it seems that mitochondria-associated F-box proteins do not act as bona fide SCF ubiquitin ligases. Rather, they may exert at least some of their functions independently of the proteasome, as it has been demonstrated for Rcy1, an F-box protein involved in regulation of vesicular trafficking (Galan et al., 2001). Apparently, Mfb1 and Mdm30 are two important components of a complex network of substrate recognition factors and proteolytic systems mediating turnover of mitochondrial proteins. Many, yet unknown, factors remain to be identified.

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Mitochondria-associated F-Box Proteins


