Mmm1p, a Mitochondrial Outer Membrane Protein, Is Connected to Mitochondrial DNA (mtDNA) Nucleoids and Required for mtDNA Stability

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Abstract. In the yeast *Saccharomyces cerevisiae*, mitochondria form a branched, tubular reticulum in the periphery of the cell. Mmm1p is required to maintain normal mitochondrial shape and in *mmm1* mutants mitochondria form large, spherical organelles. To further explore Mmm1p function, we examined the localization of a Mmm1p–green fluorescent protein (GFP) fusion in living cells. We found that Mmm1p-GFP is located in small, punctate structures on the mitochondrial outer membrane, adjacent to a subset of matrix-localized mitochondrial DNA nucleoids. We also found that the temperature-sensitive *mmm1-1* mutant was defective in transmission of mitochondrial DNA to daughter cells immediately after the shift to restrictive temperature. Normal mitochondrial nucleoid structure also collapsed at the nonpermissive temperature with similar kinetics. Moreover, we found that mitochondrial inner membrane structure is dramatically disorganized in *mmm1* disruption strains. We propose that Mmm1p is part of a connection between the mitochondrial outer and inner membranes, anchoring mitochondrial DNA nucleoids in the matrix.

Key words: mitochondria • *MMM1* • cristae • nucleoids • mitochondrial DNA

Introduction

Mitochondria are ubiquitous and essential organelles with a variety of shapes in different cell types (Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994). In the yeast *Saccharomyces cerevisiae*, mitochondria form a branched, tubular reticulum in the cell periphery. While this tubular shape is common to mitochondria in many types of eukaryotic cells, it is not clear why any of these cells establish this particular morphology. In addition, the mechanism by which mitochondrial shape is established and maintained is not well understood. To identify the proteins that mediate mitochondrial structure, yeast mutants have been isolated that are defective in normal organelle shape. We isolated one mutant, *mmm1*, in which mitochondria appear as a few large, spherical organelles (Burgess et al., 1994). Additional genetic screens identified two other mutants similarly defective in mitochondrial shape, *mdm10* (Sogo and Yaffe, 1994) and *mdm12* (Berger et al., 1997).

The Mmm1 protein resides in the mitochondrial outer membrane and is proposed to maintain mitochondria in an elongated conformation by mediating its interaction with the cytoskeleton (Burgess et al., 1994). Yeast mitochondria have been shown to interact with actin (Drubin et al., 1993; Smith et al., 1995), and *mmm1* mutants have been shown to be defective in binding to actin filaments and in actin-based motility assays (Boldogh et al., 1998). Actin binding, however, is not the sole determinant of mitochondrial shape. Disrupting the actin cytoskeleton by mutating actin (Drubin et al., 1993) or by treating cells with Latrunculin A (Ayscough et al., 1997) changes mitochondrial shape, but neither produces the large, spherical organelles seen in *mmm1*, *mdm10*, or *mdm12* mutants.

To learn more about the function of Mmm1p, we constructed a fusion between Mmm1p and the green fluorescent protein (GFP).[1] In living yeast cells, we found that Mmm1p-GFP was present in punctate spots on the surface of mitochondrial tubules, and that Mmm1p-GFP was adjacent to a subset of mitochondrial DNA (mtDNA) nucleoids. Because of the connection between Mmm1p and mtDNA, we examined mtDNA stability and structure in the temperature-sensitive *mmm1-1* mutant. We found that *mmm1-1* mutants rapidly lost nucleoid structure and were

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[1] *Abbreviations used in this paper:* 3-D, three dimensional; DIC, differential interference contrast; GFP, green fluorescent protein; HA, hemagglutinin; mtDNA, mitochondrial DNA.

immediately defective in transmission of mtDNA to daughter cells. At later times, *mmm1-1* mutants lost virtually all of their mtDNA. We also found that in mutants disrupted in *MMM1*, mitochondria showed a dramatic loss of inner membrane structure.

Materials and Methods

Strains and Relevant Genotypes

Strain YAAH1 was constructed by transforming plasmid pAA2, which contains Mmm1p-GFP, into *mmm1::URA3/MMM1 leu2/leu2* strain YSB108 (Burgess et al., 1994). After sporulation, *MATa mmm1::URA3 leu2* strain containing pAA2 was isolated. YAAH2, which lacks mtDNA, was made by treating strain YAAH1 with ethidium bromide (Guthrie and Fink, 1991). Strain YAAH3 was constructed by transforming plasmid pAA7, which contains the Mmm1p-HA fusion protein, into YSB108. After sporulation, *MATa mmm1::URA3 leu2* strain with pAA7 was isolated. *MATa mmm1-1* strain YSB105 (Burgess et al., 1994) has been described. *MMM1* strain RJ485 and *mmm1::URA3* strain RJ483 were generated by sporulation of YSB108. Standard yeast media and genetic techniques (Adams et al., 1997) were used. *MMM1* strain YHS92 was treated with ethidium bromide (Guthrie and Fink, 1991) to induce loss of mtDNA.

Plasmid Construction

pAA2, a *LEU2-CEN* plasmid that expresses Mmm1p with GFP fused to its carboxyl terminus, was constructed as follows. First, pAA1, a *CEN-LEU2* plasmid that contains GFP with a unique NotI site at its amino terminus and the *TIM23* terminator region, was constructed by PCR amplifying GFP from pQBI25 (Quantum Biotechnologies Inc.) using oligonucleotides 239 (5′-CGGGCGGCCGCATAACTAGCAAAGGAG-3′) and 246 (5′-TCCCCGCGGTCAGTTGTACAGTTCATC-3′), and then digested with SacII and NotI. The *TIM23* terminator region from pJE7 (Emtage and Jensen, 1993) was amplified using oligonucleotides 243 (5′-GCCCCGCGGGCAACACAAGAACCTACTC-3′) and 244 (5′-CGGGAGCTCGGATCCGTACATTGTAG-3′), and then digested with SacII and SacI. The two PCR fragments were inserted into NotI/SacI-digested pRS315 (Sikorski and Hieter, 1989). Second, using pSB1 (Burgess et al., 1994), oligonucleotides 245 (5′-CCGCTCGAGTCGTATTCATCACTTGTC-3′) and 106 (5′-CCAGCGGCCGCATAACTCTGTAGGCTTTTC-3′), a 1.4-kbp DNA fragment containing the *MMM1* open reading frame and 450 bp of upstream sequences, was PCR amplified, isolated, digested with NotI and XhoI, and inserted into the NotI-XhoI sites of GFP-containing plasmid pAA1.

pAA7, a *LEU2-CEN* plasmid that expresses Mmm1p with the hemagglutinin (HA) epitope fused to its carboxyl terminus, was constructed as follows. First, pAA3, a *CEN-LEU2* plasmid that contains the HA epitope with a unique NotI site at its amino terminus was constructed by amplifying the triple HA epitope from pGTEP (Tyers et al., 1992) using oligonucleotides 284 (5′-TCCCGCGGTTAGCACTGAGCAGCGTAATC-3′) and 285 (5′-ATAAGAATGGCGGCCGCATCTTTTAC-3′). The PCR product was digested with NotI and SacII, and used to replace the GFP containing NotI-SacII fragment of pAA1. pAA7 was constructed by inserting the NotI-XhoI fragment containing *MMM1* from pAA2 into NotI-XhoI–cut pAA3.

pAA11 was constructed by PCR amplifying the mitochondrial *COX2* gene from total yeast DNA (Yaffe et al., 1989) using oligonucleotides 389 (5′-GTTAGATTTATTAAGATTAC-3′) and 390 (5′-GTTCATTTAATCC-3′), and was then blunt-end ligated into the EcoRV site of Bluescript II KS$^+$ (Stratagene).

Fluorescence Microscopy

Yeast cells were grown in SD medium supplemented with the appropriate amino acids to an OD_{600} of 0.5–0.7. To stain mitochondria, cells were incubated with 0.1 μM MitoTracker™ CMX-Ros (Molecular Probes, Inc.) for 30 min. To stain mitochondrial DNA, cells were incubated with 1 μg/ml DAPI (Molecular Probes, Inc.) for 15 min. Cells were examined with a Axioskop microscope (Carl Zeiss, Inc.) using a 100× Plan Apochromat objective equipped with differential interference contrast (DIC) optics. Images were captured with a Princeton MicroMax CCD camera (Roper Industries) using IP Lab software, version 3.2 (Scanalytics). Alternatively, cells were examined using a DeltaVision system (Applied Precision Instruments) based on an Axiovert (Carl Zeiss, Inc.) with a PXL CCD camera (Roper Industries) and a 100× Plan Apochromat lens. 0.2-μm images in the z axis were taken of each cell, and each image was deconvolved using DeltaVision software to remove out of focus material. For three-dimensional (3-D) reconstructions, all 15 images were used; for figures, the indicated number of images were flattened and printed.

Immunogold Labeling and Electron Microscopy

Electron microscopy was performed as previously described (Reider et al., 1996). In brief, cells were fixed in 3% glutaraldehyde, embedded in Spurr's resin, and thin sections were cut on a Reichert Ultracut T ultramicrotome. Samples were examined using a Philips EM 410 electron microscope. Immunoelectron microscopy was performed as previously described (Reider et al., 1996). Cells growing at 30°C were fixed in suspension for 15 min by adding an equal volume of freshly prepared 8% formaldehyde in PBS. Cells were pelleted and resuspended in 4% formaldehyde in PBS and fixed for an additional 18–24 h at 4°C. Cells were then washed briefly in PBS and resuspended in 1% low temperature melting agarose. After cooling, the agarose blocks were trimmed into 1-mm^3 pieces, infiltrated with 2.3 M sucrose, 20% polyvinyl pyrrolidone (10 K), pH 7.4 for 2 h, mounted onto cryopins, and rapidly frozen in liquid nitrogen. Ultrathin cryosections were cut on a Leica UCT ultramicrotome equipped with an FCS cryoattachment and collected onto formvar-carbon–coated nickel grids. Grids were washed through several drops of PBS containing 2.5% fetal calf serum, 10 mM glycine, pH 7.4, blocked in 10% fetal calf serum for 30 min, and then incubated overnight with a 1:50 dilution of antibody to the HA epitope. After washing, the grids were incubated for 2 h in 5 nm Donkey anti–mouse antibody conjugated to 5 nm gold particles (Jackson ImmunoResearch Laboratories). Grids were then washed through several drops of PBS, followed by several drops of water, and subsequently immersed in a solution of 3.2% polyvinyl alcohol (10 K), 0.2% methyl cellulose (400 centiposes), and 0.1% uranyl acetate. Grids were examined at 80 kV using the electron microscope.

Quantitation of Mitochondrial DNA

For dot-blot analysis, total yeast DNA was isolated, and then roughly equivalent amounts (determined by agarose gel electrophoresis and ethidium bromide staining) were spotted onto Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech). For Southern blots, total DNA was digested with NotI, and then transferred to membranes as described (Maniatis et al., 1982). DNA was cross linked to membranes using a UV Stratalinker (Stratagene) with 1.2 J. For use as a probe, the mitochondrial COX2 gene was PCR amplified from plasmid pAA11 using oligonucleotides 389 and 390, ^{32}P labeled using a Random Primer Labeling kit (Amersham Pharmacia Biotech), and hybridized to nylon membranes as described (Maniatis et al., 1982). After washing, membranes were analyzed by phosphorimaging using a Molecular Dynamics Storm 860 phosphorimager (Molecular Dynamics) and quantitated with ImageQuant software, version 1.2 (Molecular Dynamics). To account for variable DNA recovery, we reprobed filters with the nuclear-encoded, ^{32}P-labeled *TIM23* on a 2.2 kbp SacI-BamHI fragment isolated from pKR1 (Ryan and Jensen, 1993). All values were normalized to the amount of hybridization to *TIM23*.

Online Supplemental Material

Quicktime movies of the three-dimensional reconstructions of Mmm1p-GFP and mitochondria to accompany Figure 1 (Video 1) and Mmm1p-GFP and mtDNA nucleoids to accompany Figure 2 (Video 2) are available at http//:www.jcb.org/cgi/content/full/152/2/401/DC1.

Results

Mmm1p-GFP Is Localized in Discrete Patches on the Mitochondrial Surface, Adjacent to mtDNA Nucleoids

Previous studies have shown that Mmm1p is a mitochondrial outer membrane protein required to maintain mitochondrial shape. To further examine Mmm1p function, we localized Mmm1p in living yeast cells by expressing the green fluorescent protein fused to the carboxyl terminus of Mmm1p in cells disrupted in *MMM1*. We found that Mmm1p-GFP rescued the growth defect of *mmm1::URA3*

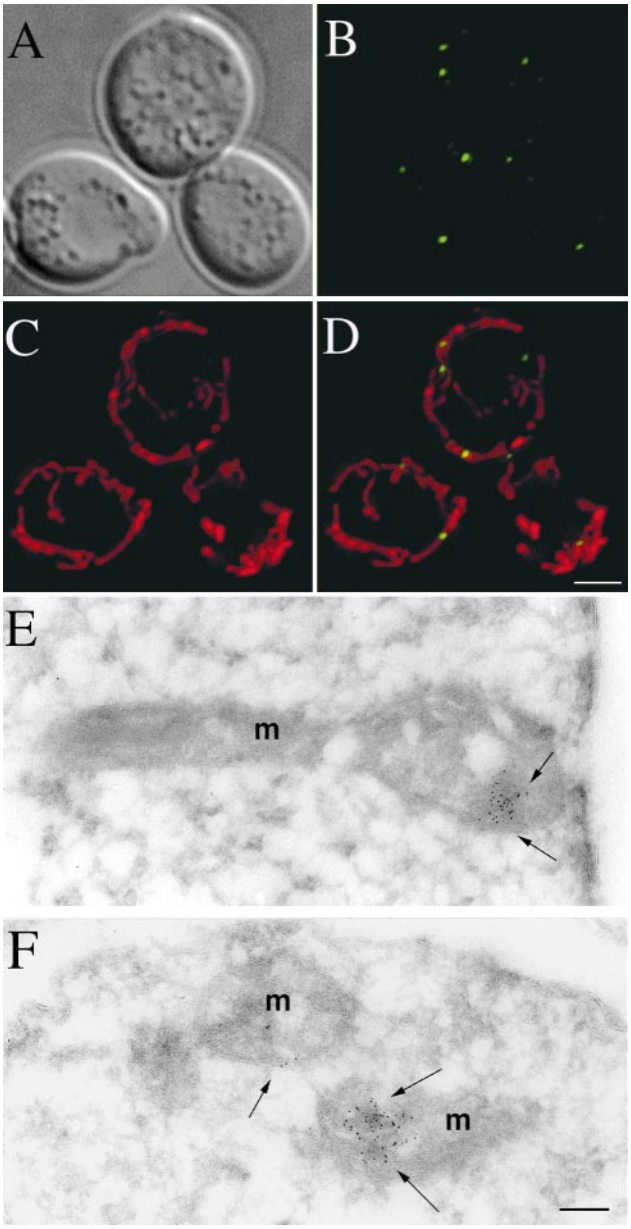

Figure 1. Mmm1p is localized in punctate structures on the mitochondrial surface. *mmm1::URA3* strain YAAH1, which expresses Mmm1p-GFP from plasmid pAA2, was grown in YEP glycerol/ethanol media to early log phase and stained with MitoTracker™ Red. Live cells were mounted on slides and examined using a DeltaVision microscope system. 15 total images were taken in the z axis through the cells, deconvolved, and 7 of the 15 optical sections near the top of the cells were flattened into a single image. (A) Differential interference contrast (DIC); (B) Mmm1p-GFP fluorescence; (C) MitoTracker fluorescence; (D) merged images of B and C. Bar: 2 μm. (E and F) Strain YAAH3, which expresses Mmm1p-HA, was fixed, embedded, and frozen. Cryosections were incubated with antibodies to the HA epitope, followed by incubation with secondary antibodies conjugated to 5-nm gold particles. After staining, sections were examined under the electron microscope. Bar: 0.1 μm.

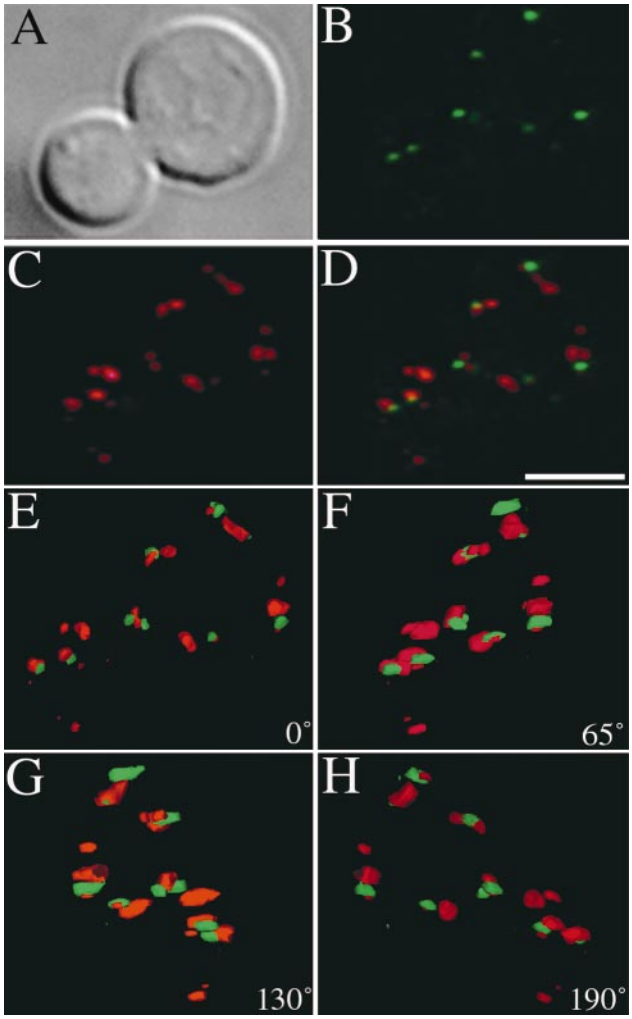

Figure 2. Mmm1p is adjacent to mitochondrial DNA nucleoids. Mmm1p-GFP–containing strain YAAH1 was grown to early log phase, stained with DAPI, and examined using a DeltaVision microscope. DAPI staining of live cells preferentially stains mtDNA, and the nucleus is only poorly stained (Williamson and Fennell, 1979). Shown are all 15 deconvolved z sections, which have been flattened to a single image. (A) DIC; (B) Mmm1p-GFP fluorescence; (C) DAPI fluorescence; (D) merged images of B and C. (E–H) The images in D were processed using the DeltaVision model building tool, which removes background and shows fluorescent signals as solid objects for easier viewing. Four views of the 3-D model, each rotated 0° (E), 65° (F), 130° (G), and 190° (H) with respect to each other. Bar: 2 μm.

cells, demonstrating that the fusion protein is functional, and Western blots confirmed that the fusion protein was intact in yeast cells (A. Aiken Hobbs, unpublished observations). By fluorescence microscopy, the Mmm1p-GFP protein was concentrated in distinct, dot-like structures that colocalized with mitochondrial tubules (Fig. 1). Cells contained on average between five and eight Mmm1p-GFP–containing dots per cell, and 3-D reconstruction of optical slices taken through yeast cells showed that each punctate structure was attached to a mitochondrion (see online movie). While the distribution of dots appeared to be random along the mitochondrial tubule, we noticed that small buds almost always contained an Mmm1p-GFP–containing dot at the bud neck. In contrast to Mmm1p, most other mitochondrial outer membrane proteins, including an OM45p-GFP fusion protein (K. Cerveny, unpublished observations), Tom6p-GFP (Okamoto et al., 1998), and Fzo1p (Hermann et al., 1998), are distrib-

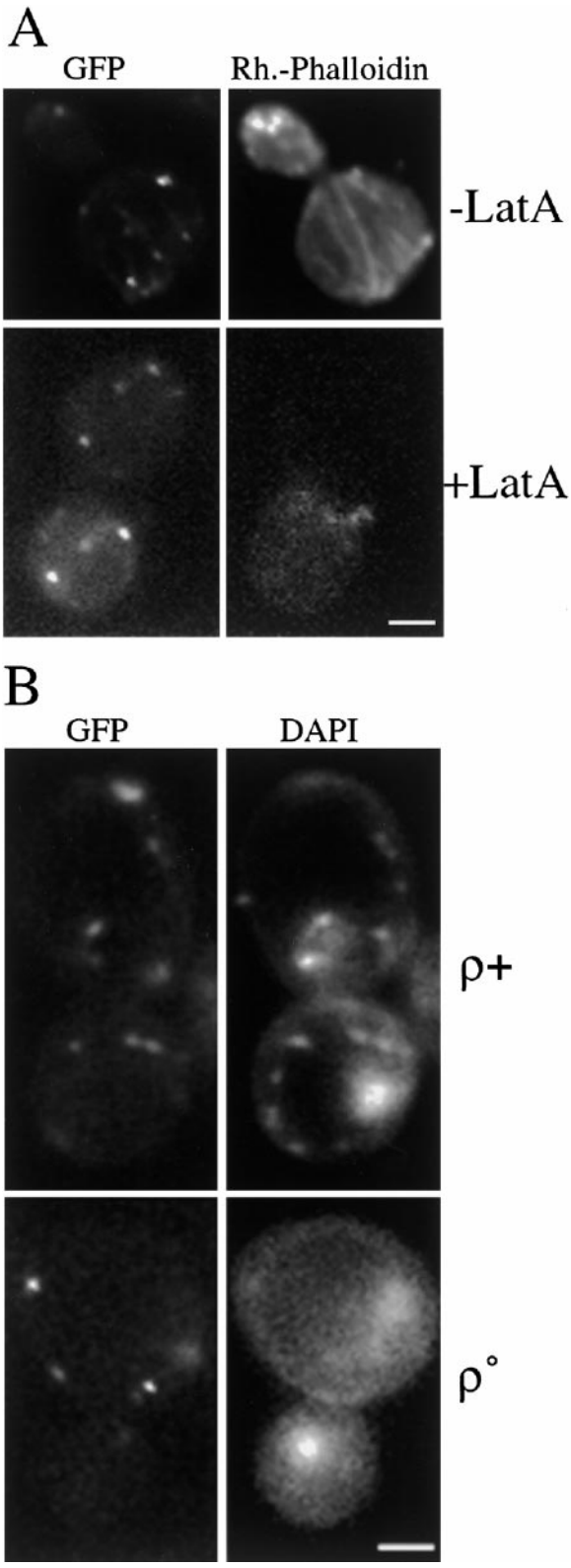

Figure 3. Mmm1p maintains a punctate localization in the absence of actin or mtDNA. (A) YAAH1, which expresses Mmm1p-GFP, was grown to early log phase and treated with Latrunculin A (+LatA), or mock treated (−LatA) at 24°C for 30 min. Cells were fixed and stained with Rhodamine-Phalloidin to visualize the actin cytoskeleton. Representative images of cells examined by fluorescence microscopy in the green (GFP) and red (Rh.-Phalloidin) channels are shown. (B) Mmm1p-GFP–expressing strains YAAH1, which contains mtDNA (ρ+) and YAAH2, which lacks mtDNA (ρ°), were stained with 1 μg/ml DAPI to visualize the mtDNA. Although DAPI treatment of living cells preferentially stains mtDNA, low-level staining of nuclear DNA can be seen when images are overexposed or no mtDNA is present. Bars: 2 μm.

uted uniformly along the organelle surface. To confirm this unusual location for Mmm1p, we examined immunogold-labeled sections of cells expressing an Mmm1p-HA fusion protein by electron microscopy (Fig. 1, E and F). Consistent with the localization of Mmm1p-GFP in live cells, Mmm1p-HA was clustered at distinct sites associated with mitochondria.

In yeast cells, mtDNA is organized into ~10–20 separate DNA-protein complexes called nucleoids (Fig. 2 C; Miyakawa et al., 1987; Kaufman et al., 2000). Since the punctate distribution of mtDNA is similar to the pattern we found for Mmm1p, we stained Mmm1p-GFP–containing cells with DAPI and examined cells by fluorescence microscopy. Strikingly, the majority of Mmm1p-GFP was associated with mtDNA nucleoids (Fig. 2, A–D; also see online movie). When a total of 32 cells were examined using the DeltaVision microscope, deconvolved images and 3-D reconstruction showed that >87% of Mmm1p-GFP structures (186 of 213 GFP-containing dots) were clearly associated with mtDNA. The remaining 27 Mmm1p-GFP structures may also have been adjacent to mtDNA, but our images did not reveal this unambiguously. It is important to note that while Mmm1p-GFP and mtDNA are adjacent, they are clearly in separate structures. In most of the merged images (Fig. 2 D), the Mmm1p-GFP and nucleoids were seen as twin, dot-like structures, one red and one green. Occasionally, a single yellow dot was seen, which probably represented a pair of dots viewed from the top or bottom, instead of from the side. Supporting this idea, 3-D reconstruction of images processed with the DeltaVision model building tool, which removes background and shows fluorescent signals as solid objects for easier viewing, also confirmed the side-by-side location of Mmm1p and mtDNA (Fig. 2, E–H).

While most or all Mmm1p-GFP was located adjacent to mtDNA, not all mtDNA nucleoids were associated with Mmm1p. About half of the nucleoids (186 of 356 DAPI-staining dots) colocalized with Mmm1p-GFP, the remaining nucleoids (170 of 356 DAPI-staining dots) had no associated Mmm1p-GFP. Mmm1p therefore associates with a subset of mtDNA nucleoids.

Actin and mtDNA Are Not Required for Punctate Localization of Mmm1p

Yeast mitochondria have been shown to interact with actin filaments (Drubin et al., 1993; Smith et al., 1995) and Mmm1p appears to be important for this interaction (Boldogh et al., 1998). Nevertheless, an intact actin cytoskeleton was not required for Mmm1p distribution. Mmm1p-GFP–containing cells were treated with Latrunculin A to disrupt the actin cytoskeleton (Ayscough et al., 1997) and examined by fluorescence microscopy (Fig. 3 A). Under conditions where normal actin cables and

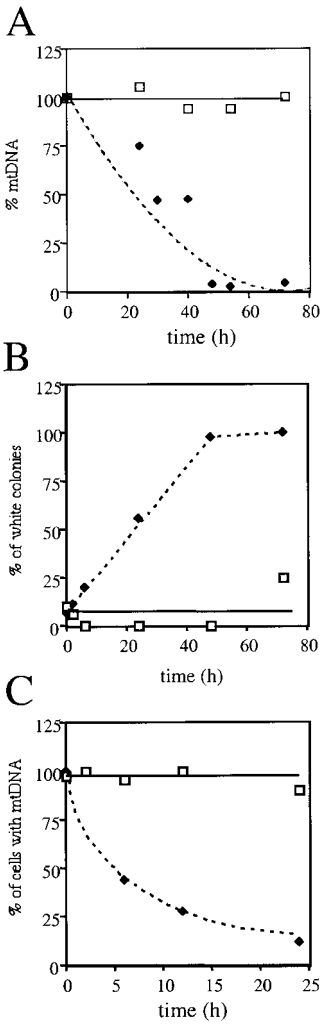

Figure 4. mmm1-1 cells lose mtDNA at the nonpermissive temperature. (A) Wild-type strain RJ485 and *mmm1-1* strain YSB105 were grown at 24°C in YEPD medium, and then shifted to 37°C for the indicated times. Total DNA was isolated, Southern blotted, and probed with ^{32}P-labeled mitochondrial *COX2* gene. After normalization to the levels of hybridization to the nuclear-encoded *TIM23* gene, relative amounts of mtDNA in wild-type (□, solid line) and *mmm1-1* (◆, dotted line) cells was plotted. (B) *ade2 MMM1* strain RJ485 and *ade2 mmm1-1* strain YSB105 were grown at 24°C in YEPD medium, and then shifted to 37°C for the indicated times and dilutions of cells were plated onto YEPD plates. After growth at 24°C for 3–5 d, the number of white (lacking functional mtDNA) and red (with functional mtDNA) colonies were determined. The percentage of white colonies using wild-type (□, solid line) and *mmm1-1* (◆, dotted line) cells is indicated. (C) Wild-type and *mmm1-1* cells were grown at 24°C, and then shifted to 37°C for the indicated times, stained with 1 μg/ml DAPI for 15 min, and examined by fluorescence microscopy. At each time point, 50 cells were examined and the percentage of cells with mtDNA is indicated. Wild-type (□, solid line) and *mmm1-1* (◆, dotted line).

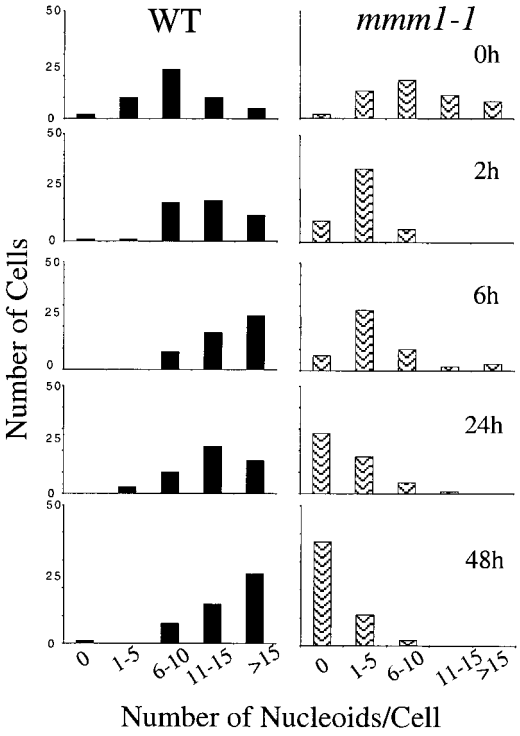

Figure 5. The number of mtDNA nucleoids rapidly decreases in *mmm1-1* cells at the nonpermissive temperature. Wild-type strain RJ485 and *mmm1-1* strain YSB105 were pregrown at 24°C, and then shifted to 37°C for the indicated times. After staining with DAPI, cells were examined by fluorescence microscopy. The number of individual mtDNA nucleoids per cell is plotted.

patches cannot be seen by rhodamine-phalloidin staining, the same number of Mmm1p-GFP dots were still present.

The organization of Mmm1p-GFP also does not require mtDNA. Cells containing Mmm1p-GFP were examined after being treated with ethidium bromide to induce loss of mtDNA (Fig. 3 B). In cells lacking mtDNA (ρ°), the size and number of Mmm1p-GFP dots was the same as that in cells containing mtDNA (ρ+).

mmm1 Mutants Are Defective in Maintenance of mtDNA and Transmission of mtDNA to Daughter Cells

Because Mmm1p-GFP is adjacent to mtDNA nucleoids, we tested the hypothesis that Mmm1p is required to maintain mtDNA, using the temperature-sensitive *mmm1-1* mutant (Burgess et al., 1994). *mmm1-1* cells have normal mitochondrial shape at 24°C, but lose mitochondrial structure at 37°C. To determine the rate of mtDNA loss in *mmm1* mutants, we grew *mmm1-1* cells in glucose-containing medium at 24°C and shifted cells to 37°C for the times indicated. At each time point, total DNA was isolated, transferred to filters by Southern blotting (Fig. 4 A) or dot blotting (A. Aiken Hobbs, unpublished observations), and probed for the mtDNA-encoded *COX2* gene (Maniatis et al., 1982). We found that *mmm1-1* cells rapidly lost mitochondrial DNA at nonpermissive temperature, whereas mtDNA content in wild-type cells remained constant. By 24 h, half of the *mmm1-1* cells no longer contained mtDNA and, by 48 h, mtDNA was undetectable by blotting. Normal mitochondrial shape was lost within 60–90 min of the temperature shift (data not shown). mtDNA loss, therefore, appeared to be secondary to the change in mitochondrial morphology.

To independently determine the rate of mtDNA loss, we examined *mmm1-1* cells for mitochondrial function after a shift to the nonpermissive temperature. As shown in Fig. 4 B, we asked whether *mmm1-1 ade2* cells grew into red- or white-colored colonies on glucose-containing medium. *ade2* mutants accumulate a red pigment if they contain functional mtDNA, but are white if cells lose their mtDNA (Reaume and Tatum, 1949). These growth assays showed that *mmm1-1* cells lost functional mtDNA with similar kinetics, as seen by Southern blotting (compare Fig. 4, A and B).

mmm1-1 mutants are defective in transmission of mtDNA nucleoids. *mmm1-1* cells were grown at 24°C, shifted to 37°C, and then examined by fluorescence microscopy after DAPI staining (Fig. 4 C). We found that, immediately after the shift to 37°C, there was a defect in the segregation of mtDNA to daughter buds. By 6 h, about

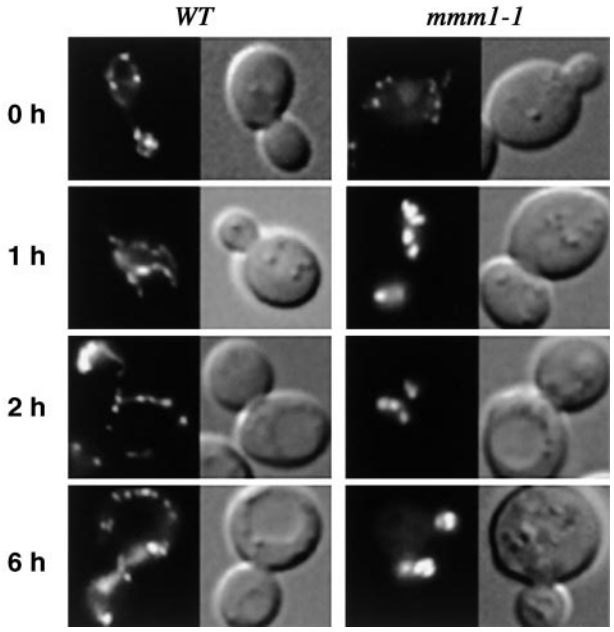

Figure 6. mtDNA nucleoids aggregate in *mmm1-1* cells at the restrictive temperature. Wild-type and *mmm1-1* cells were grown at 24°C, shifted to 37°C for the indicated times, and stained with DAPI. Cells were examined by fluorescence microscopy and representative fluorescent (left) and DIC (right) images are shown.

half of the cells in the culture no longer contained detectable mtDNA and, by 24 h, only 25% of the cells still contained mtDNA. Therefore, failure to segregate mtDNA appears to be part of the explanation for the rapid loss of mtDNA in *mmm1* mutants. Failure to segregate mtDNA is not due to a defect in mitochondrial inheritance. *mmm1-1* cells shifted to 37°C for 6 h show a defect in mtDNA transmission, but efficient mitochondrial segregation on glucose-containing medium. In 111 *mmm1-1* cells expressing a matrix-targeted COX4-GFP construct, 104 cells clearly showed mitochondria present in daughter buds (A. Aiken Hobbs, unpublished observations). These results are consistent with our previous studies (Burgess et al., 1994), which showed that *mmm1* mutants can segregate their mitochondria when grown on glucose medium. Only on nonfermentable carbon sources, such as glycerol, do *mmm1* mutants fail to transmit mitochondria to daughter cells (Burgess et al., 1994).

mmm1 Cells Rapidly Lose mtDNA Nucleoid Structure

Since Mmm1p-GFP colocalizes with mtDNA nucleoids, we asked whether Mmm1p function is required for the normal distribution of nucleoids. We used fluorescence imaging to compare wild-type and *mmm1-1* cells after the shift to 37°C. DAPI staining of 50 total cells revealed that, before the temperature shift, both wild-type and *mmm1-1* cells contained similar numbers (Fig. 5) and sizes (Fig. 6) of mtDNA nucleoids. Most wild-type and *mmm1-1* cells contained ~6–10 separate, mtDNA-containing dots. In wild-type cells, the number of separate nucleoids immediately increased after the shift to the nonpermissive temperature, and then remained constant (Fig. 5). Although the reason that nucleoids rapidly increased in wild-type cells at 37°C is not clear, it is known that number of nucleoids is affected by the growth media and other nutritional factors (MacAlpine et al., 2000). Regardless, the number and size of individual mtDNA nucleoids did not change in wild-type cells at later times at 37°C (Figs. 5 and 6). In *mmm1-1* cells, however, both the number and size of separate nucleoids changed at 37°C. Within 60 min, *mmm1-1* cells contained fewer nucleoids that stained more brightly with DAPI, and nucleoids continued to aggregate at later time points (Fig. 5). The number of separate nucleoids steadily decreased at 37°C. After 6 h, some cells contained a single mtDNA-containing structure, while other cells did not contain any mtDNA. At later times, most cells had lost all mtDNA.

mmm1 Disruptions Show Dramatic Loss of Mitochondrial Inner Membrane Structure

To further investigate the function of Mmm1p, we examined the morphology of mitochondria in *mmm1* mutants by electron microscopy. Instead of the temperature-sensitive *mmm1-1* mutant, we used cells carrying a null mutation in *MMM1*. *mmm1::URA3* disruptions show altered mitochondrial shape and fail to grow on nonfermentable carbon sources at 24°, 30°, and 37°C (Burgess et al., 1994). Wild-type and *mmm1::URA3* cells were grown on raffinose-containing medium at 24°C, fixed, stained, embedded, and sectioned. As shown in Fig. 7 A, wild-type cells contained tubular-shaped mitochondria seen cut in both longitudinal and cross section. Inner membrane cristae appeared as simple membrane invaginations or tubules. In contrast, *mmm1::URA3* cells contained very large organelles whose inner membrane morphology was drastically altered. Instead of tubular-shaped cristae with simple attachments to the outer membrane, the inner membrane in mitochondria of *mmm1::URA3* cells was collapsed, often forming stacks of membrane sheets inside the organelle (Fig. 7 C). In other *mmm1::URA3* mitochondria, circular-shaped inner membrane structures with expanded intermembrane spaces were seen (Fig. 7 C). The aberrant inner membrane structures were often very long, sometimes stretching for over a micron in the *mmm1::URA3* mitochondria. Thus, the lack of the outer membrane protein, Mmm1p, caused a dramatic alteration in the structure of the mitochondrial inner membrane. The disruption of inner membrane structure is not simply the result of mtDNA loss. Wild-type cells that lack mtDNA show elongated tubules similar to those in wild-type cells (Fig. 7 B). Although the number of cristae are reduced compared with wild-type, rho° mitochondria do not show the severe defects in inner membrane structure seen in *mmm1::URA3* cells. The mitochondrial morphology seen in *mmm1::URA3* cells differs from that seen in *mmm1-1* cells shifted to the nonpermissive temperature (Burgess et al., 1994). In our previous studies, *mmm1-1* cells were incubated for 3 h at 37°C. In these cells, mitochondria were seen as spherical organelles, but inner membrane structure was not dramatically altered. However, we find that the mitochondrial inner membrane in *mmm1-1* cells shifted for longer times to the restrictive temperature show many of the dramatic changes seen in Fig. 7 (A. Aiken Hobbs and J.M. McCaffery, unpublished observations). Why in *mmm1-1* cells the col-

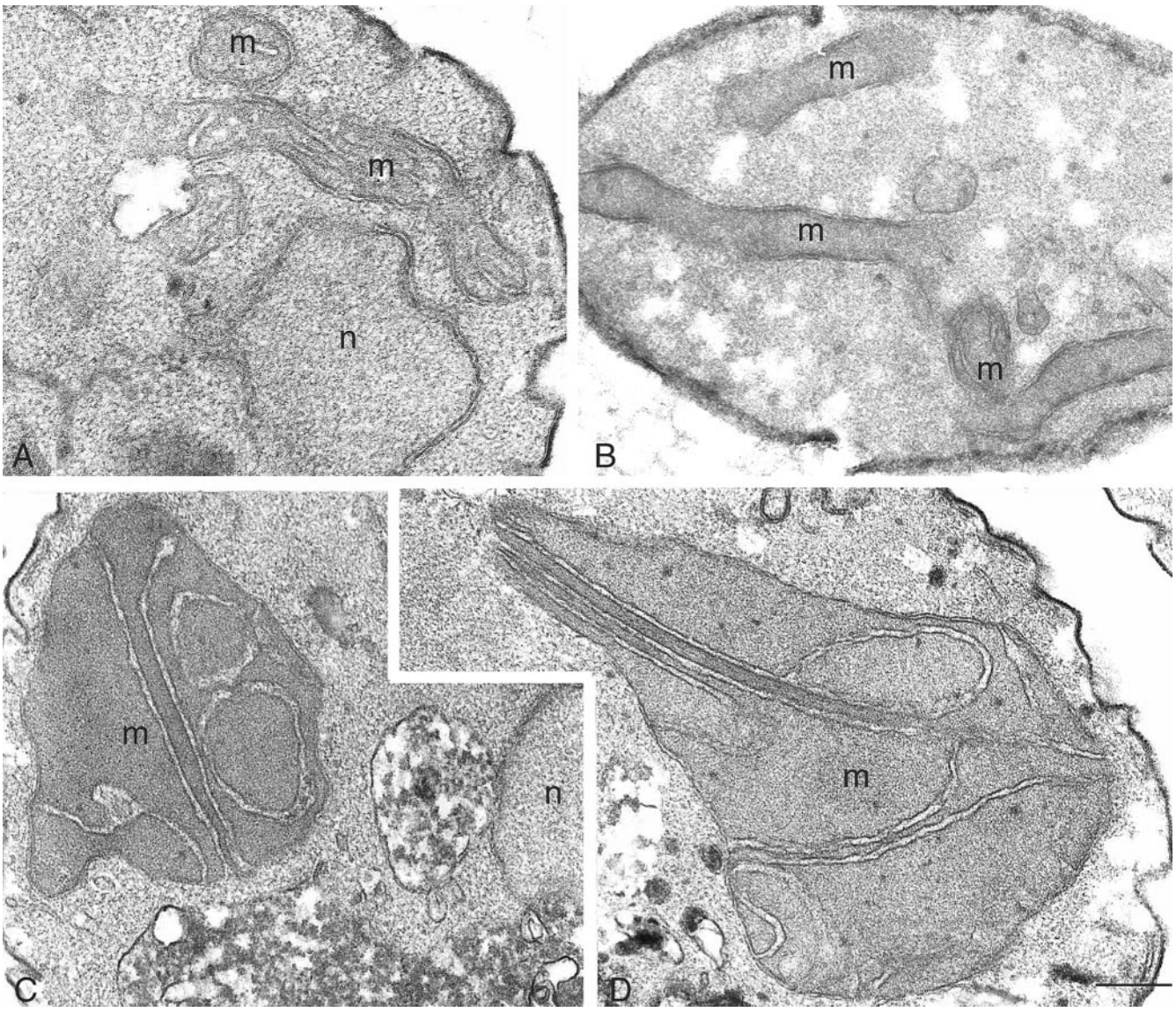

Figure 7. Normal inner membrane cristae morphology is lost in *mmm1* disruption mutants. Wild-type (RJ485), wild-type rho° (YHS92), and *mmm1::URA3* (RJ483) cells were grown at 24°C to mid-log phase, fixed, and thin sections were examined by electron microscopy. (A) Wild-type cells; (B) wild-type rho° cells; (C) *mmm1::URA3* cells. m, mitochondria; n, nucleus. Bar: 0.1 μm.

lapse in external mitochondria shape is seen within 90 min, but the loss of inner membrane structure takes many hours is unclear.

Mmm1p Is Found in Mitochondrial Membrane Vesicles Intermediate in Density to Outer and Inner Membrane Vesicles

Since we find that Mmm1p, an outer membrane protein, is located adjacent to matrix-localized mtDNA nucleoids, our results raise the possibility that Mmm1p resides in contact sites. Contact sites, or regions of close association between the mitochondrial outer and inner membranes, have long been observed in electron micrographs of mitochondria (Hackenbrock, 1968; Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994). Pon et al. (1989) showed that mitochondrial membrane vesicles enriched in contact sites could be isolated on sucrose density gradients. This fraction contained many paired vesicles, with one vesicle containing inner membrane proteins and the other derived from the outer membrane (Pon et al., 1989). Using this procedure, we find that membrane vesicles that contain Mmm1p are located in a fraction intermediate in density to outer and inner membrane vesicles (Fig. 8). Mitochondria from cells expressing an Mmm1p-HA fusion protein were sonicated, and separated on sucrose gradients. Western blots of gradient fractions showed that the outer membrane protein OM45p was located near the top of the gradient (Fig. 8, 1–9), and the β subunit of the F1-ATPase, an inner membrane protein, was found in more dense fractions (Fig. 8, 4–13). Mmm1p-HA–containing vesicles were located between the outer and inner membrane fractions (Fig. 8, 3–9). Our results thus suggest that Mmm1p is located in contact sites.

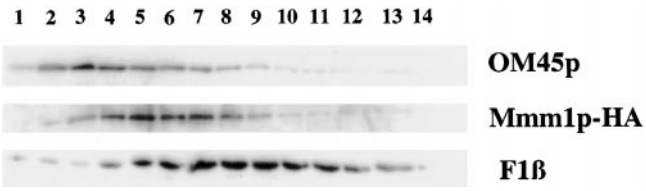

Figure 8. Mitochondrial membrane vesicles containing Mmm1p are located in a fraction intermediate in density to outer and inner membrane vesicles. Mitochondria were isolated from YSB107 (Burgess et al., 1994), which expresses the Mmm1p-HA fusion protein. Mitochondria were sonicated and membrane vesicles were separated on sucrose gradients (Pon et al., 1989). Gradient fractions were collected and analyzed by Western blotting using antibodies to Om45p, an outer membrane protein, the β-subunit of the F1-ATPase (F1β), an inner membrane protein, and HA (Mmm1p-HA). Lane 1 represents the top of the gradient.

Discussion

We have found that Mmm1p, in contrast to most other outer membrane proteins, is located in distinct, punctate structures along mitochondrial tubules. Using an Mmm1p-GFP fusion, we also find that Mmm1p is adjacent to a subset of mtDNA nucleoids. Loss of Mmm1p function causes rapid loss of mtDNA nucleoid structure and an immediate defect in mtDNA transmission during cytokinesis. Mmm1p is likely to be part of a complex of proteins in the mitochondrial outer membrane. Besides Mmm1p, two other proteins, Mdm10p and Mdm12p, are required for mitochondrial shape, mitochondrial segregation, and mtDNA stability, and also reside in the outer membrane (Sogo and Yaffe, 1994; Berger et al., 1997). Genetic and biochemical studies suggest that all three proteins may interact. For instance, double mutants between *mmm1* and either *mdm10* or *mdm12* show the same mitochondrial phenotype (Berger et al., 1997), and each mutation is suppressed by an alteration in the Sot1 protein (Berger et al., 1997). Also suggesting that Mmm1p is part of a large, oligomeric structure on the mitochondrial surface, our fluorescence images showed that Mmm1p-GFP containing dots are ~0.3 μm in diameter. The punctate distribution of Mmm1p-GFP requires the presence of both Mdm10p and Mdm12p, suggesting that all three proteins are part of the Mmm1p complex (A. Aiken Hobbs, unpublished observations). In preliminary studies, blue native gel electrophoresis of detergent-solubilized mitochondria indicates that the 48-kD Mmm1 protein migrates at >200 kD (A. Aiken Hobbs, unpublished observations).

Our results raise the possibility that Mmm1p and its associated proteins mediate a connection between the mitochondrial outer and inner membranes. Since Mmm1p is an integral protein in the mitochondrial outer membrane (Burgess et al., 1994), and since mtDNA is located in the matrix, we argue that it is highly unlikely that Mmm1p (or other outer membrane proteins) directly interacts with mtDNA. Instead, we propose that the Mmm1p-containing complex is connected to mtDNA indirectly, acting through one or more inner membrane proteins. The inner membrane proteins would then directly bind to mtDNA nucleoids. Supporting this idea, our previous work has shown that Mmm1p interacts with the inner membrane protein, Tim54, in a yeast two-hybrid screen (Kerscher et al., 1997). Moreover, *mmm1* mutants are lethal in combination with mutations in genes encoding the inner membrane proteins, Phb1p or Phb2p, suggesting an association between the three proteins (Berger and Yaffe, 1998). Although we feel it is unlikely, it is possible that Mmm1p or other members of the Mmm1p complex directly interact with mtDNA nucleoids. For example, the amino terminus of the inner membrane protein, Tim23p, protrudes through the mitochondrial outer membrane into the cytosol (Donzeau et al., 2000). In a similar manner, Mmm1p might span both the outer and inner membranes and have a domain accessible to the matrix. Further studies are clearly needed to determine the mechanism by which the Mmm1p complex is connected to mtDNA nucleoids.

Attachments between the mitochondrial outer and inner membranes, called contact sites, have been observed for decades (Hackenbrock, 1968; Bereiter-Hahn, 1990; Bereiter-Hahn and Voth, 1994), but the molecules that mediate these attachments have not been identified. Our results showing a connection between Mmm1p and mtDNA suggest that Mmm1p plays a role in contact site formation. Supporting this idea, we have found that mitochondrial vesicles obtained by sonication fractionate at a density intermediate between vesicles that contain only outer or inner membrane proteins. Earlier studies showed that this intermediate density fraction was enriched in contact site proteins, and also enriched in a protein import substrate arrested across both membranes (Pon et al., 1989). Also supporting a role for Mmm1p in contact sites, our results show that mitochondria in cells disrupted in *MMM1* show a striking loss of normal inner membrane organization. We suggest that Mmm1p-mediated attachments to the inner membrane are required for normal membrane structure.

Mmm1p is required for the structure, stability, and transmission of mtDNA nucleoids. Yeast cells contain 50–100 copies of mtDNA packaged into 10–20 separate DNA-protein complexes called nucleoids (Miyakawa et al., 1987; Kaufman et al., 2000). Several studies suggest that mtDNA nucleoids are attached to the mitochondrial inner membrane at discrete locations (Miyakawa et al., 1987; Azpiroz and Butow, 1993). For example, during yeast cell mating, mitochondria rapidly fuse and matrix proteins are immediately mixed (Azpiroz and Butow, 1993). In contrast, mtDNA nucleoids mix more slowly, suggesting that mtDNA is not freely diffusible. When DAPI-stained cells were examined, we found that transmission of mtDNA to daughter cells was immediately defective upon shift of the *mmm1-1* mutant from 24° to 37°C. Moreover, the number of distinct mtDNA nucleoids quickly decreased in *mmm1-1* cells at the restrictive temperature. Within 6 h, cells that initially contained 10–20 individual nucleoids now carried one large mtDNA aggregate. We speculate that the collapse of nucleoid structure is due to loss of a Mmm1p-based attachment. It is not clear, however, why the detached nucleoids aggregate to a single position within the mitochondria. Nonetheless, the collapsed mtDNA is not efficiently transmitted to daughter buds.

We previously reported that *mmm1* disruptions contain mtDNA (Burgess et al., 1994). However, these studies

used a sensitive mating assay in which a small fraction of mtDNA-containing cells would be detected. By DAPI-staining and fluorescence microscopy, we do not observe mtDNA in any *mmm1::URA3* cells (A. Aiken Hobbs, unpublished observations). Consequently, we argue that *mmm1::URA3* cells are strictly analogous to *mmm1-1* cells grown for long times at the restrictive temperature.

Why do *mmm1* mutants lose mtDNA? Clearly, failure to transmit mtDNA to daughter cells produces some *mmm1-1* cells lacking mtDNA, but additional processes are needed for the total population of cells to rapidly lose mtDNA. For example, *mmm1-1* mitochondria may be defective in both mtDNA replication and mtDNA transmission, which would explain the lack of mtDNA seen in *mmm1-1* cells after 48 h at 37°C. The replication defect may result from the lack of Mmm1p-mediated attachments to mtDNA. Similar to their bacterial counterparts, mtDNA nucleoids are proposed to be attached to membranes, and this connection is thought to be important for replication, recombination, transcription, and segregation (Shadel, 1999). Thus, a block in both replication and transmission may explain the loss of mtDNA in *mmm1* cells. A block in replication may also be indirect. For example, mtDNA replication in *mmm1-1* mother cells that accumulate mtDNA due to failure to transmit mtDNA to daughter cells may be inhibited by a copy-number control mechanism. Alternatively, mtDNA in *mmm1-1* cells may be incompetent for replication. The altered nucleoid structures seen in *mmm1* mutants are similar to those seen in mitochondria lacking an HMG-like mtDNA binding protein, Abf2p (Newman et al., 1996). In *abf2* cells, mtDNA recombination intermediates are thought to accumulate and interfere with replication and segregation (MacAlpine et al., 1998; Zelenaya-Troitskaya et al., 1998). A different explanation for mtDNA loss in *mmm1-1* cells is by DNA degradation. We have noticed that the collapsed nucleoids seen in *mmm1* mutants are often located adjacent to the vacuole, suggesting that autophagy of the altered organelles may be increased (A. Aiken Hobbs, unpublished observations, see also Fig. 6). Thus, a defect in mtDNA segregation to daughter cells, coupled to increased mtDNA degradation in mother cells, may lead to mtDNA loss in all *mmm1-1* cells. Although the exact mechanisms for mtDNA loss in *mmm1-1* cells requires further studies, it is interesting to note that alleles of *mmm1* have been isolated by screening for mutants that increase escape of mtDNA to the nucleus (Thorsness and Weber, 1996).

Our results indicate that only about half of the total mtDNA nucleoids are associated with Mmm1p, raising the possibility that nucleoids connected to the Mmm1p complex behave differently from nucleoids that are unattached. For example, replication of mtDNA may require connection with the Mmm1p complex, whereas nucleoids not attached to Mmm1p may be inactive for replication. Alternatively, binding to the Mmm1p complex may be required for mtDNA transmission to daughter cells, and those nucleoids not attached to Mmm1p are retained in the mother cell. Experiments to distinguish between these and other possibilities are in progress.

We speculate that Mmm1p may be a link, direct or indirect, between actin and mtDNA. For example, mtDNA may be anchored to a mitochondrial membrane protein complex that includes Mmm1p, as well as to actin filaments, and this connection may be important for mtDNA transmission. In this scenario, Mmm1p may form part of a structure analogous to the kinetochore for nuclear DNA. Actin-based movement of mtDNA attached via Mmm1p-containing "mitochores" would ensure faithful segregation of mtDNA nucleoids to daughter cells. Mmm1p-associated nucleoids may be those actively segregated to daughter cells. Alternatively, during cell division, some fraction of mtDNA needs to be maintained in the mother cell. Mmm1p attachment to actin may play an anchoring role, preventing excess mtDNA transmission to daughter cells. Although our observations indicate that lack of Mmm1p prevents transfer of mtDNA, Mmm1 protein is proposed to retain a subset of mitochondria at the base of the mother cell during cytokinesis (Yang et al., 1999).

Finally, our results suggest a mechanism by which Mmm1p may maintain mitochondrial shape. The dot-like distribution of Mmm1p does not require the actin cytoskeleton or mtDNA. Cells treated with Latrunculin A to disrupt actin or ethidium bromide to induce loss of mtDNA still contain a punctate distribution of Mmm1p-GFP on mitochondria. Therefore, the Mmm1p-containing complex may be part of an internal scaffold-like structure, holding the outer and inner membranes together. This scaffold or "mitoskeleton" may be required for normal mitochondrial shape, and loss of Mmm1p would lead to drastic alterations in mitochondrial (and mtDNA) structure. Clearly, further studies are needed to determine the role of Mmm1p in mitochondrial morphology, mtDNA stability, and in the efficient segregation of mitochondria and mtDNA to daughter cells.

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