

# A Screen for Nigericin-Resistant Yeast Mutants Revealed Genes Controlling Mitochondrial Volume and Mitochondrial Cation Homeostasis

Blanka Kucejova,\* Martin Kucej,\* Silvia Petrezselyova,<sup>†</sup> Lenka Abelovska\* and Lubomir Tomaska<sup>†,1</sup>

\*Department of Biochemistry, Faculty of Natural Sciences, Comenius University, 84215 Bratislava, Slovak Republic and

<sup>†</sup>Department of Genetics, Faculty of Natural Sciences, Comenius University, 84215 Bratislava, Slovak Republic

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## ABSTRACT

Little is known about the regulation of ion transport across the inner mitochondrial membrane in *Saccharomyces cerevisiae*. To approach this problem, we devised a screening procedure for facilitating the identification of proteins involved in mitochondrial ion homeostasis. Taking advantage of the growth inhibition of yeast cells by electroneutral  $K^+/H^+$  ionophore nigericin, we screened for genetic mutations that would render cells tolerant to this drug when grown on a nonfermentable carbon source and identified several candidate genes including *MDM31*, *MDM32*, *NDI1*, *YMR088C* (*VBA1*), *CSR2*, *RSA1*, *YLR024C*, and *YNL136W* (*EAF7*). Direct examination of intact cells by electron microscopy indicated that mutants lacking *MDM31* and/or *MDM32* genes contain dramatically enlarged, spherical mitochondria and that these morphological abnormalities can be alleviated by nigericin. Mitochondria isolated from the  $\Delta mdm31$  and  $\Delta mdm32$  mutants exhibited limited swelling in an isotonic solution of potassium acetate even in the presence of an exogenous  $K^+/H^+$  antiporter. In addition, growth of the mutants was inhibited on ethanol-containing media in the presence of high concentrations of salts (KCl, NaCl, or  $MgSO_4$ ) and their mitochondria exhibited two- ( $\Delta mdm31$  and  $\Delta mdm32$ ) to threefold ( $\Delta mdm31\Delta mdm32$ ) elevation in magnesium content. Taken together, these data indicate that Mdm31p and Mdm32p control mitochondrial morphology through regulation of mitochondrial cation homeostasis and the maintenance of proper matrix osmolarity.

**M**ITOCHONDRIA maintain a membrane potential ( $\Delta\Psi$ ), which generates a driving force for the electrophoretic influx of cations into the mitochondrial matrix (BERNARDI 1999; GARLID and PAUCEK 2003). Since the inner mitochondrial membrane is permeable to water, the organelle has to combat an increased osmotic pressure and consequent swelling. Due to the high physiological concentration of intracellular potassium and magnesium, tight regulation of these cation fluxes is essential for proper organelle volume maintenance. MITCHELL (1966) hypothesized that mitochondrial cation asymmetry could be sustained by a membrane-associated proton/cation antiporter(s), which would export cations out of the matrix using the energy of the electrochemical potential gradient. Measurements of swelling-induced potassium export and passive swelling in isotonic solutions of potassium acetate have demonstrated that mitochondria possess coupled  $K^+/H^+$  antiporter activity, possibly involved in potassium extrusion (GARLID 1980; VILLALOBO *et al.* 1981). Mammalian mitochondria contain two monovalent cation/proton antiporters; one is specific for sodium, whereas the second transporter does not discriminate between sodium and potassium ions (NAKASHIMA

and GARLID 1982). The mammalian mitochondrial  $K^+/H^+$  antiporter was purified to homogeneity, but the molecular identity of the 82-kD protein remains unknown (LI *et al.* 1990). In contrast, the yeast mitochondria exhibit only nonspecific monovalent cation antiporter activity (WELIHINDA *et al.* 1993). Recently, it was proposed that *YOL027* (*MDM38*, *MKH1*), a yeast homolog of the human *LETMI* gene deleted in patients with Wolf-Hirschhorn syndrome, might either code for a mitochondrial  $K^+/H^+$  antiporter or contribute to its activity (NOWIKOVSKY *et al.* 2004; FROSCHAUER *et al.* 2005). This concept is based on the decreased swelling ability of mitochondria in potassium acetate solution, the increased volume of mitochondria, the increased concentration of intramitochondrial potassium, as well as the abolished  $K^+/H^+$  exchange activity in submitochondrial particles of the *yol027* deletion mutant.

The information regarding the regulation of mitochondrial magnesium fluxes is even more limited. Whereas magnesium export was detected in rat heart mitochondria in the presence of respiratory substrates (RUTTER *et al.* 1990), mitochondria from *Saccharomyces cerevisiae* exhibited only negligible magnesium export (KOLISEK *et al.* 2003).  $\Delta\Psi$ -driven magnesium import is mediated by a channel encoded by the *MRS2* gene and presumably by its functional homolog, Lpe10p (GREGAN *et al.* 2001a; KOLISEK *et al.* 2003). The Mrs2p-mediated

<sup>1</sup>Corresponding author: Department of Genetics, Faculty of Natural Sciences, Comenius University, Mlynska dolina B-1, 84215 Bratislava, Slovak Republic. E-mail: tomaska@fns.uniba.sk

transport activity seems to be tightly regulated since overexpression of the channel elicits only a moderate increase in mitochondrial magnesium content (BUI *et al.* 1999). Molecular details of the regulation of Mrs2p activity are presently unknown, but characterization of several gain-of-function mutations indicates an involvement of the central part of the channel (GREGAN *et al.* 2001b).

Nigericin is a lipophilic ionophore mediating electro-neutral antiport of potassium and proton ions across a lipid membrane (NICHOLLS and FERGUSON 2001). In *S. cerevisiae*, nigericin acts preferentially on the inner mitochondrial membrane, resulting in a massive formation of *petite* colonies and inhibition of growth when propagated in media containing only nonfermentable carbon sources (KOVAC *et al.* 1982; KOVAC and KLOBUCNIKOVA 1983). With the aim of dissecting the molecular mechanism(s) regulating mitochondrial ion homeostasis in *S. cerevisiae*, we took advantage of the selective action of nigericin on the inner mitochondrial membrane. We performed a large-scale screen for yeast mutants resistant to nigericin when grown on media containing glycerol as the sole carbon source. Analysis of the resulting mutations not only provided a strategy to uncover the genetic basis of the selective action of nigericin on yeast mitochondria, but also has led to identification of novel proteins involved in the maintenance of mitochondrial ion homeostasis and organelle morphology.

## MATERIALS AND METHODS

**Strains and media:** *S. cerevisiae* strains W303-1B (*MAT $\alpha$* , *ura3-1*, *ade2-1*, *leu2-3*, *112*, *trp1-1*, *his3-11*, *15*, *can1-100*) and isogenic W303-1A (*MAT $\alpha$* , *ura3-1*, *ade2-1*, *leu2-3*, *112*, *trp1-1*, *his3-11*, *15*, *can1-100*) were used in this study. *Escherichia coli* strain DH5 $\alpha$  (Life Technologies) was used for plasmid DNA propagations. Bacteria were grown at 37° in Luria-Bertani broth (0.5% yeast extract, 1% peptone, and 1% NaCl, pH 7) supplemented with ampicillin (100 mg/liter) or kanamycin (50 mg/liter). Yeast were grown at 30° in complex yeast peptone medium (1% yeast extract, 1% peptone) supplemented with 2% glucose (YPD), 3% ethanol (YPE), or 2% glycerol plus 0.1% glucose (YPDG), and in synthetic dextrose (SD; 0.67% yeast nitrogen base without amino acids and 2% glucose) or semisynthetic glycerol (semiSG; 0.67% yeast nitrogen base without amino acids, 0.1% yeast extract, and 3% glycerol) media. Synthetic media were supplemented with appropriate amino acids and nitrogen bases. Solid media were supplemented with 2% agar. All media supplements were purchased from Difco.

**Screen for nigericin-resistant mutants:** Yeast strain W303-1B was transformed with ~0.5  $\mu$ g of *NotI*-cut mTn-*lacZ* *LEU2*-mutagenized genomic integrative library (BURNS *et al.* 1994) using the LiAc/ssDNA/PEG method (GIETZ and WOODS 2002). Transformants were selected on UTAH plates (SD supplemented with 40 mg/liter uracil, 30 mg/liter tryptophan, 40 mg/liter adenine, and 30 mg/liter histidine). Approximately 36,000 transformants were collected from the UTAH plates by washing each plate with water. The cells were concentrated by centrifugation, divided into four pools (each pool representing one genome equivalent), and used directly or stored in a form of glycerol stocks at -80°. For isolation of

nigericin-resistant (*Nig<sup>R</sup>*) transformants, the corresponding pool was streaked on a semiSG LUTAH solid media (10<sup>6</sup> cells/plate; semiSG supplemented with 30 mg/liter leucine, 40 mg/liter uracil, 30 mg/liter tryptophan, 40 mg/liter adenine, and 30 mg/liter histidine) containing nigericin [obtained from either Reanal (Hungary) or Fluka (Germany)] (40 mg/liter). The sensitivity of the wild-type strain varied between 5 to 10 mg/liter of nigericin, depending on the brand of yeast extract in the media. Colonies that appeared within 3–7 days were collected and verified by streaking on the same type of plates. The specificity of nigericin resistance of the prospective candidates was verified by the drop test method on semiSG LUTAH plates containing valinomycin (25 mg/liter), oligomycin (3 mg/liter), or erythromycin (4 g/liter) and on SD LUTAH plates containing cycloheximide (1 mg/liter). Linkage analysis of *Nig<sup>R</sup>* loci with insertion of the transposon to nuclear DNA was tested by tetrad analysis of sporulated diploids obtained by crossing of *Nig<sup>R</sup>* mutants with the W303-1A strain. Genomic DNA from verified clones was isolated as described (PHILIPSEN *et al.* 1991), except that purification by glassmilk was replaced by isopropanol precipitation in the presence of one-tenth volume of 3 M sodium acetate pH 5.2. To identify the site of the transposon insertion, ~2  $\mu$ g of genomic DNA was cut by the restriction endonuclease *AluI* and ligated to a 4- $\mu$ m “bubble” linker. The linker was prepared by annealing of the BUBBLE53 (5'-GAAGGAGAGGACGCTGTCTGTCGAAGGT AAGGAACGGACGAGAGAAGGGAGAG-3') and the BUBBLE55 (5'-GACTCTCCCTTCTCGAATCGTAACCGTTCGTAC GAGAATCGCTGTCCTCTCCTTC-3') primers in the presence of 2 mM MgCl<sub>2</sub>. Subsequently, 5  $\mu$ l of ligation mixture was added to 100  $\mu$ l of Vectorsite PCR with the M13(-47) (5'-CGCCAGGGTTTCCAGTCAAGAC-3') and the 224 (5'-CGAATCGTAACCGTTCGTACGAGAATCGCT-3') primers (ARNOLD and HODGSON 1991). Amplified DNA was purified from agarose gel using Qiaquick gel extraction kit (QIAGEN, Chatsworth, CA) and subjected to DNA-sequencing analysis primed by M13(-47).

**Preparation of deletion mutants:** Deletion cassettes  $\Delta$ *mdm31::kanMX4<sup>R</sup>* and  $\Delta$ *mdm32::kanMX4<sup>R</sup>* were isolated from genomic DNA of respective ATCC deletion strains using PCR. The set of used primers was 5'-TCCCAGAAGGAGACTTTACCTTAGT-3' and 5'-CACACACTCTCTCACTCACAAAAAT-3' for *MDM31* and 5'-CTTACAGGTGTCACAGTAGCCTTTT-3' and 5'-CGTCGTAATAAATCGATAAATAACG-3' for *MDM32*. Amplified cassettes were transformed into W303-1B and W303-1A strains using the LiAc/ssDNA/PEG method (GIETZ and WOODS 2002). Transformants were selected on YPD plates containing 300 mg/liter geneticin (G418, Invitrogen). Double  $\Delta$ *mdm31* $\Delta$ *mdm32* mutant was obtained by crossing the corresponding W303-1A and W303-1B deletion strains, sporulation of the diploid, and dissection of tetrads. All deletions were verified by PCR using combinations of primers designed to amplify 5'- or 3'-ends of genes or deletion cassettes. W303-1A mutants deleted for *MDM31* or *MDM32* were used only for preparation of double disruptant.

**Induction of *petites*:** Cells grown overnight in SD LUTAH were inoculated into SD LUTAH media at a final concentration 1  $\times$  10<sup>5</sup> cells/ml in the absence or presence of nigericin (30 mg/liter) and incubated at 30° for four to five generations. Cells were then plated on YPDG plates and *petite* colonies were identified by 2,3,5-triphenyltetrazolium chloride overlay (OGUR *et al.* 1957). At least three independent experiments were performed with each strain.

**Whole-cell oxygen consumption:** Whole-cell oxygen uptake was measured at 30° using a Clark-type oxygen electrode (Strathkelvin Instruments). Since  $\Delta$ *mdm31* and  $\Delta$ *mdm32* mutants have a greater rate of spontaneous *petite* formation than wild type, the strains employed for whole-cell oxygen

consumption experiments were grown in YPE medium. After reaching the late exponential phase, the cells were collected by centrifugation for 5 min at  $600 \times g$ , washed with distilled water, and resuspended in water. Cells were placed into the electrode chamber containing 0.55 ml of 100 mM glucose and 10 mM Tris-maleate (pH 6.2) to a final OD<sub>600</sub> of 1.0. After stabilization of the oxygen consumption rate, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma-Aldrich) was added to a final concentration of 10  $\mu$ M. The data were processed by the Strathkelvin Oxygen System software. At least three independent experiments were performed with each tested strain.

**Isolation of mitochondria:** Yeast cells were grown in 2 liters of YPE medium to the late exponential phase. Mitochondria were isolated essentially as described (KOVAC *et al.* 1972) with the following modifications. Harvested cells were suspended in a solution of 0.1 M Tris-HCl (pH 9.3), 1% 2-mercaptoethanol (20 ml/g of cells, wet weight), incubated 10 min at 37°, and washed with 0.1 M Tris-HCl pH 8.5 (20 ml/g of cells, wet weight) and 1 M sorbitol (5 ml/g of cells, wet weight). The washed cells were suspended in 1 M sorbitol (1 ml/g of cells, wet weight). Zymolyase-20T (ICN) (2 mg/g of cells, wet weight) was added and the suspension was incubated for 30–45 min at 37° with gentle shaking until ~90% of cells were converted to spheroplasts. After chilling the suspension on ice, spheroplasts were harvested by centrifugation at  $2000 \times g$  for 10 min and washed once with 1 M sorbitol (2 ml/g of cells, wet weight) and once with 1 M sorbitol containing 0.1% bovine serum albumin (BSA, Sigma-A7906) (1 ml/g of spheroplasts, wet weight). From this point, all operations were carried out at 4°. For homogenization, spheroplasts were suspended in 0.6 M mannitol containing 0.1% BSA (2.5 ml/g of spheroplasts, wet weight) and homogenized (30–35 strokes) in a hand-operated Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at  $4000 \times g$  for 10 min and the supernatant was transferred to a fresh tube. No further rehomogenization of pellet was performed. Mitochondria were collected by centrifugation at  $20,000 \times g$  for 10 min, after which the floating lipids were carefully removed by cotton wool and the supernatant was discarded. The pellet was resuspended in 0.6 M mannitol (the same volume that was used for spheroplast homogenization) and the residual cell debris was removed by centrifugation at  $4000 \times g$  for 10 min. Mitochondria from supernatant were collected by centrifugation at  $25,000 \times g$  for 10 min and suspended in 0.6 M mannitol to a final concentration of 5–10 mg protein/ml.

**Passive swelling of mitochondria:** Only freshly prepared mitochondria (<5 hr after isolation) were used in swelling measurements. Experiments were carried out essentially as described previously (KOVAC *et al.* 1972; VILLALOBO *et al.* 1981). Passive swelling was followed by absorbance time scan at 750 nm using Hitachi U-2001 spectrophotometer. Swelling was initiated by the addition of mitochondria (0.25 mg protein/ml) into a cuvette containing an isoosmotic solution consisting of 600 mOsm potassium acetate (KAc), 10 mM Tris-maleate (pH 7.0), 0.2 mM NaCN, and 2 mg/liter oligomycin. Where indicated, valinomycin (0.8 mg/liter) and CCCP (3  $\mu$ M) were added and the suspension was thoroughly mixed. At least three independent experiments were performed with each tested strain.

**Determination of intramitochondrial ion concentrations:** Mitochondria were decomposed in 1 ml of 0.5 M HCl at 25° for 90 min. Solid debris was removed by centrifugation at  $12,000 \times g$  for 10 min. The supernatant was diluted with milliQ water and the concentration of potassium and magnesium was determined by atomic absorption spectroscopy [Perkin-Elmer (Norwalk, CT) 1100]. At least three independent experiments were performed with each tested strain.

**Fluorescence microscopy:** For the visualization of mitochondria in living cells, yeast strains were grown to late ex-

ponential phase in the appropriate media as indicated in the figure legends. One hundred microliters of a cell suspension was mixed with 2  $\mu$ l of 400  $\mu$ M 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI; Molecular Probes, Eugene, OR), incubated at 25° for 15 min, and immediately examined by fluorescence microscopy (Olympus BX50). Results are representative of at least two independent experiments; for each sample, 100 cells have been inspected. The contrast of digital pictures was enhanced using the GIMP software (<http://www.gimp.org/>).

**Transmission electron microscopy:** Wild-type and mutant cells were grown overnight in YPD, fixed using potassium permanganate (McCLARY and BOWERS 1967), cross-sectioned, and then scanned by transmission electron microscopy (Centre for High Resolution Imaging and Processing, University of Dundee). Only cells with clearly distinguishable intracellular structures (*e.g.*, nucleus, vacuole, lipid particles) were selected for examination of mitochondria. The area of all recognizable mitochondrial cross sections was quantified using Quantity-One software (Bio-Rad, Hercules, CA). GIMP software was used to enhance the contrast of the electron micrographic digital images.

## RESULTS

**Genetic screen for nigericin-resistant mutants:** To perform a large-scale screen for Nig<sup>R</sup> mutants, we transformed the mTn-*lacZ* *LEU2*-mutagenized genomic integrative library into the W303-1B strain (see MATERIALS AND METHODS). After selecting for primary transformants, we looked for clones that were able to grow on semiSG media containing 40 mg/liter nigericin. To exclude the possibility that the Nig<sup>R</sup> phenotype of isolated clones was caused by activation of the pleiotropic drug resistance (PDR) machinery, each mutant was tested for its resistance to several structurally unrelated drugs. Of 86 candidate clones exhibiting nigericin resistance, none exhibited a PDR phenotype. However, 20 clones were found to be resistant not only to nigericin, but also to valinomycin and were eliminated from further studies. As a result, all mutant strains summarized in Table 1 are resistant to nigericin, but not to valinomycin, oligomycin, erythromycin, and cycloheximide. Of all the candidates, only the *yhr194* and *yor147* mutants exhibited impaired growth on YPG, indicating a mitochondria-related phenotype. Both *YHR194W* and *YOR147W* genes were identified previously in a genome-wide screen for mutants with an altered mitochondrial morphology and were designated as *MDM31* and *MDM32*, respectively (DIMMER *et al.* 2002). Analysis of the primary amino acid sequence of Mdm31p and Mdm32p by MITOPROT indicated a >99% probability of their mitochondrial localization and cleavable N-terminal mitochondrial presequence. This prediction was recently proved experimentally by DIMMER *et al.* (2005), who demonstrated that both Mdm31p and Mdm32p are located in the inner mitochondrial membrane. Since our genetic screen was aimed at identification of novel genes involved in mitochondrial ion homeostasis, we decided to pursue a more detailed study of the *MDM31* and *MDM32* genes and the proteins they encode.

**TABLE 1**  
**Genes identified in screen for Nig<sup>r</sup> mutants**

Systematic gene name	Synonym	ORF (bp)	No. of alleles	Site of insertion	Function, description	References
<i>YHR194W</i>	<i>MDM31</i>	1740	2	203, 207	Mitochondrial morphology, stabilization of mitochondrial nucleoid	DIMMER <i>et al.</i> (2002, 2005)
<i>YOR147W</i>	<i>MDM32</i>	1962	2	370, 573	Mitochondrial morphology, stabilization of mitochondrial nucleoid	DIMMER <i>et al.</i> (2002, 2005)
<i>YML120C</i>	<i>NDI1</i>	1542	1	-223	Mitochondrial NADH:ubiquinone oxidoreductase	MARRES <i>et al.</i> (1991)
<i>YMR088C</i>	<i>VBA1</i>	1689	1	117	Vacuolar basic amino acid transporter	SHIMAZU <i>et al.</i> (2005)
<i>YPR030W</i>	<i>CSR2</i>	3366	1	1617	Unknown, utilization of nonfermentable C sources	KABIR <i>et al.</i> (2000)
<i>YPL193W</i>	<i>RSA1</i>	1146	1	884	Unknown, ribosome assembly	KRESSLER <i>et al.</i> (1999)
<i>YLR024C</i>	<i>UBR2</i>	5619	1	5497	Ubiquitin-protein ligase E3	HOCHSTRASSER (1996)
<i>YNL136W</i>	<i>EAF7</i>	1278	1	342	Subunit of the NuA4 histone acetyltransferase complex	KROGAN <i>et al.</i> (2004)

**Sequence analysis of Mdm31p and Mdm32p:** BLAST search (ALTSCHUL *et al.* 1990) identified 18 fungal proteins exhibiting a significant [expect (*E*)-value <10<sup>-20</sup>] degree of sequence similarity to Mdm31p and Mdm32p. Hydropathy and dense alignment surface analyses indicated that all proteins contain one putative transmembrane domain positioned between residues 100 and 200 (KYTE and DOOLITTLE 1982; CSERZO *et al.* 1997). Some programs indicate the possibility of a second transmembrane domain at the extreme C terminus as indicated by DIMMER *et al.* (2005). All protein sequences were aligned using the ClustalX 1.83 program at its default settings (THOMPSON *et al.* 1997). The alignment revealed that the putative transmembrane region contains several conserved serines and threonines preceded by two acidic residues. This pattern is present in the majority of Mdm31p and Mdm32p homologs (Figure 1). In addition, the N-terminal hydrophobic regions and the nearby sequences of Mdm31p and Mdm32p exhibit ~50% identity; a relatively high degree of conservation compared to only 20% overall identity between these proteins.

**Characterization of *mdm31* and *mdm32* mutants:** We observed that the *mdm31* and *mdm32* mutants obtained in our original screen, as well as constructed single- and double-deletion mutants, exhibited slow growth on complex media containing glycerol as the sole carbon source. We observed that the mutants were resistant to higher concentrations of nigericin on solid media compared to the liquid media. Heterozygous diploids did not exhibit any visible phenotype. Total growth yield was decreased for all the mutants in both YPD and YPE media. Maximum cell density in YPD media reached ~90% that of the single-deletion mutants and ~70% that of the double-deletion mutant when compared to the wild type. When grown in YPE, all the mutants reached a cell density ~75% of that observed for the wild-type strain (data not shown).

Next we measured the respiration rate of intact wild-type and mutant cells and observed some differences. When grown in YPE, all of the mutants, as compared to the wild-type cells, exhibited a reduction in their respiratory rates (Figure 2A). To exclude the possibility that the reduction in respiratory rates observed in the mutants was caused by an increased fraction of *petites*, we spread aliquots from all tested strains on solid YPE and YPDG media. In each case we observed an equivalent number of colonies on both media, indicating that defects in respiration are not due to a high frequency of loss of mtDNA in the mutant strains grown on a non-fermentable carbon source.

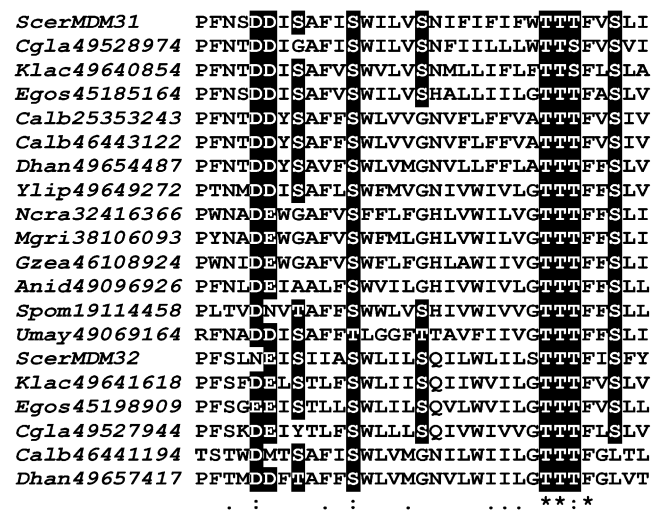


FIGURE 1.—A protein sequence alignment of putative transmembrane regions in Mdm31-related proteins. Conserved serines, threonines, and acidic residues are indicated by a solid background. Numbers following the species names represent GenBank sequence identifiers.

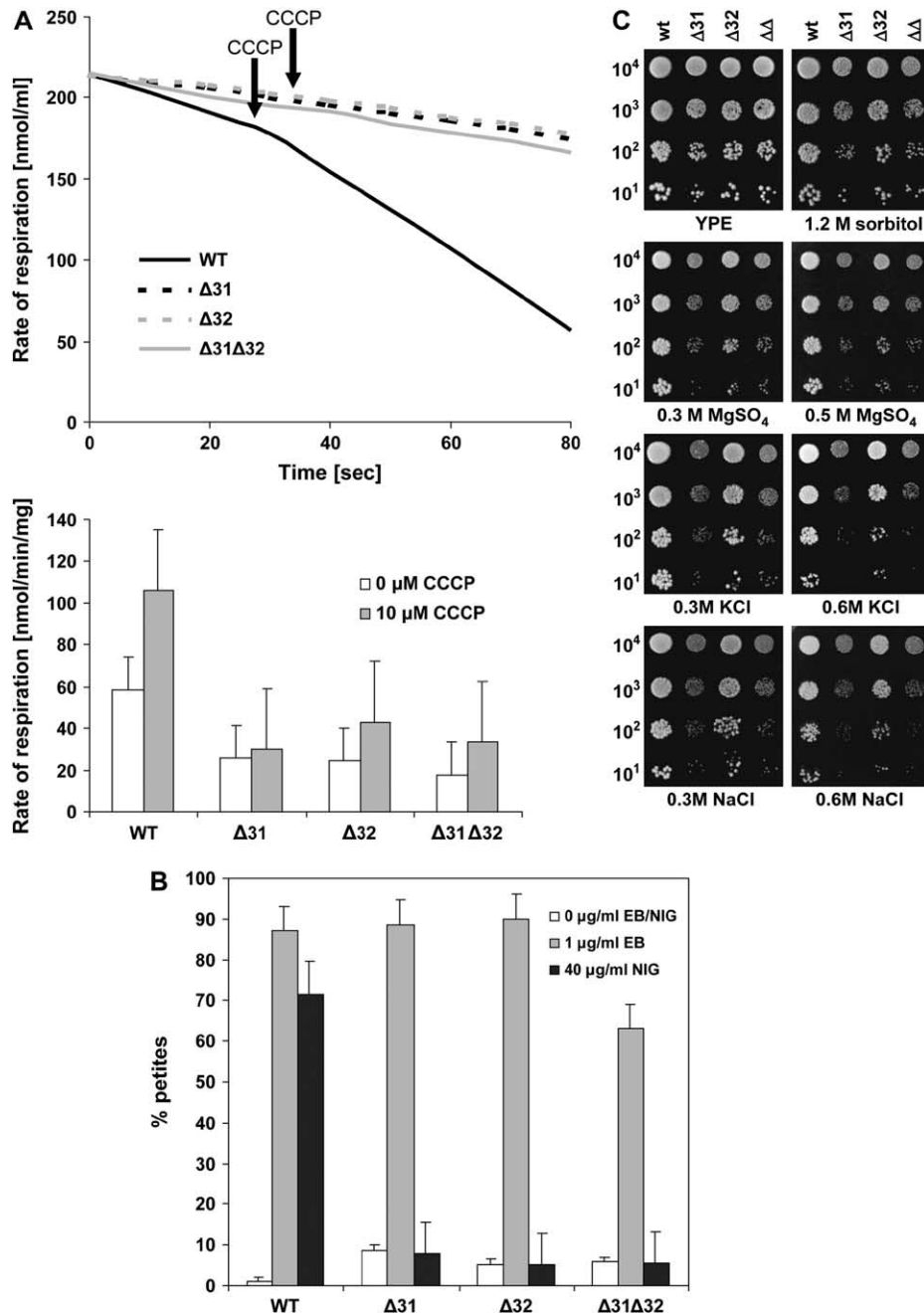


FIGURE 2.—Characterization of W303-1B (wt),  $\Delta mdm31$  ( $\Delta 31$ ),  $\Delta mdm32$  ( $\Delta 32$ ), and  $\Delta mdm31\Delta mdm32$  ( $\Delta\Delta$ ) strains. (A, top) Whole-cell oxygen consumption of cells grown in YPE measured by a Clark-type electrode. Arrows indicate the addition of CCCP to the electrode chamber. (Bottom) Rates of respiration calculated before (open bars) and after (shaded bars) addition of 10  $\mu$ M CCCP. (B) Frequency of spontaneous, ethidium-bromide- and nigericin-induced *petite* colony formation in the strains. Data are represented as the mean  $\pm$  average deviations (error bars). (C) Growth of the strains on YPE plates in the absence or presence of the compounds is indicated at the bottom. Numbers to the left represent the approximate number of cells in each drop. The plates were photographed after 5 days of incubation at 30°.

As mentioned previously, nigericin selectively acts on the inner mitochondrial membrane and induces *petite* colony formation of wild-type cells when grown on media containing a fermentable carbon source such as glucose (KOVAC and KLOBUCNIKOVA 1983). Consequently, we used nigericin to examine its effects on mtDNA in strains deleted for *MDM31* and *MDM32* (Figure 2B). Similarly as reported by DIMMER *et al.* (2005), mutant cells grown in SD medium without the ionophore formed *petite* colonies at a higher frequency (5–10%) than did the wild-type strain (<1%) (Figure 2B). However, after treatment with nigericin, the formation of *petite* colonies in the  $\Delta mdm31$  (8%),  $\Delta mdm32$  (5%), and  $\Delta mdm31\Delta mdm32$  (6%) mu-

tants was markedly lower than that in the wild-type strain (72%). To investigate if this phenomenon is specific for nigericin or if it is a general feature of these mutations, we assessed ethidium-bromide-induced *petite* formation in wild-type and mutant strains. We found no significant differences between the wild-type and single  $\Delta mdm31$  and  $\Delta mdm32$  mutants and only a slight decrease (~20%) in ethidium-bromide-induced *petite* formation in the double-mutant  $\Delta mdm31\Delta mdm32$ . This is in contrast to a dramatic (>65%) decrease in nigericin-induced *petite* formation in all mutants. The ethidium bromide treatment demonstrated that the mutant strains are capable of forming and tolerating mutations in mtDNA at a high

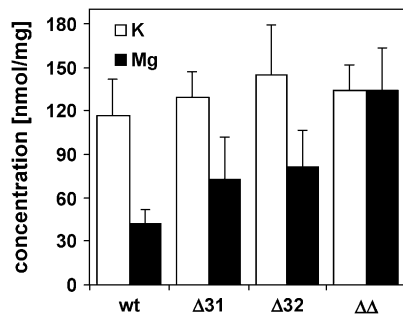


FIGURE 3.—Intramitochondrial potassium and magnesium concentrations (nanomoles per milligram of protein) obtained by atomic absorption spectroscopy measurements in mitochondria isolated from the following strains: W303-1B (wt, [K] = 117 ± 26, [Mg] = 42 ± 10);  $\Delta mdm31$  ( $\Delta 31$ , [K] = 130 ± 17, [Mg] = 72 ± 30);  $\Delta mdm32$  ( $\Delta 32$ , [K] = 145 ± 35, [Mg] = 82 ± 25); and  $\Delta mdm31\Delta mdm32$  ( $\Delta\Delta$ , [K] = 134 ± 18, [Mg] = 134 ± 29). Data are represented as the mean concentration ± average deviations.

frequency and that ethidium bromide and nigericin induce the loss of mtDNA by different means.

Since the increased tolerance to nigericin could be attributed to changes in intracellular ion homeostasis, we examined the individual effect of  $K^+$ ,  $Na^+$ , and  $Mg^{2+}$  salts on the growth of  $\Delta mdm31$ ,  $\Delta mdm32$ , and  $\Delta mdm31\Delta mdm32$  mutants. While in glucose media, elevated concentrations (0.3 and 0.6 M) of all three ions had no effect (data not shown); 0.3 M of KCl, NaCl, or  $MgSO_4$  in solid YPE media significantly impaired the growth of all the mutants (Figure 2C; similar salt sensitivity was observed in liquid YPE media; data not shown). When cells were grown in the presence of high-sorbitol-containing (1.2 M) media to test their response to increased osmolarity, subtle differences were also observed between the wild-type and mutant strains. No differences in sensitivity to other ions such as iron, copper, cobalt, manganese, and nickel were detected on media containing either glucose or respiratory substrates (data not shown).

**Increased levels of mitochondrial magnesium in the  $\Delta mdm31$ ,  $\Delta mdm32$ , and  $\Delta mdm31\Delta mdm32$  mutants:** Sensitivity to high salt of the  $\Delta mdm31$  and  $\Delta mdm32$  mutants grown on ethanol media is indicative of changes in intramitochondrial ion concentrations. Atomic absorption spectroscopy analysis of extracts from isolated mitochondria revealed that while potassium levels were comparable in all strains, the mutants contained substantially elevated concentrations of magnesium (Figure 3). Magnesium levels were about twofold higher in mitochondria in  $\Delta mdm31$  and  $\Delta mdm32$  single-deletion mutants and threefold higher in the double-deletion strain.

**Enlarged mitochondria in the  $\Delta mdm31$ ,  $\Delta mdm32$ , and  $\Delta mdm31\Delta mdm32$  mutants:** DIMMER *et al.* (2002) have shown that mitochondria of  $\Delta mdm31$  and  $\Delta mdm32$  mutants exhibit abnormal morphology as visualized by light microscopy. To analyze these mitochondrial struc-

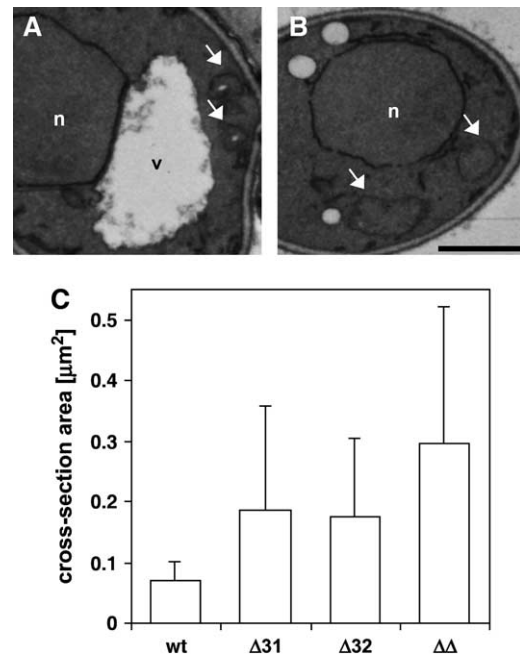


FIGURE 4.—Analysis of mitochondrial morphology by electron microscopy. Electron micrographs of cross sections of permanganate-fixed (A) W303-1B and (B)  $\Delta mdm31$  cells. Mitochondria are indicated by open arrows. Nuclei (n) and vacuole (v) are also indicated. Bar, 1  $\mu m$ . The mitochondria in  $\Delta mdm31$ ,  $\Delta mdm32$ , and  $\Delta mdm31\Delta mdm32$  cells exhibited similar morphology (not shown). (C) The quantitative analysis of mitochondrial cross-section areas in the strains W303-1B (wt,  $n = 60$ ),  $\Delta mdm31$  ( $\Delta 31$ ,  $n = 20$ ),  $\Delta mdm32$  ( $\Delta 32$ ,  $n = 70$ ), and  $\Delta mdm31\Delta mdm32$  ( $\Delta\Delta$ ,  $n = 23$ ). Data are represented as the mean area ± average deviations (error bars).

tures in greater detail, we utilized transmission electron microscopy of permanganate-fixed cells. We observed that the size of mitochondrial cross-section areas was more variable in mutant cells than in the wild-type strain. In addition, the average cross-section size was 2.5-fold larger in the  $\Delta mdm31$  and  $\Delta mdm32$  single mutants and 4-fold larger in the  $\Delta mdm31\Delta mdm32$  double mutant (Figure 4).

**Passive swelling of mutant mitochondria in potassium acetate:** Nonrespiring mitochondria undergo passive swelling in the presence of an isotonic concentration of potassium acetate. This swelling is caused by KAc influx, which is mediated by a  $K^+/H^+$  antiporter. Acetate anions can cross the mitochondrial membrane as acetic acid, thereby cotransporting a proton into the matrix. Potassium can be exchanged for a proton, resulting in electroneutral KAc translocation. Subsequently, an increased matrix concentration of KAc initiates water influx and organelle swelling, which can be monitored by measuring light scattering. The rate of spontaneous passive swelling in KAc is therefore indicative of  $K^+/H^+$  antiporter activity. In its absence the matrix would rapidly acidify due to cotransported protons and acetate influx would be counterbalanced by the pH gradient (NICHOLLS and FERGUSON 2001).

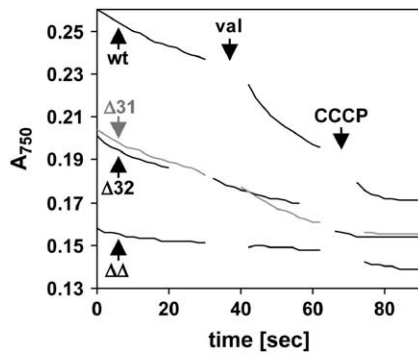


FIGURE 5.—Passive swelling of mitochondria in isotonic potassium acetate. Light absorbance time scans of mitochondrial preparations from W303-1B (wt),  $\Delta mdm31$  ( $\Delta 31$ ),  $\Delta mdm32$  ( $\Delta 32$ ), and  $\Delta mdm31\Delta mdm32$  ( $\Delta\Delta$ ) strains. At  $\sim 30$ -sec intervals (indicated by arrows), valinomycin and CCCP were added as indicated.

On the basis of this rationale we measured the passive swelling of mitochondria in 0.3 M KAc and observed that the organelles from the  $\Delta mdm31$  and  $\Delta mdm32$  single-deletion mutants exhibited decreased swelling rates (Figure 5). The effect was even more pronounced in the  $\Delta mdm31\Delta mdm32$  strain. Furthermore, the addition of exogenous  $K^+/H^+$  antiporter (valinomycin plus CCCP) revealed that the swelling capacity of the mutant mitochondria was also substantially reduced.

**The effect of nigericin on mitochondrial morphology in  $\Delta mdm31$  and  $\Delta mdm32$  mutants:** Deletion of *MDM31* and *MDM32* in the W303-1B strain affected the morphology of mitochondria in cells grown in complex media containing either glucose or raffinose as the sole carbon source (Figure 6). More than 90% of the cells in all three mutant strains ( $n = 100$  for each strain) contained mitochondria that formed clusters, exhibited a bulky shape, and did not form branched reticulum. However, in  $\geq 80\%$  of the inspected cells ( $n = 100$  for each tested strain) the addition of nigericin to the mutant strains converted their mitochondria to small dots and tubules forming branched reticulum (Figure 6).

## DISCUSSION

Our screen for nigericin-resistant mutants revealed genes whose functions in regulation of mitochondrial ion homeostasis and/or organelle morphology are largely unknown (Table 1). The results presented in this work shed some light on the possible role for *MDM31* and *MDM32* in maintaining mitochondrial morphology. We show that loss-of-function mutations in these genes render yeast tolerant to the ionophore nigericin on media containing glycerol as the sole carbon source. Also, the nigericin-induced *petite* colony formation is dramatically reduced in *mdm31* and *mdm32* mutants. This effect is specific to nigericin, as ethidium bromide treatment resulted in a high frequency of *petites* in all mutant strains (Figure 2B). The nature of our

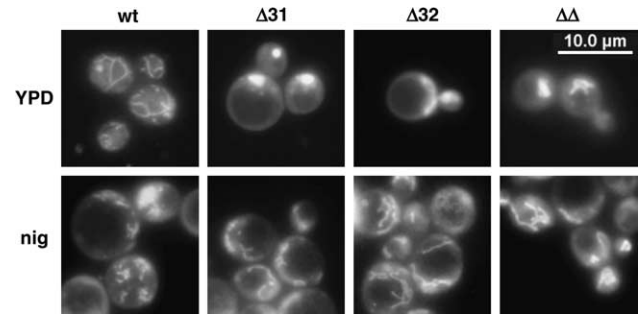


FIGURE 6.—Morphological differences between wild-type and mutant mitochondria. Mitochondrial morphology was visualized by light microscopy following DASPMI staining in the following strains grown in YPD in the absence (top row) or presence of 30 mg/liter nigericin (bottom row): W303-1B (wt),  $\Delta mdm31$  ( $\Delta 31$ ),  $\Delta mdm32$  ( $\Delta 32$ ), and  $\Delta mdm31\Delta mdm32$  ( $\Delta\Delta$ ).

screen prevented the isolation of PDR mutants, indicating that nigericin enters the mutant cell and interacts with intracellular membranes. Initially, we wanted to verify that incorporation of nigericin into mitochondrial membranes was not compromised. We found that nigericin was able to induce fast swelling of isolated mutant mitochondria in 0.3 M KAc, indicating that nigericin is indeed able to incorporate into the mutant mitochondrial membranes and operate there as a  $K^+/H^+$  antiporter (data not shown). These observations plus (i) the growth inhibition of the mutant strains by high concentrations of potassium, sodium, or magnesium and (ii) the elevated levels of magnesium in mutant mitochondria suggest that the increased tolerance to nigericin is most likely due to an imbalance in mitochondrial ion homeostasis. The two- to threefold increase of magnesium levels in mitochondrial extracts from *mdm31* and *mdm32* mutants is extraordinary. In comparison, yeast strains with the overexpressed mitochondrial magnesium channels *Mrs2* or *Lpe10* exhibit no greater than a 50% increase in mitochondrial magnesium (BUI *et al.* 1999; GREGAN *et al.* 2001a,b; KOLISEK *et al.* 2003). Hence, *Mdm31p* and *Mdm32p* seem to be involved in mitochondrial magnesium homeostasis either directly or indirectly in a regulatory capacity. Since magnesium export is negligible in yeast mitochondria (KOLISEK *et al.* 2003), *Mdm31p* and *Mdm32p* proteins are more likely involved in regulation of mitochondrial magnesium influx. Alternatively, *Mdm31p* and *Mdm32p* may regulate levels of magnesium-binding ligands (*e.g.*, phosphate, nucleotides, or citrate) in mitochondria, thus affecting magnesium content indirectly.

Computer analyses indicated that both proteins contain one putative transmembrane domain. This hydrophobic region is particularly well conserved among homologs from other fungi (Figure 1). As it was recently demonstrated that both *Mdm31p* and *Mdm32p* are integral membrane proteins (DIMMER *et al.* 2005), it is conceivable that their transmembrane domain is functionally important [interestingly, *Yo1027* protein,

recently indicated to be involved in mitochondrial  $K^+/H^+$  exchange, has only a single predicted transmembrane domain (NOWIKOVSKY *et al.* 2004; FROSCHAUER *et al.* 2005)]. Conserved serines and threonines are over-represented in the transmembrane domains of Mdm31 protein homologs, composing ~20–25% of the sequences. It was shown previously that the hydroxyl group containing amino acids can form intra- or interhelical hydrogen bonds that may be important for helix stabilization and bending or protein-protein interactions, respectively (SENES *et al.* 2001; DEUPI *et al.* 2004). Interestingly, the role for serines and threonines in substrate recognition and/or cation binding was recognized in various transporters like  $Na^+$ /sulfate cotransporter NaSi-1 (LI and PAJOR 2003), human retinal cone Na/Ca-K exchanger (WINKFEIN *et al.* 2003), neuronal vesicular monoamine transporter VMAT2 (MERICKEL *et al.* 1995), or  $Na^+K^+$ -ATPase (ARGUELLO and LINGREL 1995). In addition, the hydroxyl group containing amino acids is likely involved in transport of small cations like magnesium (MONCRIEF and MAGUIRE 1999). It is therefore conceivable that a conserved sequence in the Mdm31p transmembrane domain may be involved in a specific protein-protein interaction and/or a small cationic substrate recognition and transport.

Mitochondria in the  $\Delta mdm31$  and  $\Delta mdm32$  mutants, as assessed by light microscopy, form compact aggregates (DIMMER *et al.* 2002). Electron-microscopic analysis performed in this study revealed that mutant mitochondria are dramatically enlarged compared to those of the wild-type strain (Figure 4). Furthermore, an observed heterogeneity in the mitochondrial cross-section areas suggests that mutant cells contain spherical rather than tubular mitochondria. This observation indicates that Mdm31 and Mdm32 proteins are implicated in mitochondrial volume regulation. Giant organelles, largely devoid of cristae, were observed in mitochondria of  $\Delta mdm31$ ,  $\Delta mdm32$ , and  $\Delta mdm31\Delta mdm32$  mutants also by DIMMER *et al.* (2005). In addition, these authors observed frequent circular-shaped double-membrane structures inside the organelles. All these data indicate that lack of Mdm31p and/or Mdm32p dramatically affects the global structure of the organelle.

It has been proposed that mitochondrial volume homeostasis could be maintained by a  $K^+/H^+$  antiporter (MITCHELL 1966; BRIERLEY *et al.* 1977). Therefore, we tried to measure the activity of such a transporter in our mutant strains using passive swelling of mitochondria in an isotonic KAc solution. Indeed, mitochondria from the mutant strains exhibited decreased swelling rates (Figure 5). However, the addition of exogenous  $K^+/H^+$  antiport composed of valinomycin plus CCCP revealed that the swelling capacity of the mutant mitochondria is very low, indicating that they are already swollen. This is supported by the fact that the starting absorbance of  $\Delta mdm31$ ,  $\Delta mdm32$ , and  $\Delta mdm31\Delta mdm32$  mitochondrial preparations of the

same concentration was lower than that of the wild-type mitochondria. We propose that changes in the swelling rates are a consequence of a preswollen state of the organelles and therefore are not a reliable indicator of impaired  $K^+/H^+$  antiport activity. This precaution may extend to all mutants with defective mitochondrial morphology. Experiments using nigericin or valinomycin plus CCCP are critical in demonstrating whether the swelling capacity of the mutant mitochondria is already compromised.

What is the link between defective mitochondrial morphology and magnesium content? Accumulated magnesium *per se* may increase matrix osmolarity and facilitate water influx. Yet changes in mitochondrial volume occur naturally due to variable cation influx caused by normal fluctuations in the  $\Delta\Psi$ . These changes are believed to be counteracted by  $K^+/H^+$  antiporter activity, which exports potassium, reestablishes normal intramitochondrial osmolarity, and alleviates the generated turgor. However,  $K^+/H^+$  antiporter must be tightly regulated. If not, the antiport would dissipate the  $\Delta pH$  and create a futile cation transport cycle. Coincidentally, there is evidence for inhibition of  $K^+/H^+$  antiporter activity by magnesium from the matrix side of the mitochondrial membrane, although the  $K^+/H^+$  exchange in yeast was inhibited with lower affinity ( $K_i = 2$  mM) than in liver mitochondria ( $K_i = 0.05$ – $0.06$  mM) (WELIHINDA *et al.* 1993). In addition, it was shown that both mammalian and yeast mitochondrial  $K^+/H^+$  antiporter activities are activated upon magnesium depletion (DORDICK *et al.* 1980; NOWIKOVSKY *et al.* 2004). Recent studies from the Schweyen laboratory demonstrated that addition of magnesium to submitochondrial particles exerts a strong inhibition of  $K^+$  and  $H^+$  fluxes (FROSCHAUER *et al.* 2005). Accumulated magnesium in  $\Delta mdm31$  and  $\Delta mdm32$  mitochondria not only contributes to increased osmolarity, but also may inhibit  $K^+/H^+$  antiporter activity. Consequently, mitochondrial osmolarity and volume deregulation may lead to significant changes in mitochondrial morphology. This is supported by recent results of DE VOS *et al.* (2005), who have shown that disruption of mitochondrial functions rapidly and reversibly altered mitochondrial shape by either a cytoskeletal-dependent or a swelling-dependent mechanism, the latter indicating a close relationship between the regulation of mitochondrial volume and morphology.

Our hypothesis is that nigericin may substitute for an endogenous mitochondrial  $K^+/H^+$  antiporter activity, which is inhibited by abnormally elevated levels of magnesium. This is supported by the observation that nigericin not only affected growth of the mutants, but also alleviated the morphology defects of their mitochondria. Light microscopy of DASPMI-stained mitochondria from the mutant cells treated with nigericin revealed that the ionophore was able to restore their defective morphology to an almost normal appearance



(Figure 6). We also investigated whether nigericin is able to suppress other phenotypes associated with the  $\Delta mdm31$  and  $\Delta mdm32$  mutations, namely the growth defects in YPE media with high salt concentration. However, the addition of nigericin into YPE supplemented with (0.3 or 0.6 M) KCl, NaCl, or MgSO<sub>4</sub> did not prevent their inhibitory effect (data not shown). There are two problems with a clear-cut interpretation of these experiments. First, the growth yield of the mutants in salt-containing media is decreased by ~20–30% and partial restoration of the growth defects by nigericin would be difficult to distinguish from statistical deviations. Furthermore, although the *mdm31* and *mdm32* mutants are able to grow in the presence of nigericin, their growth in YPE containing the ionophore is still slower than in a control YPE media. In any case, we can conclude that nigericin operates as a chemical suppressor of the morphological defects observed in  $\Delta mdm31$  and  $\Delta mdm32$  mutants (Figure 6).

Recently, it was demonstrated that Mdm31p and Mdm32p are components of two distinct protein complexes in the inner mitochondrial membrane (DIMMER *et al.* 2005). The authors observed that mutations in *MDM31* and *MDM32* are synthetically lethal with mutations in *MMM1*, *MMM2*, *MDM10*, and *MDM12*. Mmm1p, Mmm2p, Mdm10p, and Mdm12p were suggested to form the outer membrane protein scaffold and the mitochore, a mitochondrial protein complex implicated in sustaining proper shape of mitochondria, the organelle attachment to the actin cytoskeleton, and an efficient segregation of mitochondrial DNA (BURGESS *et al.* 1994; SOGO and YAFFE 1994; BERGER *et al.* 1997; BOLDOGH *et al.* 1998, 2003; HOBBS *et al.* 2001; YOUNGMAN *et al.* 2004). On the basis of these genetic interactions and observed displacement of mitochondrial nucleoids, it was suggested that Mdm31p and Mdm32p are required to link mtDNA to the mitochore (DIMMER *et al.* 2005). However, since the loss of mtDNA itself cannot explain observed synthetic lethality, it was proposed that the inability to grow is caused by an exacerbated motility of mitochondria in the double mutants. Our results provide an alternative explanation for observed synthetic lethality. We showed that  $\Delta mdm31$  and  $\Delta mdm32$  mutants possess swollen, osmotically unstable mitochondria. Thus, displacement of mtDNA from mitochore structures in  $\Delta mdm31$  and  $\Delta mdm32$  mutants (DIMMER *et al.* 2005) can be explained by mechanical tension resulting from a severe mitochondrial swelling. The deletion mutant in *MDM38/MKH1* displays a similar phenotype of mitochondrial enlargement and mtDNA instability (DIMMER *et al.* 2002; NOWIKOVSKY *et al.* 2004). We suggest that *MDM31*, *MDM32*, *MKH1*, *MMM1*, *MMM2*, *MDM10*, and *MDM12* provide two independent pathways that synergistically preserve mitochondrial integrity. The synthetic lethality of mutants in *MDM31*, *MDM32*, *MMM1*, *MMM2*, *MDM10*, and *MDM12* can result from production of osmotically

destabilized mitochondria devoid of the outer membrane protein scaffold.

A study of factors regulating mitochondrial osmosis and their influence on organelle morphology may be helpful for understanding the process of apoptosis. It was demonstrated that the release of cytochrome *c* can be initiated by osmotically induced swelling, although the role for such a mechanism in apoptosis remains to be determined (HALESTRAP *et al.* 2000; GOGVADZE *et al.* 2004).

The results presented in this article support the existence of a direct link between mitochondrial cation homeostasis, mitochondrial volume control, and morphology. The emerging complex picture clearly warrants further studies in this field.

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