

Evidence for a two membrane–spanning autonomous mitochondrial DNA replisome

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The unit of inheritance for mitochondrial DNA (mtDNA) is a complex nucleoprotein structure termed the nucleoid. The organization of the nucleoid as well as its role in mtDNA replication remain largely unknown. Here, we show in *Saccharomyces cerevisiae* that at least two populations of nucleoids exist within the same mitochondrion and can be distinguished by their association with a discrete proteinaceous structure that spans the outer and inner mitochondrial membranes. Surprisingly, this two membrane–spanning structure (TMS) persists and self-replicates in

the absence of mtDNA. We tested whether TMS functions to direct the replication of mtDNA. By monitoring BrdU incorporation, we observed that actively replicating nucleoids are associated exclusively with TMS. Consistent with TMS's role in mtDNA replication, we found that Mip1, the mtDNA polymerase, is also a stable component of TMS. Taken together, our observations reveal the existence of an autonomous two membrane–spanning mitochondrial replisome as well as provide a mechanism for how mtDNA replication and inheritance may be physically linked.

Introduction

Much of our current understanding of the mitochondrial nucleoid relies on studies of the yeast *Saccharomyces cerevisiae*. In yeast, mitochondria form a continuous dynamic reticular structure, localized to the cell cortex (Hoffmann and Avers, 1973; Nunnari et al., 1997). Cytological visualization of nucleoids indicates that they are distributed in a somewhat regular pattern within the mitochondrial network, presumably through an attachment to the inner mitochondrial membrane (Miyakawa et al., 1984; Azpiroz and Butow, 1993; Nunnari et al., 1997). In addition, there is substantial genetic and cytological evidence to suggest that nucleoid inheritance is nonrandom and that mitochondrial DNA (mtDNA) diffusion within the organelle is limited (Coen et al., 1970; Birky, 1978; Strausberg and Perlman, 1978; Zinn et al., 1987; Azpiroz and Butow, 1993; Nunnari et al., 1997; Okamoto et al., 1998). These observations have led investigators to hypothesize that a membrane-bound mtDNA segregation apparatus exists to regulate nucleoid behavior. Although the exact nature of the nucleoid's membrane association is unknown, a recent study demonstrated that a subset of nucleoids within a cell is adjacent to discrete outer membrane

structures, which contain the transmembrane protein Mmm1 (Hobbs et al., 2001). Mmm1 has been shown to be required for mtDNA maintenance and also has been shown to play a role in the maintenance of mitochondrial morphology possibly by mediating attachments to extramitochondrial structures such as actin (Burgess et al., 1994; Boldogh et al., 1998; Hobbs et al., 2001).

Proteomic and genetic approaches have identified molecules directly associated with mtDNA within nucleoid structures. These include the mitochondrial-specific DNA-binding proteins Mip1, Abf2, and Mgm101 (Meeusen et al., 1999; Kaufman et al., 2000). Mip1 is a pol- γ DNA polymerase that possesses 3'-5' exonuclease proofreading activity and represents the only known yeast mtDNA polymerase (Foury, 1989). Abf2 is a relatively abundant HMG-like DNA-binding protein and is thought to function in mtDNA packaging and recombination (Diffley and Stillman, 1991, 1992). Mgm101 is a novel DNA-binding protein that is essential for mtDNA maintenance, and analysis of *mgm101* cells suggests that it is required for the repair of oxidative mtDNA damage (Chen et al., 1993; Meeusen et al., 1999). To gain insight into how nucleoids are organized and segregated within mitochondria in cells, we performed a cytological analysis of the behavior of nucleoid-associated components in vivo using fusions to fluorescent proteins.

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Key words: mitochondria; mtDNA; nucleoid; replisome; membrane–spanning

Abbreviations used in this paper: mtDNA, mitochondrial DNA; TAC, tripartite attachment complex; TMS, two membrane–spanning structure.

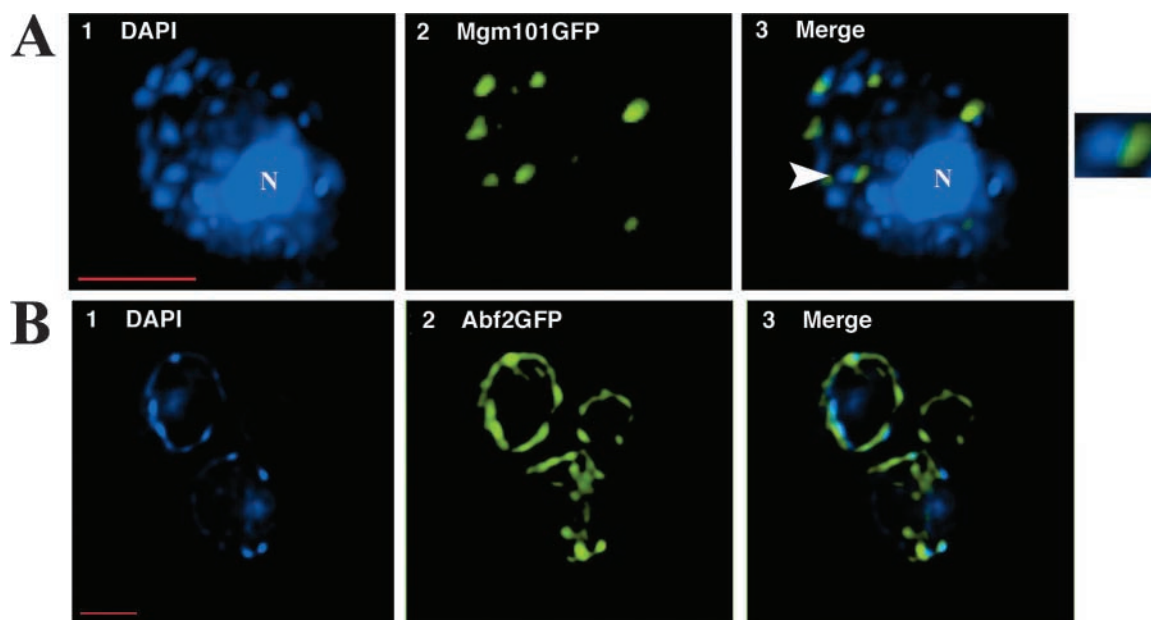


Figure 1. **Mgm101GFP labels a subset of mtDNA nucleoids within cells.** Wild-type cells expressing either (A) Mgm101GFP or (B) Abf2GFP were grown overnight in YPG to early log phase and shifted to YPDGal containing 1 $\mu\text{g/ml}$ DAPI for 20 min at 25°C to visualize mtDNA-containing nucleoids. Cells were washed into SD and imaged using a Deltavision deconvolution microscope as described in the Materials and methods. Panel A is a whole cell projection. N indicates nuclear DAPI labeling. Panel B is a 0.2- μm cell section. Arrows in A indicate the region magnified 250% in the corresponding inset. Bars, 2 μm .

Results

Mgm101 is associated with a subpopulation of nucleoids within mitochondria

Mitochondrial nucleoids are easily identified as discrete structures contained within mitochondria using the vital dsDNA-specific fluorescent dye DAPI (Williamson and Fennell, 1979; Miyakawa et al., 1984). Analysis of haploid yeast cells stained vitally with DAPI indicated that 42 ± 8 nucleoids were present in haploid cells (Fig. 4 A; Jones and Fangman, 1992). Previously, we demonstrated that the DNA-binding, matrix-localized protein Mgm101 is a constituent of nucleoid structures based on biochemical and cytological observations (Meeusen et al., 1999). Here, using the sensitive technology of deconvolution microscopy, we examined the organization and behavior of nucleoid structures using Mgm101 fused to GFP. Surprisingly, we observed that only a subset of DAPI-stained nucleoids colocalized with Mgm101GFP foci (Fig. 1 A and see Fig. 4 A). In addition, closer examination of this subset of nucleoids revealed in every case that foci labeled by Mgm101GFP only partially overlapped with the punctate region labeled by DAPI, suggesting the existence of subnucleoid organization (Fig. 1 A, arrow and inset). In contrast, analysis of a GFP-tagged version of the mitochondrial HMG-like DNA-binding protein, Abf2, revealed that Abf2 labeled the entire population of nucleoids in cells, consistent with its proposed role as a general DNA packaging protein (Fig. 1 B). These data indicate that a distinct subpopulation of nucleoids, specifically marked by matrix-localized Mgm101, exists in cells and that this subpopulation may possess an Mgm101-containing substructure associated with Abf2-packaged mtDNA.

Mgm101-containing nucleoids are part of a two membrane-spanning complex that functions in mtDNA maintenance

To gain insight into the functional significance of the population of nucleoids marked by Mgm101, we asked whether outer membrane Mmm1 puncta, previously reported to be adjacent to a subset of nucleoids, colocalized with Mgm101-labeled foci in the matrix (Hobbs et al., 2001). To assess whether these structures colocalize in cells, we visualized cells coexpressing Mgm101GFP and Mmm1dsRed using fluorescence deconvolution microscopy. Strikingly, in every case, Mmm1p outer membrane puncta were colocalized with Mgm101 foci in the matrix, and in each case, these regions of colocalization were adjacent to DAPI/Abf2-labeled mtDNA (Fig. 2 and see Fig. 4 A). Close comparison of Mgm101GFP and Mmm1dsRed in cells revealed that they only partially overlapped within each colocalized region, suggesting that although coaligned, separate Mgm101 and Mmm1 substructures exist (Fig. 2, arrow and inset). Time-lapse analysis of coaligned Mmm1dsRed puncta and Mgm101GFP foci revealed that they moved within cells together as a unit, further demonstrating their intimate association (Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200304040/DC1>). These observations suggest that separate, but tightly coupled, outer and inner membrane substructures exist, associate together, and interact with a subpopulation of DAPI-labeled, Abf2-packaged mtDNA.

To further test whether outer membrane Mmm1 and matrix-localized Mgm101 are in a complex, we performed an immunoprecipitation using anti-HA antibodies on a mitochondrial-enriched fraction from cells expressing a functional HA epitope-tagged allele of Mmm1 (Mmm1HA).

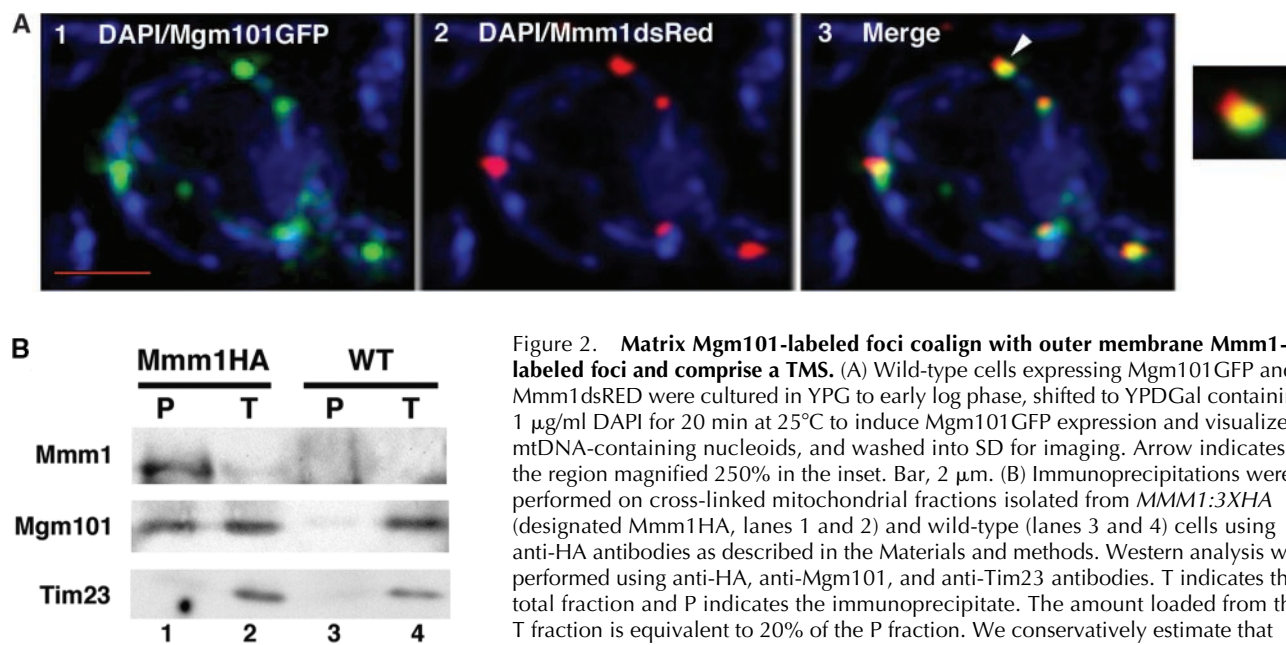


Figure 2. Matrix Mgm101-labeled foci coalign with outer membrane Mmm1-labeled foci and comprise a TMS. (A) Wild-type cells expressing Mgm101GFP and Mmm1dsRED were cultured in YPG to early log phase, shifted to YPDGal containing 1 $\mu\text{g/ml}$ DAPI for 20 min at 25°C to induce Mgm101GFP expression and visualize mtDNA-containing nucleoids, and washed into SD for imaging. Arrow indicates the region magnified 250% in the inset. Bar, 2 μm . (B) Immunoprecipitations were performed on cross-linked mitochondrial fractions isolated from *MMM1:3XHA* (designated Mmm1HA, lanes 1 and 2) and wild-type (lanes 3 and 4) cells using anti-HA antibodies as described in the Materials and methods. Western analysis was performed using anti-HA, anti-Mgm101, and anti-Tim23 antibodies. T indicates the total fraction and P indicates the immunoprecipitate. The amount loaded from the T fraction is equivalent to 20% of the P fraction. We conservatively estimate that the recovery Mmm1HA in the P fraction is 50% of the total cellular extract.

Prior to immunoprecipitation, proteins were cross-linked with the bifunctional, reversible cross-linker DSP and extracted under denaturing conditions as previously described (Wong et al., 2003). After immunoprecipitation, cross-links were reversed with reducing agents, and fractions were analyzed by SDS-PAGE and Western blotting with anti-Mgm101 and -HA antibodies. Western blot analysis of fractions from immunoprecipitations with Mmm1HA-tagged mitochondria revealed that >50% of Mmm1HA was recovered in the immunoprecipitate (Fig. 2 B, lanes 1 and 2). Significantly, we also observed that a fraction of Mgm101 from cross-linked extracts was reproducibly coimmunoprecipitated with Mmm1HA (Fig. 2 B, lanes 1 and 2). To address specificity, immunoprecipitations were performed from enriched, DSP-cross-linked mitochondrial fractions isolated from wild-type cells not expressing Mmm1HA. Under these conditions, Mgm101 was not recovered in the immunoprecipitate fraction, indicating that the ability to coimmunoprecipitate Mmm1 and Mgm101 with the anti-HA antibody is dependent on Mmm1HA (Fig. 2 B, lanes 3 and 4). In addition, Tim23 (Fig. 2 B, lanes 1 and 2) and Fis1 (not depicted) were not coimmunoprecipitated with Mmm1, further demonstrating the specificity of the Mmm1/Mgm101 interaction. These biochemical data support our cytological observations and indicate that Mmm1 and Mgm101 are present in a two membrane-spanning structure (TMS).

To test whether the observed colocalization and interaction of Mmm1 and Mgm101 is functionally relevant for the maintenance of mtDNA in vivo, we examined whether mutant alleles of the genes encoding these proteins interact genetically. Specifically, we determined whether a combination of *mmm1* (Burgess et al., 1994; mutation produces Mmm1G252A, see Materials and methods) and *mgm101* (Meeusen et al., 1999; mutation produces Mgm101D131N) temperature-sensitive alleles in haploids produces a more se-

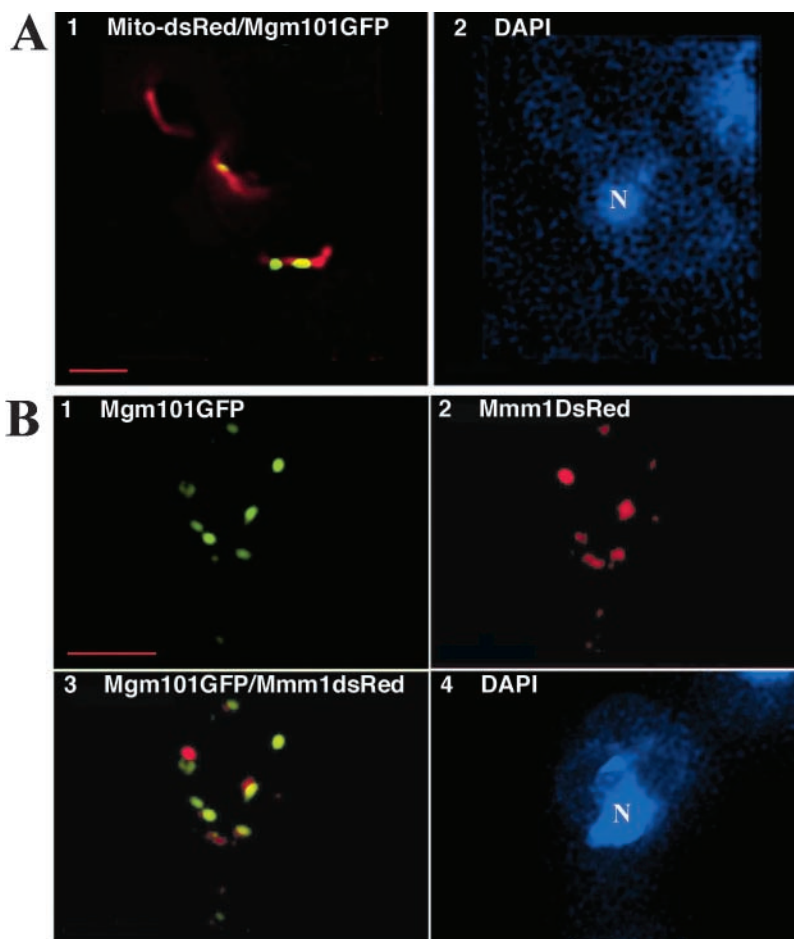
vere defect in mtDNA maintenance than observed in haploid cells harboring only a single temperature-sensitive mutation, i.e., a synthetic defect. Maintenance of mtDNA was assessed by examining the growth of cells on the nonfermentable carbon source glycerol, where mtDNA is essential for viability, and by examining mtDNA-containing nucleoids in vivo directly by staining cells with DAPI. At permissive temperatures, *mmm1^{ts}* and *mgm101^{ts}* cells grew on glycerol and contained mtDNA nucleoids, indicating that the corresponding proteins were functional (Burgess et al., 1994; Meeusen et al., 1999; Table I). As expected, at nonpermissive temperature, none of the mutants were able to grow on glycerol, and mtDNA was undetectable by DAPI (Table I). Interestingly, haploid cells harboring a combination of *mmm1^{ts}* and *mgm101^{ts}* alleles displayed a significantly more severe mtDNA maintenance defect (Table I) as compared with *mmm1^{ts}* or *mgm101^{ts}* cells. Specifically, under permissive conditions, *mmm1^{ts}mgm101^{ts}* haploids were unable to grow on glycerol-containing media and were completely devoid of DAPI-stainable mtDNA nucleoid structures. The synthetic mtDNA stability defect observed in *mmm1^{ts}mgm101^{ts}* cells, coupled with our cytological and biochemical observations, is consistent with a model where outer membrane Mmm1 and

Table I. Synthetic growth defects observed between *mmm1^{ts}* and *mgm101^{ts}* alleles

Strain	Growth on glycerol	
	25°C	37°C
<i>MMM1 MGM101</i>	+	+
<i>mmm1-1</i>	+	- ^a
<i>mgm101-2</i>	+	- ^a
<i>mmm1-1 mgm101-2</i>	- ^a	- ^a

^aContained no DAPI-stainable mtDNA nucleoids after growth on dextrose media.

Figure 3. Matrix and outer membrane components of TMS assemble in the absence of mtDNA. Cells lacking mtDNA expressing Mgm101GFP and (A) mitochondrial-targeted dsRED or (B) Mmm1 dsRED were cultured in SD to early log phase, shifted to YPDGal + 1 μ g/ml DAPI for 60 min at 25°C to induce Mgm101GFP expression and visualize mtDNA-containing nucleoids, and washed into SD for imaging. Panel A represents a 0.2- μ m z-section. Bars, 2 μ m. Panel B and DAPI panels represent whole cell projections. Bars, 3 μ m. N signifies nuclear DAPI labeling.



matrix-localized Mgm101 function together within a TMS in the maintenance of mtDNA.

Specialized nucleoid-associated structures exist in the absence of mtDNA

Our observations suggest that a population of nucleoids is specifically associated with a TMS containing resolvable Mmm1-containing outer membrane and matrix Mgm101-containing subregions. To gain insight into the assembly of TMS, we examined the localization patterns of both Mgm101 and Mmm1 in the absence of mtDNA (ρ^0 cells). Surprisingly, we observed that in ρ^0 cells, the matrix-localized DNA-binding protein Mgm101GFP labeled a similar number of foci within mitochondrial tubules as compared with cells containing mtDNA (Fig. 3 A and Fig. 4 A). A previous study had demonstrated that Mmm1 also localizes to puncta on the mitochondrial outer membrane in ρ^0 cells (Hobbs et al., 2001). Colocalization and time-lapse analysis of Mmm1 and Mgm101 revealed that in every case, outer membrane Mmm1 puncta remained tightly coupled to matrix Mgm101 foci in ρ^0 cells (Fig. 3 B and not depicted). Time-lapse analysis of Mgm101 and Mmm1 puncta in both the presence and absence of mtDNA also revealed that TMSs are not formed de novo, rather they arise through the self-replication of existing structures (Videos 2 and 3, available at <http://www.jcb.org/cgi/content/full/jcb.200304040/DC1>). Consistent with this, TMS units were faithfully inherited

into newly formed buds in 100% of the cells ($n = 100$ cells). Taken together, these data indicate that the TMS assembles in the absence of mtDNA and associates as an autonomous unit of inheritance with packaged mtDNA in cells.

Evidence that TMS functions as a mitochondrial replisome

To gain insight into the functional role of the TMS, we looked to studies of prokaryotic nucleoids. In *Bacillus subtilis*, a stationary proteinaceous replication “factory” has been identified that exists at discrete intracellular positions (Lemon and Grossman, 1998, 2000). Thus, we tested whether the TMS functions to direct the replication of mtDNA by examining the localization of active sites of mtDNA replication and determining their relationship to this structure.

To identify mtDNA replication foci, we used cells that contain an exogenous copy of a thymidine kinase gene and thus can phosphorylate and incorporate the thymidine analogue BrdU into their cellular DNA (Nunnari et al., 1997). Cells were pulse labeled for up to 30 min with BrdU, and mtDNA sites of incorporation were visualized by indirect immunofluorescence using a monoclonal anti-BrdU antibody (Nunnari et al., 1997). We have estimated by DAPI staining that a total of 42 ± 8 mtDNA-containing nucleoids are present per cell. In contrast, we detected by BrdU incorporation significantly fewer mtDNA replication foci per cell,

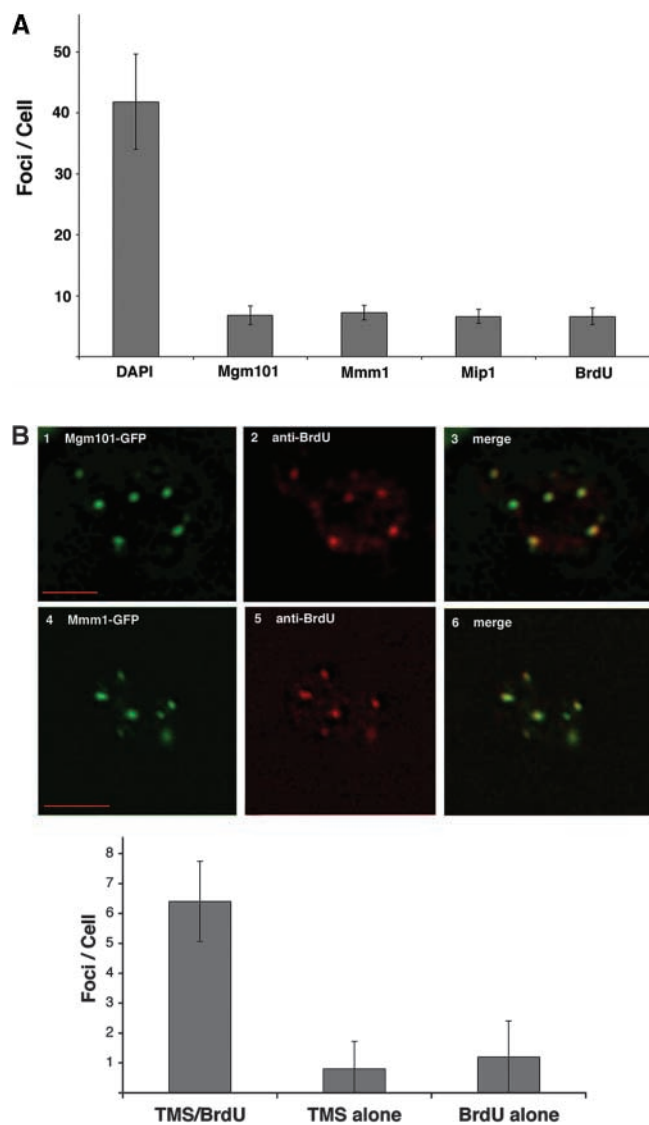


Figure 4. TMS colocalizes with actively replicating mtDNA and the mtDNA polymerase Mip1. (A) The numbers of mitochondrial-associated foci per cell as labeled by DAPI (cells = 9; total foci = 384), Mgm101 GFP (cells = 48; total foci = 333), Mmm1dsRED (cells = 38; total foci = 280), Mip1GFP (cells = 21; total foci = 141), and BrdU (cells = 86; total foci = 575) were counted, and averages were plotted for comparison. Error bars indicate the calculated standard deviation of foci per cell. (B) Wild-type cells containing an exogenous thymidine kinase gene (AFS98, see Materials and methods) expressing either Mgm101GFP (panels 1–3) or Mmm1GFP (panels 4–6) were grown overnight in YPG, pulse labeled with BrdU, and analyzed by indirect immunofluorescence as described using a secondary rhodamine-conjugated mouse antibody to the primary mouse monoclonal BrdU antibody. Bars: (panel 1–3) 2 μ m; (panels 4–6) 4 μ m. The graph represents the number of Mgm101GFP- and Mmm1GFP-labeled foci (indicated by TMS on the x axis) that colocalized with BrdU-labeled foci per cell (cells = 10; total foci = 64). The graph also contains TMS foci and BrdU foci that were alone per cell (cells = 10; total foci TMS = 8; total foci BrdU = 12). Error bars indicate the calculated standard deviation of these measurements per cell. (C) Cells expressing Mip1GFP (panels 1 and 4) under the control of its endogenous promoter in the chromosome and Mmm1dsRed (panels 2 and 5) were cultured overnight in YPG to early log phase before imaging. Two representative cells are shown. Bars, 2 μ m.

indicating that mtDNA is replicating in only a subset of nucleoids at a given time (Fig. 4, A and B). Comparison of the number of mtDNA replication sites to the number of Mgm101 and Mmm1 foci in cells demonstrated a strong correlation, suggesting that TMSs are associated specifically with replicating mtDNA (Fig. 4 A). To directly determine the relationship of the TMS to mtDNA replication, we pulse labeled cells expressing Mgm101GFP or Mmm1GFP with BrdU. Strikingly, we observed that the vast majority mtDNA replication foci were colocalized with both Mgm101GFP and Mmm1GFP foci in cells (Fig. 4 B). These observations suggest that the autonomous TMS that we have identified functions as an mtDNA replisome.

One prediction of this model is that the mtDNA replication machinery is also a constituent of TMS. Thus, we determined the localization pattern of Mip1, the mtDNA polymerase. We observed that Mip1GFP, like Mgm101GFP, localized uniquely to a subset of DAPI-stained nucleoids (Fig. 4 A and not depicted) that align with Mmm1dsRED foci in the outer mitochondrial membrane in both ρ^+ and ρ^0 cells (Fig. 4 C and not depicted). These data indicate

that the mtDNA polymerase also is a stable component of the nucleoid-associated TMS. Taken together, our findings suggest that mitochondria contain a two membrane-spanning autonomous structure that functions as a replisome.

Discussion

We have demonstrated that two essential mtDNA-binding proteins, Mgm101 and Mip1, the mtDNA polymerase, localize to discrete proteinaceous structures in the mitochondrial matrix that associate with previously identified structures in the outer membrane containing the protein Mmm1 (Hobbs et al., 2001). These structures persist and remain coupled in the absence of mtDNA, and we term them TMS for simplicity. This result is striking because the matrix TMS components, Mgm101 and Mip1, were previously assumed to localize to mtDNA-containing foci or nucleoids via their ability to bind DNA. This observation, coupled with our time-lapse analysis of TMS, indicates that there is an mtDNA-separable, self-replicating unit that is faithfully inherited during cell division. Our findings indicate that the

nucleoid-associated TMS functions as a replisome, a point that further emphasizes its unique and pivotal role in mtDNA inheritance and maintenance.

It is interesting to speculate on what other functions TMS might perform in the cell. As proposed in the case of the *B. subtilis* replisome, TMS may function as a replication factory, harnessing the energy of nucleotide incorporation to drive the segregation/distribution of mitochondrial genomes throughout the organelle to ensure faithful DNA inheritance (Lemon and Grossman, 1998, 2000). In addition, the identification of Mgm101, a protein implicated in mtDNA repair (Meeusen et al., 1999), as a component of TMS raises the possibility that it may also function as an organizational center for mtDNA metabolic enzymes, not just those required for replication, thereby increasing the efficiency of events required for the maintenance of mtDNA.

The two membrane-spanning nature of the TMS suggests that it might serve to stably position the mtDNA maintenance machinery and mtDNA within the organelle through interactions with extramitochondrial components. Consistent with this notion, mutations in the outer membrane TMS component Mmm1 cause cortically localized mitochondrial tubules to collapse into centrally localized spherical structures (Burgess et al., 1994). This morphological phenotype associated with loss of Mmm1 function has been postulated to result from a loss of mitochondrial attachment to sites located at the cortex of the cell (Burgess et al., 1994). Interestingly, Mmm1 has also been shown to be required for actin binding to mitochondria in vitro, suggesting that Mmm1-dependent attachments may be to the actin cytoskeleton (Boldogh et al., 1998). However, we and others have observed that disassembly of the actin cytoskeleton using latrunculin-A does not affect the assembly or stability of TMS (Hobbs et al., 2001) or TMS movement in vivo (Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200304040/DC1>), suggesting that F-actin is not required for the putative TMS-dependent extramitochondrial attachment. Alternatively, the TMS may possess multiple unrelated functions within the cell: organization of mtDNA maintenance machinery and regulation of organelle structure.

Although the existence and nature of TMS extramitochondrial attachment sites are unknown, we have observed that TMSs have very limited movement within cells and that when movement is observed, in the majority of cases it is coupled to the movement of the mitochondrial organelle (90%, $n = 30$ nucleoids; Videos 2 and 3). An exception to this coordinated TMS/organelle movement is where the segregation of TMS within mitochondrial tubules was observed (Videos 2 and 3). Thus, our observations suggest that the organization of components responsible for maintenance of mtDNA into discrete structures whose behavior is membrane dependent may serve to secure the faithful inheritance of both the mtDNA metabolic machinery and associated mtDNA to daughter cells.

Our findings raise the question of whether TMS will be present in mitochondria of other cell types and function as a replisome and positioning apparatus. Recently, such a structure, termed tripartite attachment complex (TAC), was reported to exist in *Trypanosoma brucei*, where the mitochon-

drial genome is organized into a single copy kinetoplast that is attached to and segregated by the cell's basal body (Robinson and Gull, 1991; Ogbadoyi et al., 2003). Specifically, a differentiated region of mitochondrial outer and inner membrane between the kinetoplast and basal body was identified by EM analysis and shown to be a part of a superstructure of three distinct morphological regions, which include extra- and intramitochondrial filamentous structures that likely attach the kinetoplast to the basal body and function to position and segregate it (Ogbadoyi et al., 2003). The structural organization of TAC and TMS is similar, suggesting that like TAC, TMS functions to help position mtDNA in addition to its function as a replisome. Although trypanosomes are a specialized case where mtDNA is organized into a single copy structure that requires stringent segregation machinery, such as a basal body, our findings in yeast and other observations make it likely that mitochondrial TMSs also exist in mammalian cells. In human cells, similar nucleoid dynamics within the organelle have been recently reported using a GFP fusion to the helicase Twinkle (Garrido et al., 2003). In addition, in the absence of mtDNA, Twinkle retains its punctate morphology within mitochondrial tubules, suggesting that human mitochondria also contain mtDNA-independent structures dedicated to mtDNA maintenance (Spelbrink et al., 2001). Identification of additional TMS components and their organization within TMSs will lend insight into the nature of the link between mtDNA replication and inheritance and will ultimately enhance our understanding of mtDNA-linked human diseases.

Materials and methods

Media and yeast genetic techniques

Standard genetic techniques and yeast media, including YPG (2% glycerol), YPDGal (2% galactose + 1% dextrose), SD (2% dextrose), and SD-Gal (2% galactose + 1% dextrose), were prepared as previously described (Guthrie and Fink, 1991). Yeast transformations were performed as described by Gietz and Schiestl (1991). Yeast cells lacking mtDNA (ρ^0 strains) were generated by culturing cells in YPD in the presence of 25 μ M EtBr for 3 d. Treated cells were subsequently plated onto YPD media, and colonies were screened by staining with DAPI (Molecular Probes) by immersion in 70% ETOH containing 1 μ g/ml DAPI for 5 min, followed by two washes in 1 \times PBS and visualization using fluorescence microscopy. Strains lacking detectable nucleoid structures were confirmed to be ρ^0 by Southern blotting using mtDNA probes to the mitochondrial genes COX2 and VAR1 (not depicted).

Strains and plasmid construction

A previously characterized episomal mitochondrial-targeted MGM101GFP fusion (pTS330MGM101GFP) was used and transformed into the wild-type strain W303, yielding JNY 970 (Meeusen et al., 1999). We previously reported that 20-fold overexpression of Mgm101GFP caused the induction of respiratory-incompetent mitochondrial genomes (ρ^-) in wild-type cells (Meeusen et al., 1999). However, in this study, we expressed Mgm101GFP in wild-type cells at a level less than twofold above endogenous Mgm101p levels (not depicted). Under these experimental conditions, full respiratory competence and growth rates comparable to wild-type cells were observed (not depicted). An episomal mitochondrial-targeted ABF2GFP fusion (pts330ABF2GFP) was constructed by replacing MGM101 in pts330MGM101GFP with ABF2 by subcloning and transformed into W303 to yield JNY 969. Abf2GFP is fully functional, as assessed by its ability to complement the temperature-sensitive respiratory defect of *abf2 Δ* cells (not depicted).

To generate an Mmm1dsRED fusion, pRS426MMM1GFP (provided by Steve Gorsich and Janet Shaw, University of Utah, Salt Lake City, UT) was digested with NheI and HpaI to drop out GFP, which was then replaced by dsRED.T1 (provided by Ben Glick, University of Chicago, Chi-

ago, IL; Bevis and Glick, 2002) with engineered compatible ends: 5'-GCTAGCGCTCCTCCGAGGACGCATCAAGG-3' and 5'-TCCCGGC-TACAGGAACAGGTGGTGG-3'. Transformation of plasmids expressing Mmm1dsRED or Mmm1GFP into cells containing *mmm1-1*, a temperature-sensitive allele of *MMM1* (provided by Rob Jensen, Johns Hopkins University, Baltimore, MD), resulted in full complementation of the glycerol growth defect at the nonpermissive temperature (Burgess et al., 1994, and not depicted). To generate *MMM1:3XHA* (JNY461), an integrating 3XHA cassette was PCR amplified with primers that were complementary to the 3' region of *MMM1* (Bahler et al., 1998). The PCR product was transformed into W303 cells, and transformants that integrated by homologous recombination at the *MMM1* locus created an expressed in frame COOH-terminal Mmm1-3XHA. Correct integration was confirmed by PCR amplification across the recombination site. Mmm1-3XHA was fully functional, as assessed by the integrant's ability to grow on nonfermentable carbon sources at rates indistinguishable from wild-type strains (not depicted).

To generate *MIP1:GFP* (JNY967), an integrating GFP cassette was PCR amplified using primers that were complementary to the 3' region of *MIP1* (Bahler et al., 1998). The PCR product was transformed into W303 cells and integrated by homologous recombination at the *MIP1* locus, creating an expressed in frame COOH-terminal Mip1GFP. Correct integration was confirmed by PCR amplification across the recombination site. Mip1GFP was fully functional, as assessed by the integrant's ability to grow on nonfermentable carbon sources at rates indistinguishable from wild-type strains (not depicted).

AFS98, a W303 strain harboring the thymidine kinase gene was constructed as reported by Nunnari et al. (1997). The plasmid containing ADH promoter-regulated mitochondrial-targeted dsRed (PADHmitodsRED) was constructed as previously reported (Wong et al., 2000).

Haploid cells harboring combinations of the temperature-sensitive *mgm101-2* (Meeusen et al., 1999) and *mmm-1* (Burgess et al., 1994) alleles were obtained by crossing, sporulation, and tetrad analysis. As previously determined, *mgm101-2* cells express a mutant Mgm101D131N protein. To determine the mutation in *mmm-1* cells, we amplified the *MMM1* locus in mutant cells by PCR using Vent polymerase (New England Biolabs, Inc.) and sequenced the products directly (Davis Sequencing, University of California, Davis). Sequencing revealed a single point mutation at the *MMM1* locus from G to A, resulting in a change in amino acid 252 from G to S. Characterization of the glycerol growth defects on solid YPD and YPG media and determination of DAPI-stainable nucleoids in mutants were performed as described by Meeusen et al. (1999).

Immunoprecipitation of cross-linked mitochondrial proteins

To enrich for mitochondrial proteins, mitochondria were isolated from W303 and JNY461 by differential centrifugation as previously described (Meeusen et al., 1999). Cross-linking of mitochondrial proteins and immunoprecipitations were conducted as described by Wong et al. (2003). Anti-HA antibodies were purchased from Covance Inc., anti-Mgm101 antibodies were prepared as previously described (Meeusen et al., 1999), and anti-Tim23 antibodies were a gift from Rob Jensen.

BrdU incorporation and detection by indirect immunofluorescence

AFS98 cells harboring pts330MGM101GFP were cultured to OD₆₀₀ 0.2 in YPG overnight and shifted to YPDGal + 1 μg/ml DAPI for 20' at 25°C to induce Mgm101GFP expression. Cells were then immediately washed into YPD containing 5 mg/ml sulfanilamide (Sigma-Aldrich), 100 μg/ml amethopterin (Sigma-Aldrich, from a 100× stock in dimethyl sulfoxide), and 500 μg/ml BrdU for 30 min at 30°C. Cells were then washed three times in YPD and fixed by resuspension in YPD + 3.7% formaldehyde for 2 h at 25°C.

Cells were then processed as previously described (Nunnari et al., 1997) with the following modifications to preserve GFP signal. After adhering cells to slides, cells were incubated with PBS containing 0.5% Tween 20 (Sigma-Aldrich) for 30 min, 0.3 N HCl for 5 min, 0.1 M sodium tetraborate, pH 8.5, for 5 min, before washing one time in PBS. Indirect immunofluorescence was conducted as previously described, substituting the fluorescein-conjugated anti-mouse secondary antibody with a rhodamine-conjugated anti-mouse secondary antibody (Molecular Probes).

Fluorescence microscopy imaging

Yeast strains were grown either in YPG or YPD, in the case of rho⁰ cells, overnight to early log phase, pelleted, and resuspended in YPDGal in either the presence or absence of 1 μg/ml DAPI for 20–60 min at 25°C before visualization using fluorescence microscopy. To visualize the mitochondrial organelle, strains were transformed with pADHmitodsRED or

stained with Mitotracker CMXR (Molecular Probes) as previously described (Nunnari et al., 1997).

Cells were viewed with an Olympus IX70 Deltavision Microscope using a 60× 1.4 N.A. objective and a 100-W mercury lamp. The following excitation wavelengths were used: DAPI, 360; FITC, 490; and Rhod, 555. Images were collected in 0.2-μm sections. Two- and three-dimensional light microscopy data collection and computational removal of out-of-focus information used an integrated, cooled CCD-based, fluorescence light microscopy data collection, processing, and visualization workstation (Applied Precision, Inc.) in the Molecular and Cellular Biology Imaging Facility, University of California, Davis. Three-dimensional datasets were processed using DeltaVision's iterative, constrained three-dimensional deconvolution method. Time-lapse analyses were done with a Princeton Micromax Camera equipped with a Sony Interline Chip. As part of our analysis of time-lapse data for Videos 2 and 3, we examined complete z-section series for every time point to rule out the possibility that movement in the z-axis of another unrelated TMS gave rise to two TMS foci as opposed to segregation of a single TMS.

Online supplemental material

The supplemental material (Videos 1–4) is available at <http://www.jcb.org/cgi/content/full/jcb.200304040/DC1>. Supplementary videos show time-lapse microscopy of the behavior of TMS components and mitochondria in live cells either containing or lacking mtDNA or treated with latrunculin.

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