Nonredundant Roles of Mitochondria-associated F-Box Proteins Mfb1 and Mdm30 in Maintenance of Mitochondrial Morphology in Yeast^D

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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 $\Delta mdm30$ cells. Mitochondrial fusion is also blocked during sporulation of homozygous 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 anabolic 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 Mühlenhoff, 2005; Youle and Karbowski, 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, 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

Karbowski, 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-

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Figure 1. Mfb1 is a novel mitochondria-associated F-box protein. (A) The F-box motifs of Mfb1 (amino acid residues 17–60) and Mdm30 (amino acid residues 16–59) are aligned with an F-box consensus sequence according to Willems *et al.* (2004). Favored sites in the consensus sequence are in uppercase letters, and moderately well tolerated residues are in lowercase letters. Residues matching the consensus sequence are in red. (B) Yeast cells expressing an Mfb1-GFP fusion protein under control of the *MFB1* promoter were grown to log phase in glucose-containing medium (YPD), and mitochondria were stained with the membrane potential-sensitive dye rhodamine B hexyl ester. Yeast cells were analyzed by fluorescence and differential interference contrast (DIC) microscopy. Rhodamine fluorescence (mito) was analyzed first to avoid photoconversion of GFP. Bar, 1 μ m.

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 α 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

Figure 2. Mfb1, Mdm30, SCF complex, and proteasome subunits are required for normal growth and mitochondrial morphology. (A) Wild-type (WT), Δ mfb1, Δ mdm30, and Δ mfb1/ Δ mdm30 ($\Delta\Delta$) cells were grown overnight in glucose-containing medium at 30°C. Then, 10-fold serial dilutions were spotted onto plates containing glucose (YPD) or glycerol (YPG) as carbon source. YPD plates were incubated for 2 d, and YPG plates were incubated for 3 d at the indicated temperatures. (B) Wild-type (WT), Δ mfb1, Δ mdm30, and Δ mfb1/ Δ mdm30 ($\Delta\Delta$) cells expressing mitochondria-targeted GFP were grown to log phase in YPD or YPG medium and analyzed by DIC (left) and fluorescence (right) microscopy. Bar, 2 μ m. (C) WT, *cdc53*, *cdc34*, and *pre1* cells expressing mitochondria-targeted GFP were grown to log phase in YPD or YPG in the presence of doxycycline (+Dox) and analyzed as described above. Magnification is as in B.

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 *GAL1* promoter in yeast, pYES-MFB1, was generated by

Table 1. Mitochondrial phenotypes during vegetative growth

Strain/medium	Mitochondrial morphology (% of cells)			No. of cells scored
	Wild-type-like	Aggregated	Fragmented	
YPD				
WT	91.1	5.1	3.8	315
$\Delta mfb1$	9.4	84.7	5.9	374
$\Delta mdm30$	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	87.5	3.0	9.5	305
WT/pYES-MDM30	85.6	6.6	7.8	408
$\Delta mfb1$ /pYES-MFB1	82.8	10.5	6.7	313
$\Delta mdm30$ /pYES-MDM30	84.8	7.6	7.6	422
$\Delta mfb1$ /pYES	38.9	56.0	5.1	553
$\Delta mfb1$ /pYES-MDM30	33.5	60.8	5.7	421
$\Delta mdm30$ /pYES	6.8	86.4	6.8	308
$\Delta mdm30$ /pYES-MFB1	9.1	80.5	10.4	307
YPD + Cu ²⁺				
$\Delta mfb1$	14.7	80.3	5.0	300
$\Delta mfb1$ /MFB1	69.5	27.2	3.3	305
$\Delta mfb1$ /MFB1 Δ Fbox	78.5	20.5	1.0	302
$\Delta mdm30$	14.3	80.0	5.7	300
$\Delta mdm30$ /MDM30	48.0	48.7	3.3	304
$\Delta mdm30$ /MDM30 Δ Fbox	59.3	38.7	2.0	300
YPD				
WT	99.0	0	1.0	300
<i>cdc53</i>	2.5	0.5	97	200
<i>cdc34</i>	100.0	0	0	100
<i>pre1</i>	97.5	0	2.5	200
YPG				
WT	91.5	0	8.5	200
<i>cdc53</i>	2.5	0	97.5	200
<i>cdc34</i>	78.0	0	22.0	100
<i>pre1</i>	90.0	0	10.0	200
YPD + Dox				
WT	99.7	0.3	0	300
<i>cdc53</i>	4.5	3.0	92.5	200
<i>cdc34</i>	14.0	0	86.0	100
<i>pre1</i>	24.7	0	75.3	300
YPG + Dox				
WT	90.0	0	10.0	200
<i>cdc53</i>	3.0	2.0	95.0	100
<i>cdc34</i>	24.0	0	76.0	100
<i>pre1</i>	31.5	0	68.5	200

PCR amplification of the mitochondria-associated F-box protein 1 (*MFB1*) coding region from genomic yeast DNA using primers 5'-AAA AAG CTT ATG ACA TTA TTT TCT TGT AGT GTA C-3' and 5'-AAA GGA TCC TTA TAT ATT AAA ATC GGT ATT AGC G-3' and cloning into the HindIII/BamHI sites of vector pYES2.0 (Invitrogen, Groningen, The Netherlands). Plasmid pYES-MDM30 for expression of Mdm30 from the *GAL1* promoter has been described previously (Fritz *et al.*, 2003). Plasmids pVT100U-mtGFP, pYX113-mtGFP (Westermann and Neupert, 2000), pRS416-*GAL1*+*PrF₀*-ATP9-RFP (Mozdy *et al.*, 2000), and pSJ55 (Jakobs *et al.*, 2003) were used for expression of mitochondria-targeted green fluorescent protein (GFP) or mitochondria-targeted DsRed. Plasmids for expression of FLAG-tagged Mdm30 and an F-box-less variant thereof under control of the *CUP1* promoter are described by Escobar-Henriques *et al.* (2006). A plasmid for expression of FLAG-tagged Mfb1 under control of the *CUP1* promoter (pJDCEX2-MFB1) was constructed by PCR amplification of the *MFB1* coding region using primers 5'-AAA AAG ATC TAT GAC ATT ATT TTC TTG TAG TGT AC-3' and 5'-AAA ACC CGG GTT ATA TAT TAA AAT CGG TAT TAG GC-3' and cloning into the BglIII/XmaI sites of pJDCEX2-MDM30 (Escobar-Henriques *et al.*, 2006). A plasmid for expression of a truncated Mfb1 protein lacking the 60

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

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 (Mnaimneh *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 *cdc4*, *cdc34*, *cdc53*, *ctf13*, *met30*, and *pre1* mutants carrying titratable promoter alleles (Mnaimneh *et al.*, 2004) was as described previously (Altmann and Westermann, 2005). Expression of FLAG-tagged Mfb1 and Mdm30 proteins and Δ F-box variants thereof was induced by addition of 100 μ M 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 (Gorsich 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 μ g/ml each of adenine, arginine, histidine, methionine, and tryptophan; and 30 μ g/ml each of isoleucine, leucine, lysine, phenylalanine, threonine, and valine).

Microscopy

Yeast cultures were grown at 30°C in liquid YPD medium to mid-logarithmic growth phase, if not indicated otherwise. Mitochondria were stained with plasmid-expressed mitochondria-targeted GFP or DsRed (see above) or rhodamine B hexyl ester (Invitrogen, Eugene, OR) as described previously (Dimmer *et al.*, 2002). Filamentous actin, vacuoles, and endoplasmic reticulum (ER) were stained as described previously (Fritz *et al.*, 2003). Epifluorescence microscopy was performed using an Axioplan 2 microscope (Carl Zeiss Lichtmikroskopie, Göttingen, Germany) equipped with a Plan-Neofluar 100 $\times$ /1.30 Ph3 oil objective (Carl Zeiss Lichtmikroskopie). Images were recorded with an Evolution VF Mono Cooled monochrome camera (Intas, Göttingen, Germany) and processed with Image-Pro Plus 5.0 and Scope Pro4.5 software (Media Cybernetics, Silver Spring, MD).

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

To examine Fzo1 degradation upon α factor-induced cell cycle arrest (Neutzner and Youle, 2005), early log phase cells grown on YPD medium were treated with 20 mM sodium citrate and 10 μ M α factor. 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 Mdm30 Binding to Mitochondria

Yeast cells expressing FLAG-tagged Mdm30 and Mdm30- Δ F-box were grown to logarithmic growth phase in SC medium lacking leucine to select for maintenance of the plasmid. The experiment was performed at intermediate Mdm30 expression levels, i.e., without the addition of copper to the medium. Total cell extracts were prepared as described above. Crude mitochondria were isolated by differential centrifugation, and soluble proteins were precipitated from the supernatant with TCA (Escobar-Henriques *et al.*, 2006). Total cell proteins, mitochondrial proteins, and cytosolic proteins were analyzed by SDS-PAGE and immunoblotting.

Electron Microscopy

Yeast cells were prepared for electron microscopy according to published protocols (Bauer *et al.*, 2001) with the exception that embedding was in Spurr's resin (Spurr, 1969). Ultrathin sections (70 nm) were cut with a diamond knife (MicroStar, Huntsville, TX) on a Leica Ultracut UCT microtome (Leica Microsystems, Vienna, Austria) and mounted on pioloform-coated copper grids. Sections were viewed with a Zeiss CEM 902 A transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at 80 kV. Micrographs were taken using SO-163 EM film (Eastman Kodak, Rochester, NY). Three-dimensional reconstructions were prepared from scanned films using IMOD software, version 3.5.5 (<http://bio3d.colorado.edu/imod/>; Kremer *et al.*, 1996).

RESULTS

Mfb1 Is a Novel Mitochondria-associated F-Box Protein

The *MFB1* gene (mitochondria-associated F-box protein 1; systematic name *YDR219c*) encodes a protein of 465 amino acid residues ($\approx$ 53 kDa). Similar to Mdm30, the Mfb1 protein contains an F-box motif at its N terminus (Figure 1A). Outside the F-box, the Mfb1 amino acid sequence does not show significant homology to any other known protein domain or sequence motif. In a large-scale analysis aimed at the systematic identification of protein complexes by mass spectrometry, Mfb1 was found in a complex with Skp1 (Ho *et al.*, 2002). A large-scale analysis aimed at the systematic

subcellular localization of the yeast proteome suggested that Mfb1 is associated with mitochondria, because expression of an Mfb1-GFP fusion protein yielded a staining pattern resembling mitochondria (Huh *et al.*, 2003). To prove a mitochondrial location of Mfb1, we stained live yeast cells expressing Mfb1-GFP under control of its endogenous promoter with a mitochondria-specific fluorescent dye, rhodamine B hexyl ester. Because Mfb1-GFP staining overlapped with mitochondrial staining (Figure 1B), we conclude that a major fraction of Mfb1 is associated with mitochondria.

Mfb1, Mdm30, and SCF Core Subunits Play Distinct and Separable Roles in Mitochondrial Distribution and Morphology

To investigate a role of Mfb1 in mitochondrial biogenesis and behavior, and to address the question whether its function overlaps with Mdm30, we constructed and analyzed mutant strains lacking or overexpressing the two mitochondria-associated F-box proteins in various combinations. Cells lacking Mfb1 showed a slight growth defect on the nonfermentable carbon source glycerol at elevated temperature (Figure 2A), which was less pronounced than the growth defect of cells lacking Mdm30 (Figure 2A; Fritz *et al.*, 2003). The growth behavior of the Δ *mfb1*/ Δ *mdm30* double mutant was identical to that of the Δ *mdm30* single mutant (Figure 2A). We conclude that both Mfb1 and Mdm30 are important for maintenance of respiratory functions at elevated temperature, but yeast cells can tolerate the simultaneous loss of both proteins without the loss of vital functions.

To examine whether Mfb1 is required for normal mitochondrial distribution and morphology, we performed a comprehensive analysis of mitochondria in cells lacking Mfb1 and/or Mdm30. Logarithmically growing wild-type cells showed a characteristic tubular network, which is more ramified on the nonfermentable carbon source glycerol than on the fermentable carbon source glucose (Figure 2B). More than 80% of the cells in Δ *mfb1*, Δ *mdm30*, and Δ *mfb1*/ Δ *mdm30* cultures displayed aggregated mitochondria that were clumped together and deposited at random locations within the cells on glucose-containing medium (Figure 2B and Table 1). In contrast, on the nonfermentable carbon source glycerol, the majority of mutant cells contained fragmented mitochondria that were more or less evenly distributed in the cell (Figure 2B and Table 1). Because mitochondrial behavior observed by light microscopy seems very similar in Δ *mfb1* and Δ *mdm30* cells, we considered the possibility that Mfb1 and Mdm30 function in related pathways and might have overlapping substrate specificity. Thus, we tested whether overexpression of one component might rescue the loss of the other. The *MFB1* and *MDM30* genes were placed under control of the strong and inducible *GAL1* promoter and expressed from a multicopy plasmid in wild type, Δ *mfb1*, and Δ *mdm30* cells. Strains transformed with the empty vector served as controls. Δ *mfb1* cells expressing the *MFB1* gene from the *GAL1* promoter and Δ *mdm30* cells expressing the *MDM30* gene from the *GAL1* promoter showed wild-type-like mitochondria (Table 1), indicating that plasmid-expressed F-box proteins are functional and suggesting that overexpression of these genes does not have negative consequences for mitochondrial morphology. In contrast, Δ *mfb1* cells expressing the *MDM30* gene from the *GAL1* promoter and Δ *mdm30* cells expressing the *MFB1* gene from the *GAL1* promoter were indiscernible from the vector controls (Table 1). This suggests that loss of one mitochondria-associated F-box protein cannot be compensated by overexpression of the other. We conclude that Mfb1 and Mdm30 are both required for maintenance of a

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

Strain/medium	Mitochondrial morphology (% of cells)			No. of cells scored
	Wild-type-like	Aggregated	Fragmented	
YPD				
WT	91.1	5.1	3.8	315
<i>Δamn1</i> (<i>Δybr158w</i>)	100.0	0	0	100
<i>Δcos111</i> (<i>Δybr203w</i>)	70.0	25.0	5.0	100
<i>Δdia2</i> (<i>Δyor080w</i>)	100.0	0	0	100
<i>Δela1</i> (<i>Δynl230c</i>)	95.0	3.0	2.0	100
<i>Δgrr1</i> (<i>Δyjr090c</i>)	100.0	0	0	100
<i>Δhrt3</i> (<i>Δylr097c</i>)	100.0	0	0	100
<i>Δmdm30</i> (<i>Δylr368w</i>)	10.4	88.4	1.2	346
<i>Δmfb1</i> (<i>Δydr219c</i>)	9.4	84.7	5.9	374
<i>Δrcy1</i> (<i>Δyjl204c</i>)	95.0	5.0	0	100
<i>Δskp2</i> (<i>Δynl311c</i>)	95.0	0	5.0	100
<i>Δufo1</i> (<i>Δym1088w</i>)	74.0	0	26.0	100
<i>Δybr280c</i>	100.0	0	0	100
<i>Δydr131c</i>	98.0	0	2.0	100
<i>Δydr306c</i>	95.0	5.0	0	100
<i>Δyjl149w</i>	97.0	2.0	1.0	100
<i>Δylr224w</i>	100.0	0	0	100
<i>Δylr352w</i>	100.0	0	0	100
<i>Δymr258c</i>	100.0	0	0	100
YPD				
WT	99.0	0	1.0	300
<i>cdc4</i> (<i>yfl009w</i>)	93.0	0	7.0	100
<i>ctf13</i> (<i>ymr094w</i>)	100.0	0	0	100
<i>met30</i> (<i>yil046w</i>)	100.0	0	0	100
YPD + Dox				
WT	99.7	0.3	0	300
<i>cdc4</i> (<i>yfl009w</i>)	67.0	12.0	21.0	100
<i>ctf13</i> (<i>ymr094w</i>)	100.0	0	0	100
<i>met30</i> (<i>yil046w</i>)	91.0	4.0	5.0	100

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 Δ F-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; Altmann 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: $\Delta amn1$, $\Delta cos111$, $\Delta dia2$, $\Delta ela1$, $\Delta grr1$, $\Delta hrt3$, $\Delta rcy1$, $\Delta skp2$, $\Delta ufo1$, $\Delta ybr280c$, $\Delta ydr131c$, $\Delta ydr306c$, $\Delta yjl149w$, $\Delta ylr224w$, $\Delta ylr352w$, and $\Delta ymr258c$. Three genes encoding F-box proteins, *CDC4*, *CFT13*, and *MET30*, are essential for viability in yeast. Mitochondrial 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 $\Delta mfb1$ cells. Because these cellular structures seemed normal in $\Delta mfb1$ (Supplemental Figure 2) and $\Delta mdm30$ cells (Fritz *et al.*, 2003), we conclude that organellar morphology defects in $\Delta mfb1$ and $\Delta mdm30$ mutants primarily affect mitochondria.

Mfb1 Is Not Required for Mitochondrial Fusion and Turnover of Fzo1

Mitochondrial fusion is blocked in $\Delta mdm30$ 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 $\Delta mfb1$ cells (Figure 3A). Consistent with this finding, the steady-state level of Fzo1 in vegetatively growing cells is not elevated in $\Delta mfb1$ cells in comparison with the wild type, and deletion of the *MFB1* gene in the $\Delta mdm30$ mutant does not elevate Fzo1 levels further in comparison with the $\Delta mdm30$ single mutant (Figure 3B). We conclude that Mfb1 is not required for mitochondrial fusion or turnover of Fzo1 during vegetative growth.

Interestingly, Neutznier and Youle (2005) reported that Mdm30 is not required for proteasome-dependent degradation of Fzo1 after G_1 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 (Escobar-Henriques *et al.*, 2006). We considered the possibility that in G_1 cell cycle-arrested cells Mfb1 might serve as an alternative F-box protein to target Fzo1 to degradation. To test this, wild-type, $\Delta mfb1$, and $\Delta mdm30$ cells were treated for different times with α factor, and turnover of Fzo1 was assayed by immunoblotting of cell extracts. Consistent with previous findings, we observed efficient degradation of Fzo1 in α factor-treated wild-type and $\Delta mdm30$ cells. However, efficient turnover of Fzo1 was also observed in $\Delta mfb1$ cells (Figure 3C). Hence, degradation of Fzo1 after mating pheromone-induced cell cycle arrest depends neither on Mdm30 nor on Mfb1.

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 (Escobar-Henriques *et al.*,

Figure 3. Mfb1 is not required for mitochondrial fusion or turnover of Fzo1. (A) WT or $\Delta mfb1$ cells of opposite mating types containing mitochondria preloaded with GFP (mtGFP) or DsRed (mtDsRed) were mated, and zygotes were analyzed by fluorescence and DIC microscopy. DsRed fluorescence was analyzed first to avoid photoconversion of GFP. Bar, 5 μ m. (B) WT, $\Delta mfb1$, $\Delta mdm30$, and $\Delta mfb1/\Delta mdm30$ cells were grown to log phase in YPD medium. Steady-state concentrations of Fzo1 in total cell extracts were analyzed by immunoblotting using Fzo1-specific antibodies (α Fzo1). A cross-reacting protein (CR), which is invariantly expressed in all four strains, served as a loading control. (C) WT, $\Delta mfb1$, and $\Delta mdm30$ cultures were treated for the indicated time periods with mating pheromone α factor. Turnover of Fzo1 was analyzed by immunoblotting of total cell extracts as in B.

2006). Intriguingly, deletion of the *MDM30* gene in a $\Delta fzo1$ background produces a synthetic growth defect, suggesting that Mdm30 may have other yet unknown target proteins in

Figure 4. Binding of Mdm30 to mitochondria does not require Fzo1. Wild-type (*FZO1*) and $\Delta fzo1$ strains were transformed with plasmids expressing N-terminally FLAG-tagged full-length Mdm30 (*Mdm30^{FLAG}*) and Mdm30 lacking its F-box (Δ F-box^{FLAG}) from the *CUP1* promoter. Total cell extracts (T) and subcellular fractions were prepared from logarithmically growing cultures. Crude mitochondria contained in the pellet fraction (P) and cytosolic proteins contained in the supernatant (S) were isolated by differential centrifugation and analyzed by immunoblotting using antibodies against the FLAG epitope, the mitochondrial protein Tom40, and the cytosolic protein triose phosphate isomerase Tpi1.

Figure 5. Ultrastructure of cells lacking Mfb1 or Mdm30. (A) WT, $\Delta mfb1$, $\Delta mdm30$, and $\Delta mfb1/\Delta mdm30$ 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 $\Delta mfb1$ cell from 27 sections, the $\Delta mdm30$ cell from 30 sections, and the $\Delta mfb1/\Delta mdm30$ 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 $\Delta mfb1/\Delta mdm30$ 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).

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 $\Delta fzo1$ cells expressing FLAG-tagged Mdm30 (Mdm30^{FLAG}) and FLAG-tagged Mdm30 lacking the F-box ($\Delta F\text{-box}^{\text{FLAG}}$). 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 bind-

ing 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, $\Delta mfb1$, $\Delta mdm30$, and $\Delta mfb1/\Delta mdm30$ cells by electron microscopy. To obtain high-resolution images of the three-dimensional organization of mitochondria, we prepared serial ultrathin

Figure 6. Mdm30 is required for mitochondrial remodeling and inheritance during sporulation. (A) Homozygous diploid WT, $\Delta mfb1$ and $\Delta mdm30$ 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 μ m. (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; $\Delta mfb1$, 422; and $\Delta mdm30$, 264). (C) Percentage of cells is indicated that had formed asci with two to four spores at the late tetrad stage (number of cells scored: 400 for each strain).

sections (70 nm), analyzed them by transmission electron microscopy, and generated three-dimensional models (Figure 5, A and B). Although aggregated and clumped mitochondria of cells grown in glucose-containing medium looked very similar in all three mutant strains when observed by fluorescence microscopy (compare Figure 2B and Table 1), electron microscopic analyses revealed striking differences. When the three-dimensional structure of mitochondria was reconstructed from 31 consecutive 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 $\Delta mfb1$ 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 $\Delta mdm30$ cell revealed an aggregate of seven distinct organelles, and 18 sections (corresponding to ≈ 1260 nm) of a $\Delta mfb1/\Delta mdm30$ 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.

Intriguingly, mitochondria in $\Delta mdm30$ (our unpublished data) and $\Delta mfb1/\Delta mdm30$ 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 $\Delta mfb1$ 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.

Tubular, aggregated mitochondria in $\Delta mfb1$ cells that were clearly different from fragmented, aggregated mitochondria in $\Delta mdm30$ cells were also observed by Kondo-Okamoto *et al.* (2006). It should be noted that these researchers found that short tubules in $\Delta mfb1$ cells were less interconnected in the W303 genetic background than the structures we observed in BY4741-related $\Delta mfb1$ strains. We consider it likely that these differences are due to strain-specific effects.

Mdm30 Is Required for Remodeling of Mitochondria during Sporulation

Mitochondria undergo extensive remodeling during sporulation (Miyakawa *et al.*, 1984; Gorsich and Shaw, 2004). Interestingly, the *MFB1* and *MDM30* genes are induced during sporulation (Chu *et al.*, 1998), raising the intriguing

Table 3. Mitochondrial phenotypes during sporulation

Stage/strain	Mitochondrial morphology (% of cells)			No. of cells or asci scored
	Tubular	Aggregated	Fragmented	
Stationary phase				
WT	13.0	0.6	86.4	828
$\Delta mfb1 / \Delta mfb1$	5.0	0.6	94.4	813
$\Delta mdm30 / \Delta mdm30$	1.5	1.3	97.2	800
Pretetrad				
WT	75.5	2.5	22.0	405
$\Delta mfb1 / \Delta mfb1$	62.8	3.0	34.2	404
$\Delta mdm30 / \Delta mdm30$	0.8	5.3	93.9	400
Early tetrad				
WT	91.6	3.5	4.9	286
$\Delta mfb1 / \Delta mfb1$	85.3	5.3	9.4	266
$\Delta mdm30 / \Delta mdm30$	4.1	14.2	81.7	169
Late tetrads				
WT	7.1	3.9	89.0	127
$\Delta mfb1 / \Delta mfb1$	6.4	1.3	92.3	156
$\Delta mdm30 / \Delta mdm30$	0	2.1	97.9	95

possibility 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 spores in early tetrads and fragment once again in late tetrads (Miyakawa *et al.*, 1984; Gorsich and Shaw, 2004; Figure 6A and Table 3). Although the behavior of mitochondria during sporulation is virtually identical in wild-type cells and cells lacking Mfb1, formation of a tubular mitochondrial network in pretetrads is blocked in the absence of Mdm30, and mitochondria remain fragmented throughout the entire sporulation process (Figure 6A and Table 3).

We asked whether the aberrant behavior of mitochondria in homozygous diploid $\Delta mdm30$ cells would result in a mitochondrial inheritance defect. To test this, spores devoid of mitochondria (Figure 6A, bottom) were counted in early and late tetrads. In wild-type cells, 6.5% of asci contained at least one spore without any visible mitochondrion. This number was slightly increased to 10.4% in $\Delta mfb1$ asci. Interestingly, the mitochondrial inheritance defect was much more severe in $\Delta mdm30$ asci, where 23.1% of the asci contained one or more spores devoid of mitochondria (Figure 6B). At the same time, sporulation efficiency was decreased in cells lacking Mdm30. Although 15.3% of wild-type cells and 15.8% of homozygous diploid $\Delta mfb1$ cells formed tetrads, only 5.3% of homozygous diploid $\Delta mdm30$ cells formed asci (Figure 6C). We conclude that Mdm30-dependent remodeling of mitochondria is important for mitochondrial inheritance during sporulation.

DISCUSSION

Even in a simple unicellular organism, such as yeast, 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 $\Delta mfb1 / \Delta mdm30$ double mutant does not show a more severe phenotype than a $\Delta mdm30$ 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 $\Delta fzo1/\Delta mdm30$ 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 $\Delta mdm30$ 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 $\Delta mdm30$ 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 (Neutznier and Youle, 2005) and mutations in proteasome subunit-encoding genes (Escobar-Henriques *et al.*, 2006) reduce the rate of degradation of Fzo1 in α 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 (Kinner and Kölling, 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 assembles 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 large-scale identification of yeast protein complexes by mass spectrometry (Ho *et al.*, 2002) and in coimmunoprecipitation 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 depletion 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 α factor-arrested cells does not require Mdm30 (Neutznier 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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REFERENCES

- Altmann, K., and Westermann, B. (2005). Role of essential genes in mitochondrial morphogenesis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 16, 5410–5417.
- Bauer, C., Herzog, V., and Bauer, M. F. (2001). Improved technique for electron microscope visualization of yeast membrane structure. *Microsc. Microanal.* 7, 530–534.
- Bereiter-Hahn, J. (1990). Behavior of mitochondria in the living cell. *Int. Rev. Cytol.* 122, 1–63.
- Bleazard, W., McCaffery, J. M., King, E. J., Bale, S., Mozdy, A., Tieu, Q., Nunnari, J., and Shaw, J. M. (1999). The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat. Cell Biol.* 1, 298–304.
- Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14, 115–132.
- Chen, H., and Chan, D. C. (2004). Mitochondrial dynamics in mammals. *Curr. Top. Dev. Biol.* 59, 119–144.
- Chen, H., Detmer, S. A., Ewald, A. J., Griffin, E. E., Fraser, S. E., and Chan, D. C. (2003). Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J. Cell Biol.* 160, 189–200.
- Chen, X. J., and Butow, R. A. (2005). The organization and inheritance of the mitochondrial genome. *Nat. Rev. Genet.* 6, 815–825.
- Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O., and Herskowitz, I. (1998). The transcriptional program of sporulation in budding yeast. *Science* 282, 699–705.
- Dimmer, K. S., Fritz, S., Fuchs, F., Messerschmitt, M., Weinbach, N., Neupert, W., and Westermann, B. (2002). Genetic basis of mitochondrial function and morphology in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 13, 847–853.
- Egner, A., Jakobs, S., and Hell, S. W. (2002). Fast 100 nm resolution 3D-microscope reveals structural plasticity of mitochondria in live yeast. *Proc. Natl. Acad. Sci. USA* 99, 3370–3375.
- Escobar-Henriques, M., Westermann, B., and Langer, T. (2006). Regulation of mitochondrial fusion by the F-box protein Mdm30 involves proteasome-independent turnover of Fzo1. *J. Cell Biol.* 173, 645–650.
- Eura, Y., Ishihara, N., Yokota, S., and Mihara, K. (2003). Two mitofusin proteins, mammalian homologues of FZO, with distinct functions are both required for mitochondrial fusion. *J. Biochem.* 134, 333–344.
- Fisk, H. A., and Yaffe, M. P. (1999). A role for ubiquitination in mitochondrial inheritance in *Saccharomyces cerevisiae*. *J. Cell Biol.* 145, 1199–1208.
- Fritz, S., Weinbach, N., and Westermann, B. (2003). Mdm30 is an F-box protein required for maintenance of fusion-competent mitochondria in yeast. *Mol. Biol. Cell* 14, 2303–2313.
- Galan, J. M., Wiederkehr, A., Seol, J. H., Haguenuer-Tsapis, R., Deshaies, R. J., Riezman, H., and Peter, M. (2001). Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. *Mol. Cell Biol.* 21, 3105–3117.
- Giaever, G., *et al.* (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 418, 387–391.
- Gietz, D., Jean, A. S., Woods, R. A., and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* 20, 1425.
- Gorsich, S. W., and Shaw, J. M. (2004). Importance of mitochondrial dynamics during meiosis and sporulation. *Mol. Biol. Cell* 15, 4369–4381.
- Griparic, L., and van der Bliek, A. M. (2001). The many shapes of mitochondrial membranes. *Traffic* 2, 235–244.
- Hales, K. G., and Fuller, M. T. (1997). Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell* 90, 121–129.
- Herlan, M., Bornhövd, C., Hell, K., Neupert, W., and Reichert, A. S. (2004). Alternative topogenesis of Mgm1 and mitochondrial morphology depend on ATP and a functional import motor. *J. Cell Biol.* 165, 167–173.

- Herlan, M., Vogel, F., Bornhövd, C., Neupert, W., and Reichert, A. S. (2003). Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. *J. Biol. Chem.* 278, 27781–27788.
- Hermann, G. J., Thatcher, J. W., Mills, J. P., Hales, K. G., Fuller, M. T., Nunnari, J., and Shaw, J. M. (1998). Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. *J. Cell Biol.* 143, 359–373.
- Ho, Y., et al. (2002). Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* 415, 180–183.
- Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature* 425, 686–691.
- Jagasia, R., Grote, P., Westermann, B., and Conradt, B. (2005). DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in *C. elegans*. *Nature* 433, 754–760.
- Jakobs, S., Martini, N., Schauss, A. C., Egner, A., Westermann, B., and Hell, S. W. (2003). Spatial and temporal dynamics of budding yeast mitochondria lacking the division component Fis1p. *J. Cell Sci.* 116, 2005–2014.
- Kinner, A., and Kölling, R. (2003). The yeast deubiquitinating enzyme Ubp16 is anchored to the outer mitochondrial membrane. *FEBS Lett.* 549, 135–140.
- Kondo-Okamoto, N., Ohkuni, K., Kitagawa, K., McCaffery, J. M., Shaw, J. M., and Okamoto, K. (2006). The novel F-box protein Mfb1p regulates mitochondrial connectivity and exhibits asymmetric localization in yeast. *Mol. Biol. Cell* 17, 3756–3767.
- Koshiba, T., Detmer, S. A., Kaiser, J. T., Chen, H., McCaffery, J. M., and Chan, D. C. (2004). Structural basis of mitochondrial tethering by mitofusin complexes. *Science* 305, 858–862.
- Kremer, J. R., Mastronarde, D. N., and McIntosh, J. R. (1996). Computer visualization of three-dimensional image data using IMOD. *J. Struct. Biol.* 116, 71–76.
- Kus, B. M., Caldon, C. E., Andorn-Broza, R., and Edwards, A. M. (2004). Functional interaction of 13 yeast SCF complexes with a set of yeast E2 enzymes in vitro. *Proteins* 54, 455–467.
- Lill, R., and Mühlenhoff, U. (2005). Iron-sulfur-protein biogenesis in eukaryotes. *Trends Biochem. Sci.* 30, 133–141.
- McQuibban, G. A., Saurya, S., and Freeman, M. (2003). Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. *Nature* 423, 537–541.
- Miyakawa, I., Aoi, H., Sando, N., and Kuroiwa, T. (1984). Fluorescence microscopic studies of mitochondrial nucleoids during meiosis and sporulation in the yeast, *Saccharomyces cerevisiae*. *J. Cell Sci.* 66, 21–38.
- Mnaimneh, S., et al. (2004). Exploration of essential gene functions via titratable promoter alleles. *Cell* 118, 31–44.
- Mozdy, A., McCaffery, J. M., and Shaw, J. M. (2000). Dnm1p GTPase-mediated mitochondrial fusion is a multi-step process requiring the novel integral membrane component Fis1p. *J. Cell Biol.* 151, 367–379.
- Mozdy, A. D., and Shaw, J. M. (2003). A fuzzy mitochondrial fusion apparatus comes into focus. *Nat. Rev. Mol. Cell Biol.* 4, 468–478.
- Muratani, M., Kung, C., Shokat, K. M., and Tansey, W. P. (2005). The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell* 120, 887–899.
- Neutzner, A., and Youle, R. J. (2005). Instability of the mitofusin Fzo1 regulates mitochondrial morphology during the mating response of the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280, 18598–18603.
- Nunnari, J., Marshall, W. F., Straight, A., Murray, A., Sedat, J. W., and Walter, P. (1997). Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol. Biol. Cell* 8, 1233–1242.
- Okamoto, K., Perlman, P. S., and Butow, R. A. (1998). The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. *J. Cell Biol.* 142, 613–623.
- Okamoto, K., and Shaw, J. M. (2005). Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. *Annu. Rev. Genet.* 39, 503–536.
- Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* 6, 9–20.
- Rapaport, D., Brunner, M., Neupert, W., and Westermann, B. (1998). Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 20150–20155.
- Rinaldi, T., Ricci, C., Porro, D., Bolotin-Fukuhara, M., and Frontali, L. (1998). A mutation in a novel yeast proteasomal gene, RPN11/MPR1, produces cell cycle arrest, overreplication of nuclear and mitochondrial DNA, and an altered mitochondrial morphology. *Mol. Biol. Cell* 9, 2917–2931.
- Rojo, M., Legros, F., Chateau, D., and Lombes, A. (2002). Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. *J. Cell Sci.* 115, 1663–1674.
- Santel, A., Frank, S., Gaume, B., Herrler, M., Youle, R. J., and Fuller, M. T. (2003). Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. *J. Cell Sci.* 116, 2763–2774.
- Scheffler, I. E. (2000). A century of mitochondrial research: achievements and perspectives. *Mitochondrion* 1, 3–31.
- Scott, S. V., Cassidy-Stone, A., Meeusen, S. L., and Nunnari, J. (2003). Staying in aerobic shape: how the structural integrity of mitochondria and mitochondrial DNA is maintained. *Curr. Opin. Cell Biol.* 15, 482–488.
- Sesaki, H., and Jensen, R. E. (1999). Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J. Cell Biol.* 147, 699–706.
- Sesaki, H., and Jensen, R. E. (2001). UGO1 encodes an outer membrane protein required for mitochondrial fusion. *J. Cell Biol.* 152, 1123–1134.
- Sesaki, H., and Jensen, R. E. (2004). Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. *J. Biol. Chem.* 279, 28298–28303.
- Sesaki, H., Southard, S. M., Hobbs, A. E., and Jensen, R. E. (2003). Cells lacking Pcp1p/Ugo2p, a rhomboid-like protease required for Mgm1p processing, lose mtDNA and mitochondrial structure in a Dnm1p-dependent manner, but remain competent for mitochondrial fusion. *Biochem. Biophys. Res. Commun.* 308, 276–283.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* 194, 3–21.
- Sherman, F., and Hicks, J. (1991). Micromanipulation and dissection of asci. *Methods Enzymol.* 194, 21–37.
- Sickmann, A., et al. (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. USA* 100, 13207–13212.
- Simon, V. R., Karmon, S. L., and Pon, L. A. (1997). Mitochondrial inheritance: cell cycle and actin cable dependence of polarized mitochondrial movements in *Saccharomyces cerevisiae*. *Cell Motil. Cytoskeleton* 37, 199–210.
- Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26, 31–43.
- Stevens, B. (1981). Mitochondrial structure. In: *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, ed. E. W. Strathern, E. W. Jones, and J. R. Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 471–504.
- Tatsuta, T., and Langer, T. (2006). Studying proteolysis within mitochondria. *Curr. Topics Gen. (in press)*.
- Uetz, P., et al. (2000). A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. *Nature* 403, 601–603.
- Visser, W., van Spronsen, E. A., Nanninga, N., Pronk, J. T., Gijs Kuenen, J., and van Dijken, J. P. (1995). Effects of growth conditions on mitochondrial morphology in *Saccharomyces cerevisiae*. *Antonie Van Leeuwenhoek* 67, 243–253.
- Westermann, B. (2002). Merging mitochondria matters. Cellular role and molecular machinery of mitochondrial fusion. *EMBO Rep.* 3, 527–531.
- Westermann, B., and Neupert, W. (2000). Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. *Yeast* 16, 1421–1427.
- Willems, A. R., Schwab, M., and Tyers, M. (2004). A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim. Biophys. Acta* 1695, 133–170.
- Wong, E. D., Wagner, J. A., Gorsich, S. W., McCaffery, J. M., Shaw, J. M., and Nunnari, J. (2000). The dynamin-related GTPase, Mgm1p, is an intermembrane space protein required for maintenance of fusion competent mitochondria. *J. Cell Biol.* 151, 341–352.
- Wong, E. D., Wagner, J. A., Scott, S. V., Okreglak, V., Holewinski, T. J., Cassidy-Stone, A., and Nunnari, J. (2003). The intramitochondrial dynamin-related GTPase, Mgm1p, is a component of a protein complex that mediates mitochondrial fusion. *J. Cell Biol.* 160, 303–311.
- Yaffe, M. P. (1999). The machinery of mitochondrial inheritance and behavior. *Science* 283, 1493–1497.
- Yang, H. C., Palazzo, A., Swayne, T. C., and Pon, L. A. (1999). A retention mechanism for distribution of mitochondria during cell division in budding yeast. *Curr. Biol.* 9, 1111–1114.
- Youle, R. J., and Karbowski, M. (2005). Mitochondrial fission in apoptosis. *Nat. Rev. Mol. Cell Biol.* 6, 657–663.