# Mdm31 and Mdm32 are inner membrane proteins required for maintenance of mitochondrial shape and stability of mitochondrial DNA nucleoids in yeast

Kai Stefan Dimmer,[1] Stefan Jakobs,[2] Frank Vogel,[3] Katrin Altmann,[4] and Benedikt Westermann[1,4]

[1]Institut für Physiologische Chemie, Universität München, 81377 München, Germany
[2]Department of NanoBiophotonics, Max-Planck-Institut für Biophysikalische Chemie, 37077 Göttingen, Germany
[3]Electron Microscopy Group, Max-Delbrück-Centrum für Molekulare Medizin, 13092 Berlin, Germany
[4]Zellbiologie, Universität Bayreuth, 95440 Bayreuth, Germany

The *MDM31* and *MDM32* genes are required for normal distribution and morphology of mitochondria in the yeast *Saccharomyces cerevisiae*. They encode two related proteins located in distinct protein complexes in the mitochondrial inner membrane. Cells lacking Mdm31 and Mdm32 harbor giant spherical mitochondria with highly aberrant internal structure. Mitochondrial DNA (mtDNA) is instable in the mutants, mtDNA nucleoids are disorganized, and their association with Mmm1-containing complexes in the outer membrane is abolished. Mutant mitochondria are largely immotile, resulting in a mitochondrial inheritance defect. Deletion of either one of the *MDM31* and *MDM32* genes is synthetically lethal with deletion of either one of the *MMM1*, *MMM2*, *MDM10*, and *MDM12* genes, which encode outer membrane proteins involved in mitochondrial morphogenesis and mtDNA inheritance. We propose that Mdm31 and Mdm32 cooperate with Mmm1, Mmm2, Mdm10, and Mdm12 in maintenance of mitochondrial morphology and mtDNA.

## Introduction

Mitochondria are ubiquitous and essential organelles of eukaryotic cells. Because they cannot be generated de novo, they have to be inherited during cell division (Warren and Wickner, 1996). Inheritance of mitochondria involves active transport of the organelles along cytoskeletal tracks, concomitant with frequent membrane division and fusion events (Yaffe, 1999). The mitochondrial genome, which encodes a small subset of mitochondrial proteins, has to be partitioned to the daughter cell in an active and ordered manner (Azpiroz and Butow, 1993; Okamoto et al., 1998; Berger and Yaffe, 2000; Garrido et al., 2003; Meeusen and Nunnari, 2003). As mitochondria are double membrane-bounded organelles, transport processes occurring at the mitochondrial surface and partitioning events of matrix components must be coordinated across two membranes. For example, mitochondrial DNA (mtDNA) is located in protein-containing complexes, termed nucleoids, in the matrix. It has been suggested that inheritance of these nucleoids requires a segregation machinery in the cytosol (Berger and Yaffe, 2000; Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003). Moreover, it is conceivable that maintenance of the structure of the inner membrane depends on an intimate coordination with the behavior of the outer membrane, involving interactions of proteins in both membranes. However, the molecular processes coordinating the behavior of the double membranes during mitochondrial inheritance are not well understood.

Mitochondria form highly dynamic interconnected networks in many cell types from yeast to man (Bereiter-Hahn, 1990; Nunnari et al., 1997; Jakobs et al., 2003). In recent years a growing number of proteins controlling mitochondrial motility and behavior have been identified, mainly in the baker's yeast *Saccharomyces cerevisiae* (Hermann and Shaw, 1998; Jensen et al., 2000; Scott et al., 2003). In yeast, establishment, maintenance, and motility of the branched mitochondrial network depend on the actin cytoskeleton (Boldogh et al., 2001). Some mitochondrial outer membrane proteins have been suggested to play a role in microfilament-dependent inheritance of mitochondria and mtDNA. Yeast mutants lacking Mdm10, Mdm12, or Mmm1 have giant spherical mitochondria (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997), which show severely compromised intracellular motility (Boldogh et al.,

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Correspondence to Benedikt Westermann: benedikt.westermann@uni-bayreuth.de

Abbreviations used in this paper: AAC, ADP/ATP carrier; mtDNA, mitochondrial DNA; mtGFP, mitochondria-targeted GFP; PK, proteinase K.


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1998, 2003). As these proteins are often localized next to mtDNA nucleoids, and as mtDNA nucleoids are disorganized in mutants, it has been proposed that Mdm10, Mdm12, and Mmm1 are parts of a cytoskeleton-dependent double membrane-spanning transport machinery required for inheritance of mitochondria and mtDNA (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003). Mmm2 (alternative name Mdm34) has been identified as another protein that participates in this process (Dimmer et al., 2002; Youngman et al., 2004). Mmm2 is located in a separate complex in the outer membrane, and mutants lacking Mmm2 harbor aberrant mitochondria and disorganized mtDNA nucleoids (Youngman et al., 2004).

It can be predicted that there must be partners in the inner membrane that physically and/or functionally interact with the outer membrane proteins Mmm1, Mmm2, Mdm10, and Mdm12 in mediating the inheritance of mitochondrial membranes and mtDNA nucleoids. It has been suggested that Mmm1 in yeast spans both mitochondrial membranes and exposes a small $NH_2$-terminal segment to the matrix (Kondo-Okamoto et al., 2003). However, the $NH_2$-terminal extension is absent in other homologous proteins, such as MMM1 in *Neurospora crassa* (Prokisch et al., 2000), and it is not required for maintenance of normal tubular networks and mtDNA nucleoids in yeast (Kondo-Okamoto et al., 2003). Thus, there must be other, yet unknown, inner membrane proteins participating in these processes. By screening a comprehensive yeast gene deletion library, we recently isolated several novel genes important for mitochondrial distribution and morphology, MDM (Dimmer et al., 2002). Here, we show that *MDM31* and *MDM32* encode novel components of the mitochondrial inner membrane. We propose that Mdm31 and Mdm32 functionally cooperate with the outer membrane machinery mediating maintenance of mitochondrial morphology and inheritance of mtDNA.

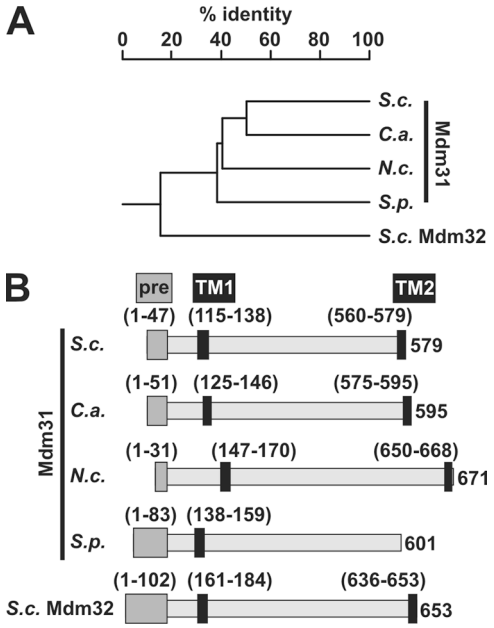



Figure 1. **Mdm31 and Mdm32 are members of a novel protein family.** (A) Homology tree of the Mdm31 protein family. Homologous proteins were identified by BLAST search (Altschul et al., 1997), and the tree was constructed using DNAMAN software (Lynnon BioSoft). Genome annotation numbers are Ca49C10.08 for *Candida albicans*, NCU07955.1 for *N. crassa*, and SPAC3H1.04c for *S. pombe*. (B) Domain structure of Mdm31 protein family members. Mitochondrial presequences (pre; indicated by gray boxes) were predicted using the MitoProt II program (Claros and Vincens, 1996). Transmembrane helices (TM; indicated by black boxes) were predicted using the TMpred program (Hofmann and Stoffel, 1993). Numbers of amino acid residues defining the borders of predicted domains are indicated; domains are drawn to scale.

## Results

### *MDM31* and *MDM32* encode two members of a novel protein family

The *MDM31* (systematic name *YHR194W*) and *MDM32* (systematic name *YOR147W*) genes encode two related proteins of 66.7 and 75.6 kD, respectively. Both proteins share 16.4% amino acid identity with each other. Related genes encoding homologous proteins can be found in the genomes of *Candida albicans*, *Schizosaccharomyces pombe*, *N. crassa*, and other ascomycetes fungi (Fig. 1 A; for an alignment see online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200410030/DC1). Remarkably, these more distantly related fungi have only one homologous gene, which is more closely related to *MDM31* (between 27.8% amino acid identity for *S. pombe* and 52.3% for *C. albicans*). Other species of the family *Saccharomycetaceae* have two related isoforms (Cliften et al., 2003; Kellis et al., 2003). Thus, the second isoform apparently has arisen by a relatively recent gene duplication event.

All members of the Mdm31 protein family have a similar domain structure (Fig. 1 B). The $NH_2$ termini have the characteristics of typical mitochondrial presequences. They are rich in positively charged residues, lack acidic charges, and have a high content of hydroxylated residues. Computational prediction of mitochondrial presequences by the MitoProt II program (Claros and Vincens, 1996) gives very high probabilities for mitochondrial targeting (between 0.9518 for *S. pombe* Mdm31 and 0.9989 for Mdm32). Hydropathy analysis (Hofmann and Stoffel, 1993) predicts two transmembrane segments, one close to the $NH_2$ terminus of the matured protein and another one at the very COOH terminus. The predicted domain structure is very similar for all five family members with the exception of the *S. pombe* protein that lacks a hydrophobic segment at its COOH terminus.

### Mdm31 and Mdm32 are located in the mitochondrial inner membrane

To determine the intracellular location of Mdm31 and Mdm32, wild-type yeast cells were fractionated into mitochondria, microsomes, and cytosol. Cell fractions were analyzed by Western blotting using specific antisera against Mdm31 and Mdm32. Both proteins cofractionated with the mitochondrial ADP/ATP carrier (AAC; Fig. 2 A), demonstrating a mitochondrial location. To determine the intramitochondrial location, isolated mitochondria were subfractionated. When intact mitochondria were treated with proteinase K (PK), both Mdm31 and Mdm32 were protected against proteolytic degradation (Fig. 2 B, lane 2), indicating that they are located in the interior

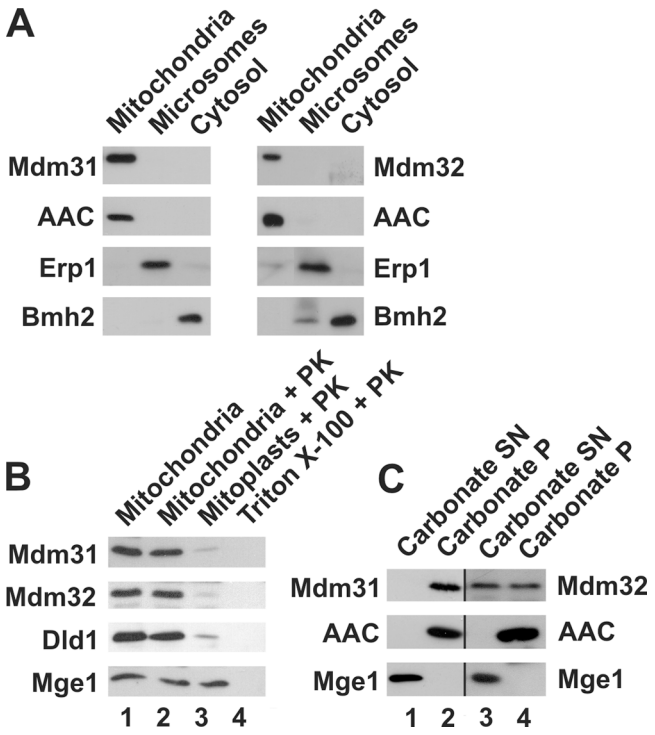



Figure 2. **Mdm31 and Mdm32 are located in the mitochondrial inner membrane.** (A) Subfractionation of yeast cells. Wild-type cells were subfractionated by differential centrifugation into mitochondria, microsomes, and cytosol. Mitochondria were further purified on a sucrose gradient, microsomes were purified on a percoll gradient. 50 μg of protein of each fraction was analyzed by Western blotting. The ADP/ATP carrier (AAC) served as a marker for mitochondria, Erp1 for ER, and Bmh2 for soluble cytosolic proteins. White lines indicate that intervening lanes have been spliced out. (B) Subfractionation of mitochondria. Isolated wild-type mitochondria were subfractionated, proteins were precipitated with TCA, and 50 μg of each fraction was analyzed by Western blotting. Lane 1, intact mitochondria; lane 2, intact mitochondria treated with proteinase K (PK); lane 3, mitoplasts generated by hypotonic swelling and treated with PK; lane 4, mitochondria solubilized with Triton X-100 and treated with PK. Markers used were Dld1 as an inner membrane protein exposed to the intermembrane space and Mge1 as a soluble matrix protein. (C) Carbonate fractionation of mitochondria. Isolated wild-type mitochondria were extracted with carbonate, proteins were precipitated with TCA, and 50 μg of each fraction was analyzed by Western blotting. Lanes 1 and 3, soluble protein–containing fraction after carbonate extraction (supernatant, SN); lanes 2 and 4, membrane protein–containing fraction after carbonate extraction (P, pellet). Markers used were AAC as an integral inner membrane protein and Mge1 as a soluble matrix protein.

of the organelle. When the outer membrane was selectively opened by hypotonic swelling, Mdm31 and Mdm32 were accessible to PK (Fig. 2 B, lane 3), indicating that a major domain is exposed to the intermembrane space. When the mitochondrial membranes were lysed with detergent, the proteins were completely degraded by PK (Fig. 2 B, lane 4). Upon carbonate extraction, all of Mdm31 and about half of Mdm32 cofractionated with mitochondrial membranes (Fig. 2 C), demonstrating that they are integral membrane proteins. It should be noted that partial extraction by carbonate has been observed also for other mitochondrial membrane proteins (Mokranjac et al., 2003). We conclude that Mdm31 and Mdm32 are located in the mitochondrial inner membrane. Protected fragments in protease-treated mitoplasts could never be observed in immunoblots of endogenous protein or after in vitro import of radiolabeled protein (unpublished data). We suggest that major parts of Mdm31 and Mdm32 are located in the intermembrane space, and the short $NH_2$ termini are exposed to the matrix.

### Cells lacking Mdm31 and Mdm32 show severe defects in mitochondrial distribution and morphology

To examine the role of Mdm31 and Mdm32 in mitochondrial distribution and morphology, Δ*mdm31* and Δ*mdm32* deletion mutants and a Δ*mdm31*/Δ*mdm32* double mutant were constructed. All mutants were viable, both on fermentable and nonfermentable carbon sources (see section Mdm31 and Mdm32 are required for organization of mtDNA nucleoids) and showed identical phenotypes. Examination of mutant strains expressing mitochondria-targeted GFP (mtGFP) by confocal microscopy revealed highly aberrant mitochondrial structures (Fig. 3 A). Most of the cells harbored one or few giant spherical mitochondria (Fig. 3 A, b, c, e, and f). Often, the organelles contained one or few small hollow inclusions (Fig. 3 A, e and f). Some cells contained several, relatively small mitochondria (Fig. 3 A, d). Branched tubular mitochondrial networks resembling the wild type (Fig. 3 A, a) were not observed in the mutants. A quantification of mitochondrial phenotypes is given in Table I.

Many mutant cells generated buds that were devoid of mitochondria. Occasionally, a giant mitochondrion was positioned at the bud neck, which it could not pass (Fig. 3 A, b). To quantify these effects, logarithmically growing cultures of mtGFP-expressing cells were analyzed by fluorescence microscopy. Cells were counted that showed mitochondria both in the mother and the daughter cell, mitochondria stuck at the bud neck, or mitochondria-free buds. For comparison, we included in this analysis a Δ*mmm1* strain, which has giant spherical mitochondria (Burgess et al., 1994) similar to the Δ*mdm31* and Δ*mdm32* mutants. Only 50–60% of the buds contained mitochondria in Δ*mdm31*, Δ*mdm32*, Δ*mdm31*/Δ*mdm32*, and Δ*mmm1* cells. In most of the remaining cells, mitochondria were somewhere deposited in the mother cell. A few percent of the cells showed mitochondria positioned directly at the bud neck (Table II).

To exclude the possibility that the observed phenotypes were caused by defects in the organization of the actin cytoskeleton, or that deletion of the *MDM31* and *MDM32* genes has pleiotropic effects on the structure of several cell organelles, we stained filamentous actin, the ER, and vacuoles. All these structures appeared normal in the mutants (Fig. 3 B). We conclude that Mdm31 and Mdm32 play an important and specific role in controlling mitochondrial distribution and morphology.

It has been speculated that Mdm31 and Mdm32 might be novel components of the mitochondrial membrane fusion machinery (Mozdy and Shaw, 2003). To test this possibility, we monitored fusion of mitochondria in vivo by mating of mutant cells preloaded with different fluorescent mitochondrial markers (Nunnari et al., 1997). Mixing of the markers could be observed in zygotes lacking Mdm31 or Mdm32, as well as in zygotes lacking both proteins (Fig. 3 C). This demonstrates that

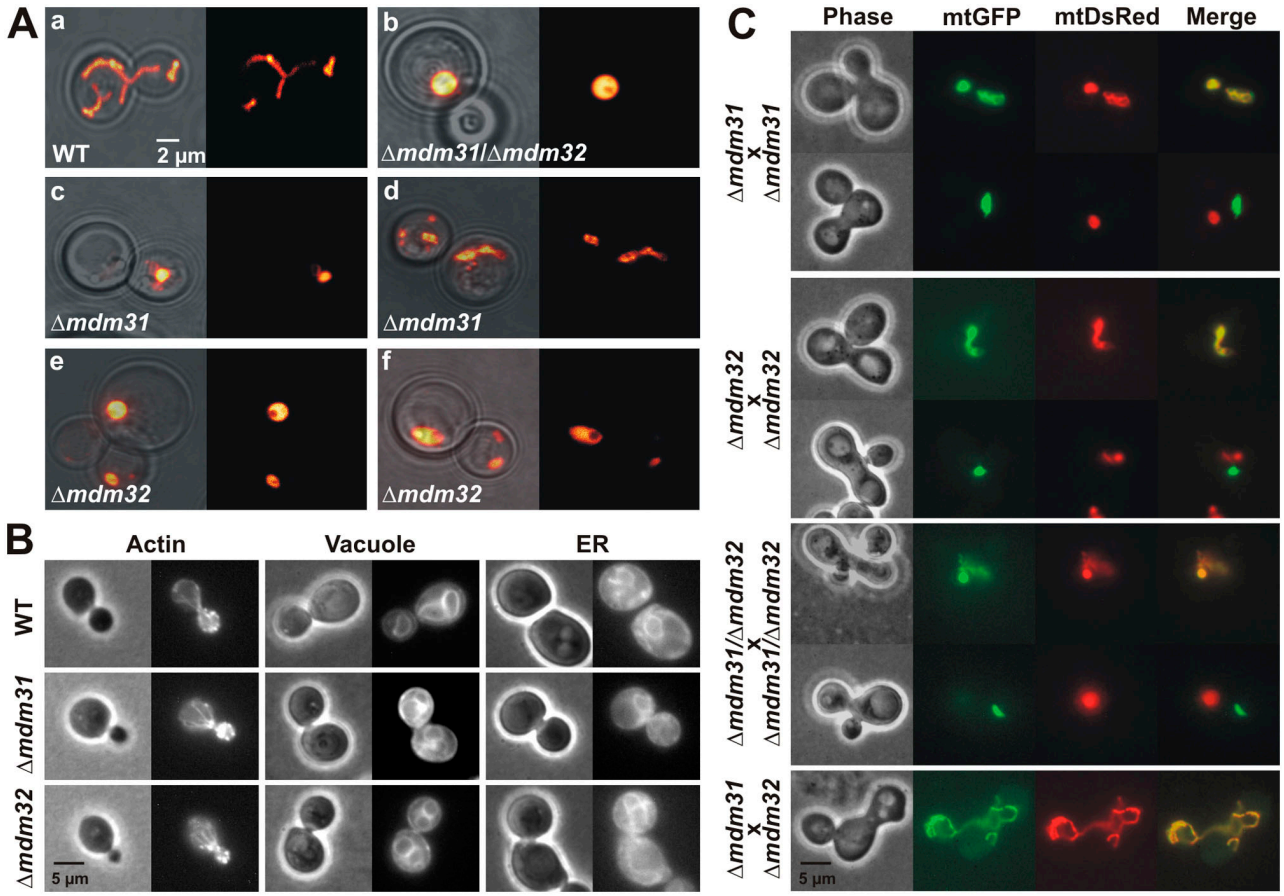



Figure 3. **Cells lacking Mdm31 and Mdm32 harbor aberrant mitochondria.** (A) Mitochondrial morphology. Wild-type (a), Δ*mdm31* (c and d), Δ*mdm32* (e and f), and Δ*mdm31*/Δ*mdm32* (b) cells expressing mtGFP were grown to log phase in YPD (yeast extract, peptone, and glucose) medium and analyzed by confocal fluorescence microscopy. (left) Maximum intensity projections of several optical planes covering the entire cell, merged with a bright field transmission image. (right) A representative single optical plane. (B) Morphology of microfilaments, vacuole, and the ER. Cells were grown to log phase in glucose-containing medium. Then, they were either fixed and stained with rhodamine-phalloidin (for actin), or living cells were stained with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (for vacuole), or cells expressing ER-targeted GFP were examined directly. Left, phase-contrast image; right, fluorescence microscopy. (C) Mitochondrial fusion. Cells of opposite mating type preloaded with mtGFP or mitochondria-targeted DsRed (mtDsRed) were mated, and zygotes were analyzed by phase-contrast and fluorescence microscopy.

Mdm31 and Mdm32 do not play an essential role in mitochondrial fusion. Interestingly, several zygotes were found in which the fluorescently labeled mitochondria of both parental cells remained separate (Fig. 3 C). However, these nonfused mitochondria were never seen close together. This observation suggests that in the latter cases fusion did not occur because the mitochondria did not approach each other. Heterologous crosses of Δ*mdm31* and Δ*mdm32* single deletion mutants showed complementation in zygotes, i.e., mitochondria looked like wild type and fused in an efficient manner (Fig. 3 C). We suggest that the function of Mdm31 and Mdm32 is required for efficient fusion in cells, even though these proteins are not integral components of the mitochondrial fusion machinery.

### Mitochondria lacking Mdm31 and Mdm32 show dramatically altered internal structure

As Mdm31 and Mdm32 are inner membrane proteins, we considered it likely that also the internal structure of mutant mitochondria is altered. To examine this possibility, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells were examined by electron microscopy and compared with the wild type. Electron micrographs of wild-type cells grown on glucose-containing medium showed characteristic cross sections of tubular mitochondria containing cristae as invaginations of the inner membrane (Fig. 4 A). In contrast, the ultrastructure of Δ*mdm31* (Fig. 4 C), Δ*mdm32* (Fig. 4, B and D), and Δ*mdm31*/Δ*mdm32* (Fig. 4, E–H) mutant cells was dramatically altered. The organelles were generally very large. These giant organelles were largely devoid of cristae. Only in some organelles a few small cristae were found (Fig. 4 E, arrows). Frequently, circular-shaped double membrane structures were seen inside the organelles (Fig. 4, B–G). These structures were of varying sizes, but the spacing between the membranes was remarkably constant and was identical to the size of the intermembrane space. This finding suggests that the double membranes were derived from the mitochondrial outer and inner membranes, and that the compartment surrounded by the circular membranes topologically corresponds to the exterior of the organelle. Consistently, these structures appeared as holes in sections obtained by confo-

Table I. **Quantification of mitochondrial morphology in Δ*mdm31* and Δ*mdm32* mutant cells**

| Strain | Mitochondrial morphology (percentage of cells) | | | | | |
|---|---|---|---|---|---|---|
| | Wild type–like | Spherical | Ring-like/with holes | Aggregated/fragmented | Elongated/tubular | Net-like |
| WT | 100 | - | - | - | - | - |
| Δ*mdm31* | - | 54 | 32 | 6 | 8 | - |
| Δ*mdm32* | - | 54 | 39 | - | 7 | - |
| Δ*mdm31*/Δ*mdm32* | - | 73 | 21 | - | 6 | - |
| Δ*mdm33* | - | 7 | 77 | 7 | 9 | - |
| Δ*mdm31*/Δ*mdm33* | - | 68 | 27 | 1 | 4 | - |
| Δ*mdm32*/Δ*mdm33* | - | 78 | 21 | 1 | - | - |
| Δ*dnm1* | - | - | - | - | 3 | 97 |
| Δ*mdm31*/Δ*dnm1* | - | 75 | 12 | 4 | 9 | - |
| Δ*mdm32*/Δ*dnm1* | - | 58 | 35 | - | 6 | 1 |
| Δ*fzo1* | - | 1 | - | 99 | - | - |
| Δ*mdm31*/Δ*fzo1* | - | 67 | 17 | 14 | 2 | - |
| Δ*mdm32*/Δ*fzo1* | - | 66 | 26 | 7 | 1 | - |

$n > 100$.

cal microscopy (Fig. 3 A). Occasionally, an internal membrane was connected with the inner membrane surrounding the organelle (Fig. 4, D, G, and H). In these cases, the intermembrane space was continuous with the space between the membranes of the circular inclusion (Fig. 4 G, arrows; enlarged image in Fig. 4 H). We conclude that deletion of the *MDM31* and *MDM32* genes has dramatic consequences on the organization of the mitochondrial membranes and the global structure of the organelle.

### Mdm31 and Mdm32 are required for normal mitochondrial motility

Two lines of evidence suggested that motility of mitochondria is compromised in cells lacking Mdm31 and Mdm32. First, mutant cells often carried buds devoid of mitochondria (Fig. 3 A and Table II), and second, in many mutant zygotes, mitochondria did not fuse because they did not approach each other (Fig. 3 C). To examine mitochondrial movement directly, the behavior of mitochondria was followed over time by confocal time-lapse microscopy of mtGFP-expressing cells. Cells grown logarithmically in glucose-containing medium were transferred to a microscope chamber that was continuously flushed with fresh medium. Wild-type mitochondria are highly dynamic under these experimental conditions (Jakobs et al., 2003). Their shapes and positions were observed to change completely within a few minutes (Fig. 5 A). In contrast, mutant mitochondria of Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* strains were almost immotile. They hardly changed their positions within time periods of 15 to 30 min (Fig. 5, B–D). However, sometimes subtle shape changes were observed in the mutant mitochondria. Generally, these shape changes started with the occurrence of small protrusions (Fig. 5, B–D, arrows), probably by a force pulling on the organelle. In most cases, these protrusions were retracted soon afterwards (Fig. 5, C and D). Together, these results demonstrate that mitochondrial motility is severely compromised in Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutants.

Sometimes, the initial deformation resulted in a successful translocation event of the entire organelle (Fig. 5 B). To test whether or not the residual translocation activity might be mediated by the actin-dependent transport machinery of mitochondria, we examined the binding of mitochondria to actin filaments in vitro. Isolated mitochondria of wild type, Δ*mdm31*, and Δ*mdm32* strains were incubated with filamentous actin in the presence or absence of ATP. Then, mitochondria were sedimented by centrifugation through a sucrose cushion, and bound actin was detected by immunoblotting. Mitochondria lacking Mdm31 or Mdm32 were able to interact with actin filaments in an ATP-dependent manner, similar to wild-type mitochondria (Fig. 5 E). This suggests that mitochondrial motility defects in the mutants are due to structural aberrations of the organelle rather than defects of the machinery mediating interactions with the cytoskeleton.

Table II. **Quantification of mitochondria-free buds in Δ*mdm31* and Δ*mdm32* mutant cells**

| Strain | Bud with mitochondria | Mitochondria stuck at bud neck | Mitochondria-free buds |
|---|---|---|---|
| | *% of cells* | *% of cells* | *% of cells* |
| WT | 99 | - | 1 |
| Δ*mdm31* | 55 | 4 | 41 |
| Δ*mdm32* | 67 | 2 | 31 |
| Δ*mdm31*/Δ*mdm32* | 60 | 8 | 32 |
| Δ*mmm1* | 54 | 10 | 36 |

$n > 100$.

### Δ*mdm31* and Δ*mdm32* mutations are epistatic to Δ*fzo1*, Δ*dnm1*, and Δ*mdm33* mutations

To investigate functional relationships of *MDM31* and *MDM32* with other genes encoding components important for mitochondrial structure and behavior, we constructed a series of double mutants. Δ*mdm31* and Δ*mdm32* strains were crossed with the following deletion strains: Δ*fzo1*, a mutant defective in mitochondrial fusion (Hermann et al., 1998; Rapaport et al., 1998); Δ*dnm1*, a mutant defective in outer membrane division (Otsuga et al., 1998); and Δ*mdm33*, a mutant defective in inner mem-

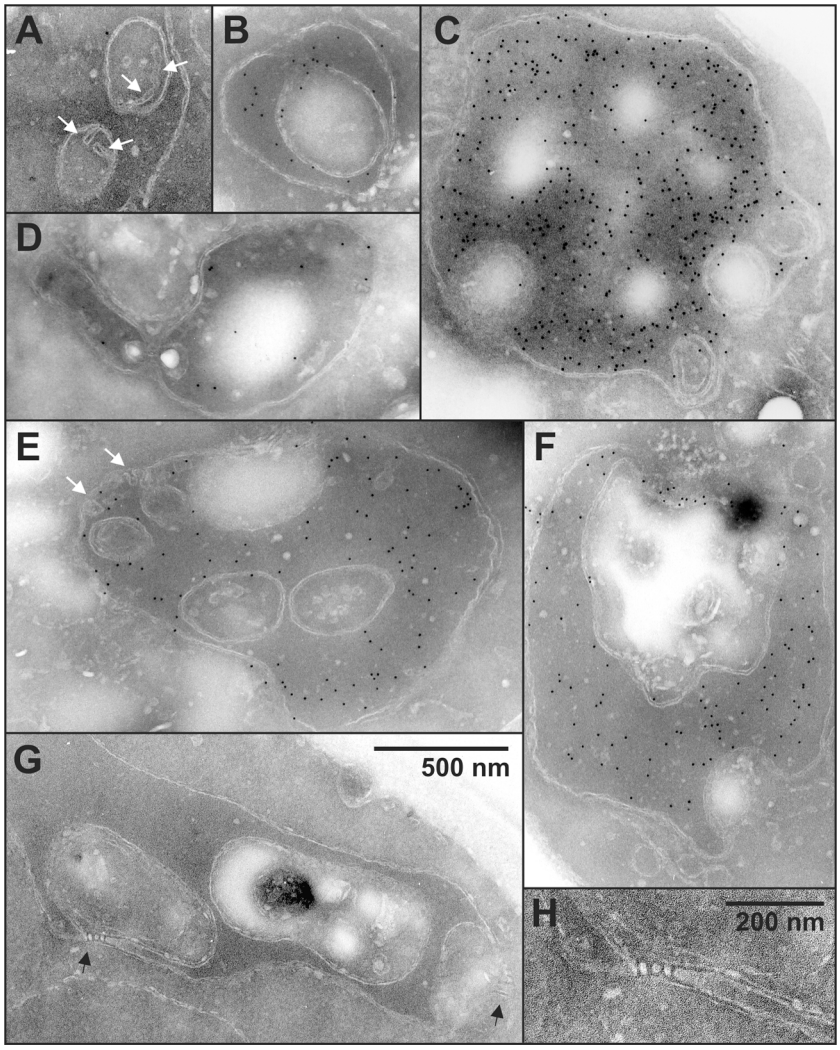



Figure 4. **Ultrastructure of mitochondria in cells lacking Mdm31 and Mdm32.** (A) Cross section of mitochondria in wild-type cells. (C) Cross section of a giant mitochondrion in a Δ*mdm31* cell. (B and D) Cross sections of mitochondria in Δ*mdm32* cells. (E–H) Cross sections of mitochondria in Δ*mdm31*/Δ*mdm32* cells. (A–F) mtGFP was labeled with immunogold to identify the matrix compartment. White arrows in A and E point to inner membrane cristae; black arrows in G point to membrane bridges between the inner membrane und circular inclusions. All images are displayed at the same magnification with the exception of H, which is an enlargement of G.

brane division (Messerschmitt et al., 2003). Resulting diploids were subjected to tetrad dissection, and mitochondrial morphology of haploid progeny was analyzed by fluorescence microscopy. In all cases, the parental mutants had clearly distinguishable phenotypes. Double mutants obtained from all crosses displayed mitochondria indistinguishable from their Δ*mdm31* and Δ*mdm32* parents (Table I). This finding indicates that the Δ*mdm31* and Δ*mdm32* mutations are epistatic to Δ*fzo1*, Δ*dnm1*, and Δ*mdm33* mutations; i.e., in the absence of Mdm31 or Mdm32, mitochondrial morphology does not depend on Fzo1, Dnm1, or Mdm33. We propose that the function of Mdm31 and Mdm32 is superior to mitochondrial fusion and division.

### Δ*mdm31* and Δ*mdm32* mutations are synthetically lethal with Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* mutations

We asked if *MDM31* and *MDM32* have overlapping functions with *MMM1*, *MMM2*, *MDM10*, and *MDM12*, because mutants lacking these genes have very similar phenotypes (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998, 2003; Aiken Hobbs et al., 2001; Youngman et al., 2004). Δ*mdm31* and Δ*mdm32* mutants were crossed with Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* strains. Upon tetrad dissection, we observed in all crosses a 1:1:4 segregation into parental ditype tetrads, nonparental ditype tetrads, and tetratype tetrads (Table III). Spores containing both deleted alleles were not viable; i.e., Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* mutations are synthetically lethal with Δ*mdm31* and Δ*mdm32* mutations. Synthetic lethality of two mutations in different genes often indicates that the gene products are required for the same cellular processes (Guarente, 1993; Hartman et al., 2001). The synthetic lethal phenotype was confirmed in a plasmid shuffling experiment using the Δ*mdm32*/Δ*mmm1* double mutant (unpublished data). These results show that the function of Mdm31 and Mdm32 is essential for cell viability in the absence of Mmm1, Mmm2, Mdm10, and Mdm12.

### Mdm31 and Mdm32 are required for organization of mtDNA nucleoids and localization of mtDNA adjacent to Mmm1 foci

Using strains obtained from the yeast gene deletion collection, we reported previously that Δ*mdm31* and Δ*mdm32* mutants are

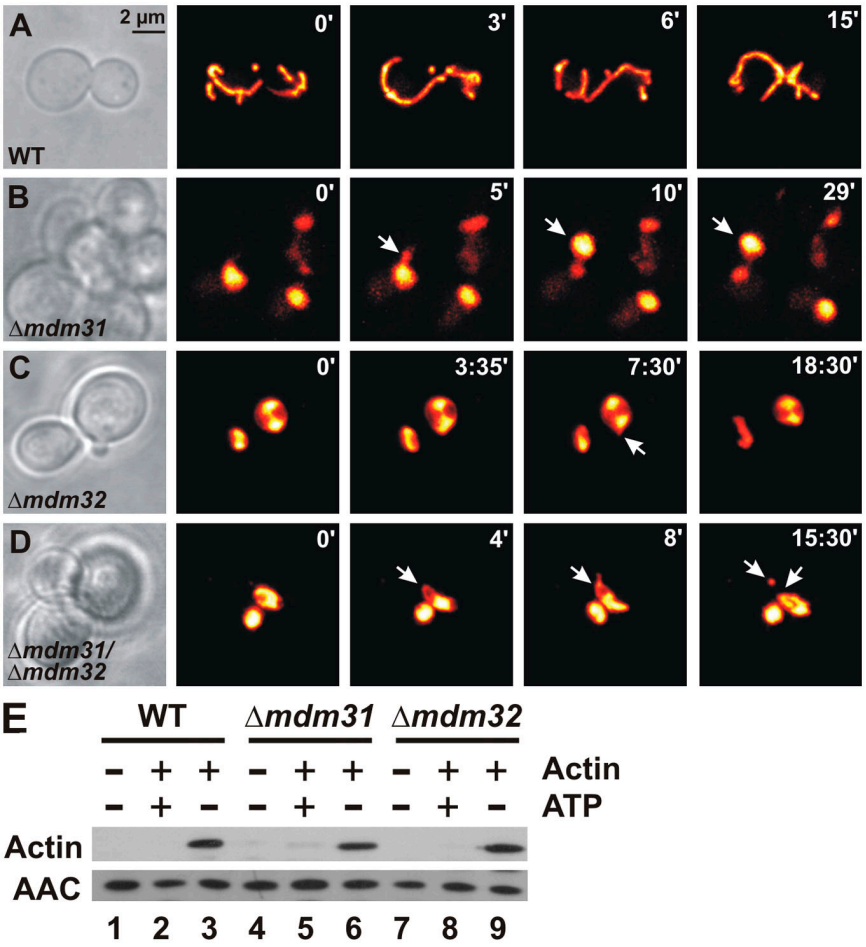



Figure 5. **Movement of mitochondria is compromised in cells lacking Mdm31 and Mdm32.** (A) Wild-type cells expressing mtGFP were grown to log phase in glucose-containing medium, transferred to a microscope chamber that was continuously flushed with fresh medium, and analyzed by confocal time-lapse microscopy. Left, bright field image; right, representative time points of the remodelling process shown as maximum intensity projections of several optical planes. Δ*mdm31* (B), Δ*mdm32* (C), and Δ*mdm31*/Δ*mdm32* (D) cells were analyzed as in A. Arrows point to shape changes of aberrant organelles. (E) Interaction of mitochondria with actin filaments in vitro. Isolated mitochondria of wild-type (WT), Δ*mdm31*, and Δ*mdm32* cells were incubated without or with isolated actin filaments in the presence or absence of ATP. After centrifugation of mitochondria through a sucrose cushion, bound actin was detected by immunoblotting. The mitochondrial protein AAC served as a loading control.

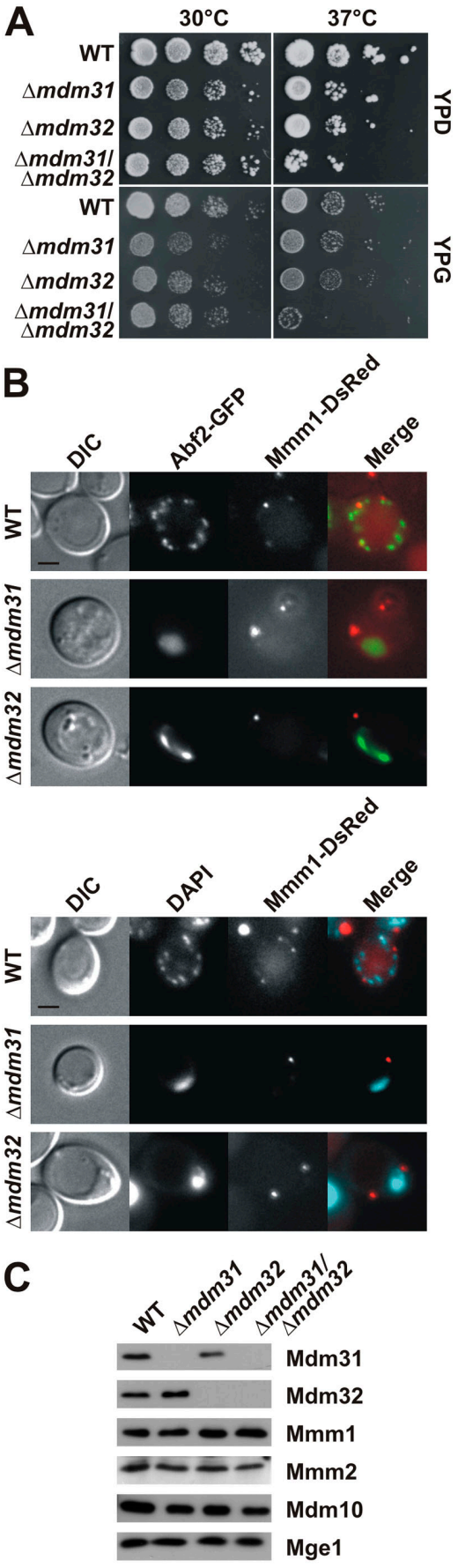
respiratory-deficient (Dimmer et al., 2002). Here, we observed that it is possible to grow newly made Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutants on nonfermentable carbon sources. Serial dilutions of wild-type and mutant cultures were spotted onto plates containing either glucose or glycerol as carbon source and incubated at 30 or 37°C. Mutant strains showed a moderate growth defect under most conditions, and the Δ*mdm31*/Δ*mdm32* double mutant showed a more severe growth defect on nonfermentable carbon sources at elevated temperature (Fig. 6 A). As inheritance of mtDNA depends on the integrity of the mitochondrial compartment (Berger and Yaffe, 2000), we reasoned that our initial observation of a petite phenotype in the mutants may be due to the gradual loss of the mitochondrial genome over several generations. To test this idea, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* strains were grown in liquid medium containing glucose to allow for loss of mtDNA. Cultures were maintained in the logarithmic growth phase at 30°C. At different time points, aliquots were taken and plated at an appropriate dilution onto glucose-containing medium. Subsequently, colonies were replica-plated onto glycerol-containing medium, and the percentage of colonies able to grow was determined as a measure of the fraction of respiratory-competent cells in the initial culture. After 3 d in glucose-containing medium, only ~50% of the mutant cells were respiratory-competent (very similar numbers were obtained for all three mutant strains). We conclude that Mdm31 and Mdm32 are required for normal inheritance of mtDNA.

We asked whether or not mtDNA is normally organized and distributed in cells lacking Mdm31 and Mdm32. mtDNA was stained in living cells by expressing an Abf2-GFP fusion protein, which binds to mtDNA nucleoids (Okamoto et al., 1998), or with a DNA-specific dye, DAPI. Wild-type cells contained normal mtDNA nucleoids, seen as 10–20 small fluores-

Table III. **Tetrad analyses**

| | Parental ditype | Nonparental ditype | Tetratype |
|---|---|---|---|
| Expected | 1 | 1 | 4 |
| Δ*mdm31* × Δ*mmm1* | 0.92 (*n* = 4) | 0.92 (*n* = 4) | 4.16 (*n* = 18) |
| Δ*mdm32* × Δ*mmm1* | 1.37 (*n* = 8) | 0.69 (*n* = 4) | 3.94 (*n* = 23) |
| Δ*mdm31* × Δ*mmm2* | 0.9 (*n* = 9) | 1.5 (*n* = 15) | 3.6 (*n* = 36) |
| Δ*mdm32* × Δ*mmm2* | 0.86 (*n* = 5) | 0.34 (*n* = 2) | 4.78 (*n* = 28) |
| Δ*mdm31* × Δ*mdm10* | 1.24 (*n* = 6) | 1.03 (*n* = 5) | 3.72 (*n* = 18) |
| Δ*mdm32* × Δ*mdm10* | 0.5 (*n* = 3) | 1.33 (*n* = 8) | 4.17 (*n* = 25) |
| Δ*mdm31* × Δ*mdm12* | 0.77 (*n* = 4) | 0.58 (*n* = 3) | 4.65 (*n* = 24) |
| Δ*mdm32* × Δ*mdm12* | 0.6 (*n* = 3) | 1.2 (*n* = 6) | 4.2 (*n* = 21) |

Parental ditype, two wild-type spores and two non-viable double mutant spores; nonparental ditype, four single mutant spores (two of each type), all four spores viable; tetratype, one wild-type spore, two single mutant spores (one of each type), and one non-viable double mutant spore. The ratios of the observed classes and the total number of tetrads for each class are indicated.




cent dots (Fig. 6 B). In contrast, Δ*mdm31* and Δ*mdm32* mutants contained only one or two misshapen mtDNA-containing structures per cell. The staining pattern of these structures was diffuse, and they were generally rather large (Fig. 6 B). Very similar results were obtained after DAPI staining of methanol-fixed cells (unpublished data). We conclude that Mdm31 and Mdm32 are required for establishment and/or maintenance of mtDNA nucleoid structure.

It has been reported that Mmm1 is located in distinct foci on the mitochondrial outer membrane. These foci are often found next to mtDNA nucleoids (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003), and their formation depends on the presence of the outer membrane protein Mmm2 (Youngman et al., 2004). It is thought that Mmm1-containing foci (in cooperation with yet unknown inner membrane proteins) contribute to the structural organization and inheritance of mtDNA nucleoids (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003; Youngman et al., 2004). The aberrant mtDNA nucleoids seen in Δ*mdm31* and Δ*mdm32* mutants and the genetic interactions with Δ*mmm1* prompted us to investigate whether the formation of Mmm1 foci and/or their localization next to mtDNA depends on the presence of Mdm31 and Mdm32. First, we tested whether or not the steady-state level of Mmm1 is altered in mitochondria of cells lacking Mdm31 and Mdm32. Immunoblot analysis showed that Mmm1 was present in similar amounts in mitochondria isolated from wild-type, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells (Fig. 6 C). The same result was obtained for Mmm2 and Mdm10 (Fig. 6 C). Mdm12 is required for localization of Mmm1 to mitochondria (Boldogh et al., 2003). As the level of Mmm1 was not changed in Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutant mitochondria, we conclude that also Mdm12 must be present in sufficient amounts. Thus, synthesis, mitochondrial targeting, and stability of Mmm1, Mmm2, Mdm10, and Mdm12 are not compromised in Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* mutants.

Next, we analyzed the intracellular distribution of Mmm1 and mtDNA by fluorescence microscopy. Consistent with previous reports (Aiken Hobbs et al., 2001; Meeusen and Nunnari,

Figure 6. **Mdm31 and Mdm32 are required for maintenance of normal mtDNA nucleoids.** (A) Growth phenotypes. Wild-type, Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells were grown overnight in glucose-containing medium. 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 for 3 d at the indicated temperatures. (B) Nucleoid structure and localization of Mmm1-containing complexes. Wild-type, Δ*mdm31*, and Δ*mdm32* cells expressing an Mmm1-DsRed fusion protein under control of the *MMM1* promoter were grown to log phase in glucose-containing medium. For staining of mtDNA nucleoids, expression of an Abf2-GFP fusion protein under control of a *GAL*-promoter was induced by shifting the cells to galactose-containing medium for 1 h (top), or cells were incubated in the presence of 1 μg/ml DAPI for 15 min. It should be noted that nuclear DNA is not stained under these conditions (Aiken Hobbs et al., 2001). Cells were washed in glucose-containing medium and analyzed by differential interference contrast (DIC) and fluorescence microscopy. Bars, 2 μm. (C) Steady-state levels of mitochondrial proteins. Mitochondria were isolated from wild-type (WT), Δ*mdm31*, Δ*mdm32*, and Δ*mdm31*/Δ*mdm32* cells, and equal amounts of mitochondrial protein were analyzed by immunoblotting using the indicated antisera. The mitochondrial matrix protein Mge1 served as a loading control.

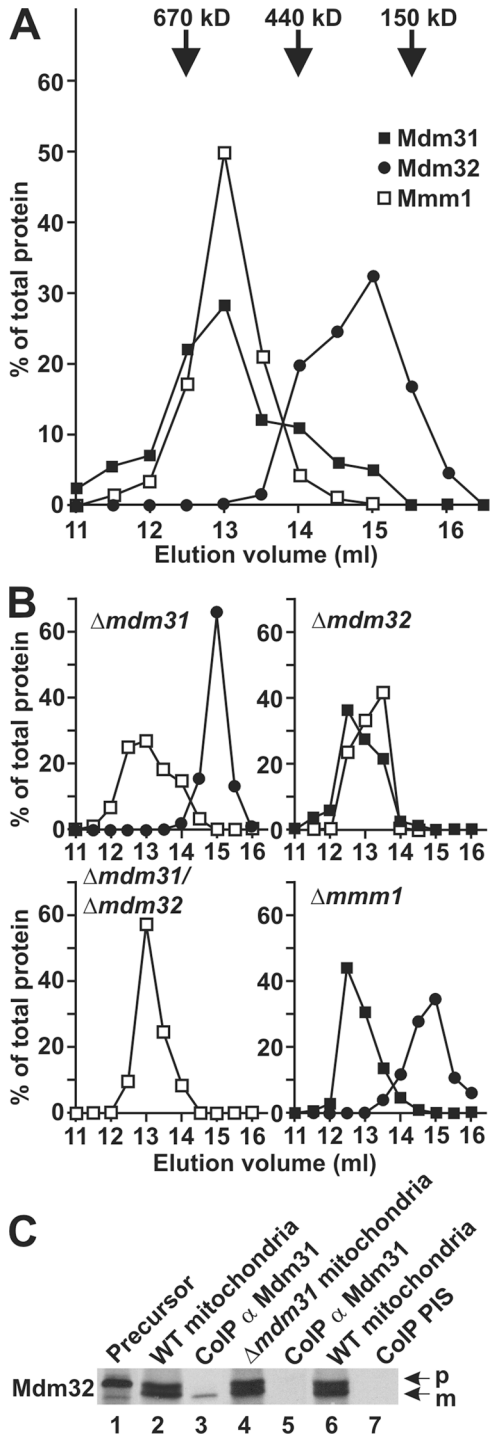



Figure 7. **Mdm31 and Mdm32 are parts of two separate complexes in the inner membrane.** (A) Gel filtration analysis of wild-type mitochondria. Isolated mitochondria were solubilized in digitonin-containing buffer and loaded onto a gel filtration column. After chromatography, proteins were precipitated with TCA, and fractions from the same column run were analyzed by Western blotting using affinity-purified antisera against Mdm31 (closed squares), Mdm32 (closed circles), and Mmm1 (open squares). Signals were quantified by densitometry and plotted as a percentage of total Mdm31, Mdm32, and Mmm1 protein in the extract. Arrows indicate molecular masses of calibration standards. (B) Δ*mdm31*, Δ*mdm32*, Δ*mdm31*/Δ*mdm32*, and Δ*mmm1* mitochondria were analyzed as in A. (C) Coimmunoprecipitation of Mdm32 with Mdm31. Radiolabeled Mdm32 (lane 1) was imported into wild-type mitochondria (lane 2). Mitochondria were lysed with Triton X-100 and subjected to coimmunoprecipitation with affinity-purified antibodies against Mdm31 (lane 3). In control reactions, coimmunoprecipitation was analyzed in Δ*mdm31* mitochondria (lanes 4 and 5), and preimmuneserum was used after import of Mdm32 into wild-type mitochondria (lanes 6 and 7). Signals were analyzed by SDS-PAGE and autoradiography. The amount of precursor protein in lane 1 corresponds to 10% of the material that was used for the import reactions; the amount of import reactions loaded in lanes 2, 4, and 6 corresponds to 10% of the material that was used for coimmunoprecipitation. p, precursor form of Mdm32; m, mature form of Mdm32. White lines indicate that intervening lanes have been spliced out.

2003; Youngman et al., 2004), wild-type cells expressing an Mmm1-DsRed fusion protein showed a punctate staining pattern. The majority of Mmm1 foci was located next to mtDNA nucleoids stained by Abf2-GFP or DAPI (Fig. 6 B). Mmm1 punctae were seen also in Δ*mdm31* and Δ*mdm32* cells, demonstrating that Mdm31 and Mdm32 are not required for Mmm1 foci formation (Fig. 6 B). However, mtDNA nucleoids were disorganized and Mmm1 foci were only rarely seen in the vicinity of mtDNA. Most Mmm1-DsRed–expressing mutant cells showed Mmm1 foci distantly located from diffusely organized mtDNA (Fig. 6 B). We conclude that Mdm31 and Mdm32 are required for localization of Mmm1 foci next to mtDNA.

## Mdm31 and Mdm32 are present in distinct complexes in the mitochondrial membranes

We determined whether or not Mdm31 and Mdm32 are subunits of larger protein complexes. Isolated wild-type mitochondria were solubilized with a mild detergent, digitonin. Protein complexes were separated by gel filtration and analyzed by Western blotting. Interestingly, Mdm31 and Mdm32 reside in separate complexes. Mdm31 was eluted at ~600 kD, which was clearly larger than the size of the Mdm32 complex at ~175 kD (Fig. 7 A). The size of the Mdm31 complex was not changed in the absence of Mdm32, and vice versa (Fig. 7 B).

We considered the possibility that Mmm1 forms a double membrane-spanning protein complex together with Mdm31 or Mdm32. To test this possibility, we asked if Mmm1 cofractionates with Mdm31 or Mdm32 in gel filtration. Consistent with a previous study (Youngman et al., 2004), Mmm1 was found in a complex slightly larger than 600 kD, the size of which was similar to the Mdm31 complex (Fig. 7 A). If Mdm31 and Mmm1 were subunits of the same complex, it could be expected that the size of this complex would change in the absence of one of the subunits. Therefore, we performed gel filtration experiments with mitochondria isolated from Δ*mdm31*, Δ*mdm32*, Δ*mdm31*/Δ*mdm32*, and Δ*mmm1* mutant cells. Neither deletion had a significant effect on the size of the other complexes (Fig. 7 B). We also noticed that the peak fractions of Mdm31 and Mmm1 were sometimes shifted by one fraction, with the Mdm31 complex being slightly larger (Fig. 7 B). Furthermore, we could not detect a direct interaction of Mdm31 or Mdm32 with Mmm1 in coimmunoprecipitation and cross-linking experiments. Thus, Mdm31, Mdm32, and Mmm1 are subunits of separate complexes in the mitochondrial membranes.

The structural and functional similarities of Mdm31 and Mdm32 point to a close collaboration of these proteins. Even

though they assemble into separate complexes, they might still interact in a weak or transient manner. To test this possibility, we imported radiolabeled Mdm32 into mitochondria and performed coimmunoprecipitation experiments with specific antibodies directed against endogenous Mdm31. Upon translation in vitro of Mdm32 in the presence of [$^{35}$S]methionine, SDS-PAGE, and autoradiography, a single band corresponding to the size of the precursor protein was observed (Fig. 7 C, lane 1). Upon incubation with isolated mitochondria, a slightly smaller form was generated by processing of the presequence by the matrix processing peptidase (Fig. 7 C, lanes 2 and 6). After import into wild-type mitochondria, a fraction of matured Mdm32 could be coimmunoprecipitated with Mdm31 antibodies (Fig. 7 C, lane 3). No precursor protein was found associated with Mdm31, demonstrating that the reaction was specific for the imported protein. Furthermore, no signal was obtained with mitochondria lacking Mdm31 (Fig. 7 C, lane 5) when preimmune serum was used (Fig. 7 C, lane 7) or when nonrelated inner membrane proteins were imported (not depicted). Thus, Mdm31 and Mdm32 interact with each other in a specific manner. The observation that only a small fraction of imported Mdm32 was coimmunoprecipitated with Mdm31 is consistent with a rather weak or transient interaction. We propose that Mdm31 and Mdm32 are subunits of two distinct protein complexes in the inner membrane that cooperate in establishing mitochondrial distribution and morphology.

## Discussion

Mutants lacking the inner membrane proteins Mdm31 and Mdm32 display phenotypes that are strikingly similar to mutants lacking either one of the outer membrane proteins Mmm1, Mmm2, Mdm10, and Mdm12 (Burgess et al., 1994; Sogo and Yaffe, 1994; Berger et al., 1997; Boldogh et al., 1998, 2003; Aiken Hobbs et al., 2001; Youngman et al., 2004). First, mutant cells harbor giant spherical mitochondria; second, aberrant mitochondria are largely immotile; third, the internal structure of mitochondria is dramatically altered; fourth, mtDNA is unstable; and fifth, mtDNA nucleoids are disorganized. Deletion of either one of the *MDM31* and *MDM32* genes is synthetically lethal with deletion of either one of the *MMM1*, *MMM2*, *MDM10*, and *MDM12* genes, suggesting that the gene products are required for the same cellular processes. We propose that Mdm31 and Mdm32 cooperate with Mmm1, Mmm2, Mdm10, and Mdm12 in maintaining mitochondrial morphology.

What might be the role of Mdm31 and Mdm32 in mitochondrial biogenesis? It has been proposed that Mmm1, Mmm2, Mdm10, and Mdm12 are involved in the attachment of mtDNA to the mitochondrial membranes and provide a link to a segregation machinery on the cytosolic side of the organelle. This hypothesis is based mainly on two findings. First, disordered nucleoids are seen in Δ*mmm1*, Δ*mmm2*, Δ*mdm10*, and Δ*mdm12* mutants (Boldogh et al., 2003; Youngman et al., 2004). Similar structures are also found in the Δ*abf2* mutant, which lacks a mitochondrial member of the nonhistone high mobility group protein family (Newman et al., 1996). Thus, disordered nucleoids are indicative of a defect of mtDNA packaging and/or attachment to the membrane. Second, GFP fusion proteins of Mmm1, Mmm2, Mdm10, and Mdm12 form foci, a subset of which is located next to a subset of mtDNA nucleoids (Aiken Hobbs et al., 2001; Boldogh et al., 2003; Meeusen and Nunnari, 2003; Youngman et al., 2004). However, the identity of inner membrane proteins that might link matrix-localized nucleoids to the putative segregation machinery in the outer membrane remained obscure. Here, we show that steady-state levels of Mmm1 in mitochondria, Mmm1 foci formation, and assembly of Mmm1 into a high molecular weight complex are not affected in mutants lacking Mdm31 and Mdm32. However, Mmm1-containing complexes lose their ability to interact with mtDNA nucleoids in Δ*mdm31* and Δ*mdm32* mutants. We propose that Mdm31 and Mdm32 are required to link mtDNA nucleoids to an Mmm1-containing segregation machinery in the mitochondrial outer membrane.

Respiratory functions of mitochondria are dispensable in *S. cerevisiae* when cells are grown on fermentable carbon sources. Hence, a defect in mtDNA inheritance is not sufficient to explain the observed synthetic lethal phenotypes of mdm mutants. Besides their role in respiration, mitochondria execute a variety of different metabolic functions, including biogenesis of iron sulfur clusters, which are essential for life (Lill and Kispal, 2000). Thus, the inheritance of the organelle is an essential process. We observed that mitochondria lacking Mdm31 and Mdm32 are almost immotile, similar to mitochondria lacking Mmm1, Mdm10, and Mdm12. Consequently, compromised mitochondrial motility leads to the appearance of mitochondria-free buds in the mutants. It is conceivable that a combination of the defects in double mutants lacking Mmm1 (or Mmm2, or Mdm10, or Mdm12) and Mdm31 (or Mdm32) results in a complete block of mitochondrial transport, and thus causes inviability of daughter cells.

We consider it unlikely that deletion of *MDM31* and *MDM32* directly influences the ability of mitochondria to bind to the actin-dependent transport machinery because mitochondria lacking Mdm31 and Mdm32 were found to be able to interact with actin in an ATP-dependent manner in vitro. It has been suggested that Mmm1 is required for coupling of mitochondria to the actin cytoskeleton (Boldogh et al., 1998). However, several lines of evidence suggest that Mmm1 is not directly acting as a receptor for actin-dependent motility factors. The function of Mmm1 has been conserved in the filamentous fungus *N. crassa*, which uses microtubules for mitochondrial transport (Prokisch et al., 2000; Westermann and Prokisch, 2002). Mitochondria isolated from loss-of-function mutants in *N. crassa* are still able to bind to the cytoskeleton (Fuchs et al., 2002), and the *N. crassa* protein complements the yeast mutant (Kondo-Okamoto et al., 2003). Similarly, homologues of Mdm31 are found in organisms that rely on microtubules for mitochondrial transport, such as *N. crassa* and *S. pombe*. This finding suggests that the main function of Mdm31, Mdm32, and Mmm1 is independent of the cytoskeletal system used by the cell for mitochondrial motility.

Mitochondria lacking Mdm31 and Mdm32 show dramatic changes in the organization of their internal membranes. This is not merely due to a defect in cristae formation, because

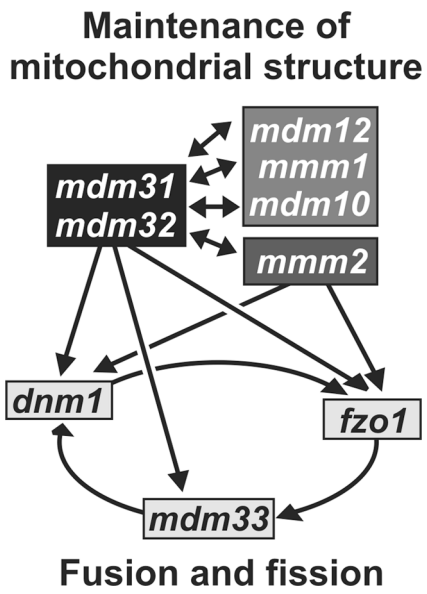



Figure 8. **Genetic relationships of components involved in mitochondrial dynamics.** Arrows indicate epistasis of mutant alleles, and double-headed arrows indicate synthetic lethality of mutations. Mutations that do not produce synthetic phenotypes are in the same box. A cycle of epistatic relationships between Δ*fzo1*, Δ*dnm1*, and Δ*mdm33* has been proposed by Messerschmitt et al. (2003). See text for further details.

some cristae are formed in glucose-grown cells (Fig. 4 E), and cristae are quite numerous in glycerol-grown cells (unpublished data). Interestingly, Aiken Hobbs et al. (2001) reported a similar phenotype for Δ*mmm1* mitochondria. Also in this mutant, cristae were lost and large extended or ring-shaped membrane inclusions were seen. These authors suggested that Mmm1 may be part of an internal scaffold-like structure required for normal mitochondrial shape and attachment of mtDNA. Our observations support a model in which Mdm31 and Mdm32 perform a similar function in the inner membrane. They may cooperate with Mmm1 in maintaining this scaffold-like structure and coordinate the behavior of the outer and inner membrane and provide anchoring sites for mtDNA nucleoids. When this function is lost, the internal structure of the organelle becomes disorganized, mitochondria lose their elongated shape, mtDNA nucleoids are destabilized, and mitochondrial motility is compromised as a consequence of aberrant mitochondrial shape.

Based on their genetic interactions and biochemical data, we can now propose at least three distinct functional entities involved in mitochondrial inheritance, the action of which is superior to the machineries of fusion and fission (summarized in Fig. 8). Mmm1, Mdm10, and Mdm12 have been proposed to be subunits of the same complex in the outer membrane (Boldogh et al., 2003). As combined deletion of their genes does not produce synthetic phenotypes (Berger et al., 1997; Hanekamp et al., 2002), these components likely share the same function. Mmm2 is a subunit of a separate complex in the outer membrane. Even though Δ*mmm1*/Δ*mmm2* double mutants are viable on fermentable carbon sources, conditional *mmm1* and *mmm2* alleles produce a synthetic lethal phenotype on nonfermentable carbon sources (Youngman et al., 2004). Hence, Mmm1 and Mmm2 act in functionally separable parallel pathways. Mdm31 and Mdm32 are functionally interdependent subunits of two novel complexes in the inner membrane, which might interact in a transient and dynamic manner. They are the first known inner membrane proteins that cooperate with the outer membrane proteins in inheritance of mitochondria and mtDNA. The functional characterization of novel components involved in these processes that were reported by Youngman et al. (2004) and herein revealed an unanticipated complexity of the machinery controlling mitochondrial behavior. It is a challenge for the future to reveal the precise molecular interactions of these complexes with components in the matrix and on the cytosolic face of the organelle that contribute to the complex process of mitochondrial inheritance.

## Materials and methods

### Plasmid and yeast strain constructions

Standard methods were used for cloning of DNA and growth and manipulation of yeast strains. Cloning procedures and strain constructions are described in detail in the online supplemental Materials and methods.

### Microscopy

Mitochondria were labeled with mtGFP (Westermann and Neupert, 2000) or mitochondria-targeted DsRed (Mozdy et al., 2000). Staining of the actin cytoskeleton with rhodamine-phalloidin (Amberg, 1998) and DAPI staining of mtDNA in living cells (Aiken Hobbs et al., 2001) was performed according to published procedures. Staining of the vacuole with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (Molecular Probes) was performed according to the manufacturer's instructions. The ER was visualized with ER-targeted GFP (Prinz et al., 2000). Abf2-containing structures were labeled with a chimeric protein consisting of Abf2 and a GFP moiety derived from mtGFP. Mmm1-containing structures were stained with Mmm1 fused to DsRed.T4 (Bevis and Glick, 2002).

Epifluorescence microscopy was performed using a microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Neofluar 100×/1.30 Ph3 oil objective (Carl Zeiss MicroImaging, Inc.). Images were recorded either with a SPOT cooled color camera (Diagnostic Instruments) and processed with Lite Meta-Morph imaging software (Universal Imaging Corp.) or with an Evolution VF Mono Cooled monochrome camera (Intas) and processed with Image Pro Plus 5.0 and ScopePro 4.5 software (MediaCybernetics). Confocal images were taken with a confocal microscope (model TCS SP1; Leica) equipped with a 1.2 NA 63× water immersion lens (Leica; 63×, Planapo). For imaging, living cells were embedded in 1% low melting point agarose and observed at RT. Quantification of mitochondrial morphology defects was performed without prior reference to strain identity.

EM and immunocytochemistry were performed as described previously (Kärgel et al., 1996; Messerschmitt et al., 2003).

### Analysis of mitochondria–actin interactions in vitro

Actin filaments were prepared by polymerizing nonmuscular human actin (tebu-bio GmbH) according to the manufacturer's instructions. Binding of filamentous actin (at a concentration of 100 μg/ml) to isolated mitochondria and cosedimentation of actin with mitochondria were performed as described previously (Lazzarino et al., 1994). Actin was detected by immunoblotting with monoclonal anti-actin antibodies (c4d6; Lessard, 1988).

### Gel filtration analysis

Isolated mitochondria (1 mg) were pelleted by centrifugation for 10 min at 10,000 *g* and resuspended in 200 μl buffer A (1% digitonin, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 10 mM Tris-HCl, pH 7.4). After incubation for 1 h at 4°C under agitation, mitochondrial extracts were centrifuged for 30 min at 90,000 *g* in a rotor (model TLA45; Beckman Coulter) at 4°C. The supernatant was loaded on a Superose 6 gel filtration column (25-ml column volume; Amersham Biosciences) and chromatographed in buffer A with 0.05% digitonin (flow rate 0.5 ml/min). 0.5-ml fractions were collected, and proteins were precipitated with TCA and analyzed by SDS-PAGE and Western blotting. Calibration standards were as follows: thyroglobulin, 670 kD; apoferritin, 440 kD; alcohol dehydrogenase, 150 kD; carboanhydrase, 29 kD.

### Miscellaneous

Antigens were expressed using the pQE system (QIAGEN) according to the manufacturer's instructions. Antisera were generated by injection of inclusion bodies into rabbits. Subfractionation of yeast cells and isolation, purification, and subfractionation of mitochondria were performed as described previously (Rowley et al., 1994). Mitochondrial fusion was examined according to published procedures (Nunnari et al., 1997; Fritz et al., 2003). Import of radiolabeled Mdm32 and coimmunoprecipitation was performed as described previously (Messerschmitt et al., 2003).

### Online supplemental material

An alignment of Mdm31 protein family members is available as Fig. S1. Cloning procedures and yeast strain constructions are described in supplemental Materials and methods. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200410030/DC1.

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