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Transmembrane segments of the dynamin Msp1p uncouple its functions in the control of mitochondrial morphology and genome maintenance

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Summary

Mitochondrial morphology depends on the equilibrium between antagonistic fission and fusion forces acting on mitochondrial membranes. Inactivation of fusion induces the loss of mtDNA. When both fusion and fission are simultaneously inactivated, the loss of mtDNA is alleviated, along with mitochondrial fragmentation. Mechanisms involved in mtDNA maintenance thus seem to depend on a coordinated regulation of fusion and fission forces. We have studied the role of the dynamin Msp1p, a fusion effector in mitochondrial morphology, in relation to the maintenance of mtDNA. Two hydrophobic regions of Msp1p, predicted to be transmembrane segments, were shown to anchor the long form of the protein into mitochondrial membranes, whereas the short form, lacking these two domains, behaved as a peripheral membrane protein. Both domains were essential for the fusogenic activity of Msp1p, but deletion of the second domain alone induced loss of mtDNA and thus lethality. Our results demonstrate that the role of Msp1p in the control of mitochondrial morphology is distinct from that required for genome maintenance, and that only the latter function is essential for cell viability. This parallels recent observations that have distinguished the role of OPA1, the human orthologue of Msp1p, in mitochondrial dynamics from that in cristae organization and apoptosis. Furthermore, our observations may contribute to our understanding of the pathological mechanisms resulting from mutations in OPA1 that give rise to the ADOA syndromes.

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Key words: Mitochondrial dynamics, mtDNA, Mitochondria, Msp1, OPA1, Mgm1

Introduction

The metabolic pathways that occur in the mitochondria have been studied for many years, but it is only recently that the implications of mitochondrial morphology in these processes have been reconsidered. Advances in this domain have been made possible by the isolation of yeast mutants deficient for the transmission, maintenance and organization of the mitochondria. Their characterization has led to the proposal that the morphology of the mitochondrial network depends on the equilibrium between antagonistic fission and fusion forces acting on mitochondrial membranes (Hoppins et al., 2007; Okamoto and Shaw, 2005). Most of the proteins involved in these processes are conserved in higher eukaryotes where they fulfil similar functions. In yeast, the state of the mitochondrial outer membrane is determined by both fission, driven by the dynamin Dnm1p (DRP1 in mammals), and fusion, controlled by the GTPase Fzo1p (mammalian orthologues MFN1 and MFN2). Identification of genetic and biochemical partners of Dnm1p and Fzo1p has led to the characterization of mitochondrial division and fusion machineries. Yeast Fis1p, for which the human orthologue is FIS1, functions as the receptor for cytoplasmic Dnm1p via the peripheral adaptor proteins Mdv1p and Caf4p, which are not conserved in other eukaryotes. The mechanisms that underlie fusion and modeling of the mitochondrial inner membrane, particularly of its dynamic tubular projections that constitute the cristae (Mannella, 2006), are not as well characterized. OPA1 and its counterparts in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, Mgm1p and Msp1p, respectively, are believed to control these processes (Olichon et al., 2006). In addition, mitofilin (John et al., 2005) and ATP synthase (Paumard et al., 2002) have been implicated to be crucial organizers of cristae morphology. Ugo1p is involved in mitochondrial fusion and possibly coordinates the dynamics of the outer and inner membrane by physically linking Fzo1p to Mgm1p. No mammalian homologue of Ugo1p has been found to date, possibly because MFN1 and OPA1 interact directly with each other (Guillery et al., 2008). Furthermore, several mitochondrial-shaping proteins have been identified in mammals and yeast, such as Mdm33p, endophilin B1, MTP18, GDAP1 (Karbowski et al., 2004; Messerschmitt et al., 2003; Niemann et al., 2005; Tondera et al., 2005).

In addition to the crucial role of mitochondrial dynamics in mitochondrial morphology, their involvement in both mitochondrial and cellular functions is beginning to be evaluated. The importance of mitochondrial dynamics has been highlighted by the discovery that inherited human diseases are caused by mutations in mitochondrial fusion and fission genes. Mutations in *MFN2* cause a peripheral motor neuropathy (CMT2A), which presents with progressive distal muscle weakness followed by muscular atrophy (Zuchner et al., 2004). Mutations in *OPA1* are associated with an optic neuropathy (ADOA-1) (Delettre et al., 2000), a leading cause of blindness. Finally, mutations in *GDAP1*, which encodes an

integral protein of the outer membrane involved in mitochondrial division, are associated with CMT4A (Baxter et al., 2002; Cuesta et al., 2002; Niemann et al., 2005).

Surprisingly, Fzo1p and Mgm1p/Msp1p, the yeast orthologues of the two human pro-fusion proteins MFN2 and OPA1, are also linked to mtDNA maintenance (Hermann et al., 1998; Jones and Fangman, 1992; Pelloquin et al., 1998). This might not be a coincidence because other yeast genes involved either in mitochondrial membrane dynamics (*UGO1*) or in mitochondrial movement and tethering (*MDM10*, *MDM12* and *MMM1*) also affect nucleoid inheritance in *S. cerevisiae* (Chen and Butow, 2005). Interestingly, the loss of mtDNA observed in fusion mutants is alleviated together with the fragmented mitochondrial phenotype when fission is inactivated (Bleazard et al., 1999; Fekkes et al., 2000; Guillou et al., 2005; Sesaki and Jensen, 2001; Wong et al., 2000). Mechanisms involved in mtDNA maintenance thus seem to depend on a coordinated regulation of fusion and fission forces that control the morphogenesis of the mitochondria.

The question of the nature of the precise links between mitochondrial membrane dynamics and nucleoid inheritance thus arises. Is the loss of mtDNA observed upon dysregulation of membrane dynamics a mere consequence of this primary alteration or is there a specific mechanism linked to membrane structure and involved in mtDNA maintenance? We took advantage of the *S. pombe* model, which is closer to the situation in mammalian cells than the *S. cerevisiae* model (Chiron et al., 2007), to study the role of the dynamin Msp1p in mitochondrial morphology and dynamics with respect to the maintenance of mitochondrial nucleoids. To this end we have characterized mutants of Msp1p deleted for one of its two hydrophobic regions that represent potential transmembrane segments (referred herein as TM1 and TM2). We show that both TM1 and TM2 are involved in the association of the long form of Msp1p with mitochondrial membranes, whereas the short form of the dynamin, lacking the two hydrophobic domains, acts as a peripheral membrane protein. Furthermore, we show that both TM1 and TM2 are involved in the fusogenic activity of Msp1p but that only modifications of TM2 induce a dominant-negative loss of mtDNA and ensuing Rho zero (ρ^0) lethality. These results demonstrate for the first time that this dynamin has distinct roles in mitochondrial morphology and in genome maintenance. It further shows that one essential function of Msp1p is to maintain the mitochondrial genome, whereas its function in mitochondrial dynamics has no effect on cell viability.

Results

The two isoforms of Msp1p differ in their association with mitochondrial membranes

To gain insight into the association of Msp1p with mitochondrial membranes the susceptibility of the long form of the dynamin (l-Msp1p) to extraction by high ionic strength, basic pH, or detergent was compared with that of the short form (s-Msp1p) (Fig. 1). In buffer-treated mitochondria, the matrix peptidase-matured l-Msp1p (Pelloquin et al., 1999) was associated with membranes, and the association was resistant to extraction by 1 M NaCl or 0.1 M Na_2CO_3, pH 11 (Fig. 1A). The shorter form, probably generated by a mitochondrial rhomboid-like protease (Herlan et al., 2003; McQuibban et al., 2003), was also mostly bound to the membranes in control mitochondria, although a certain percentage was present in the supernatant. This soluble fraction was increased by treatments with high salt or basic pH (Fig. 1A). As expected, CoxIIp, an integral membrane component of the electron transport complex IV, was

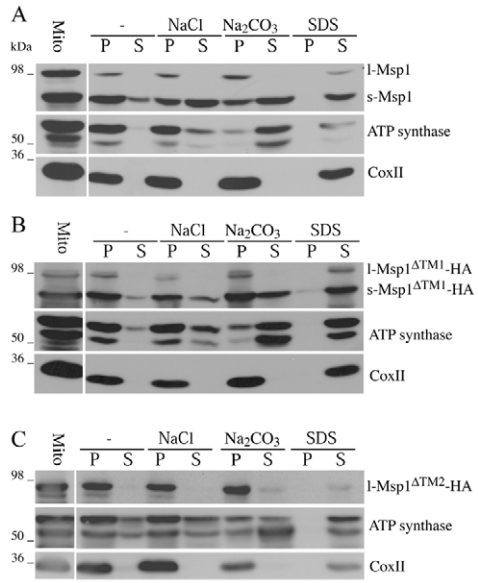

Fig. 1. TM1 and TM2 anchor l-Msp1p to mitochondrial membranes. Mitochondria (Mito) were prepared from an *msp1*$^+$ strain (A) or from the same strain expressing C-terminally HA-tagged Msp1p$^{\Delta TM1}$ (B) or Msp1p$^{\Delta TM2}$ (C) and disrupted by sonication. Intact organelles were eliminated and membrane-bound (P) and soluble (S) proteins were separated either directly (–) or following treatment with 1 M NaCl, 0.1 M Na_2CO_3, pH 11 or 0.5% SDS. Following SDS-PAGE and western blotting, proteins were visualized with anti-Msp1p, or anti-HA antibodies, and with anti F1-ATP synthase and anti-CoxIIp antibodies for control peripheral or integral membrane proteins, respectively.

fully resistant to extraction by carbonate and salts, whereas the α and β subunits of the peripheral F1-ATP synthase were partially or readily extracted with NaCl or Na_2CO_3, respectively. Each protein analyzed was totally solubilized by detergent. In these experiments, s-Msp1p and l-Msp1p thus behave as peripheral and membrane-anchored proteins, respectively.

The l-Msp1p form, which is cleaved after the mitochondrial import sequence at residue 59 (Pelloquin et al., 1999), contains the two predicted membrane-spanning segments (residues L86-A103; TM1 and P193-V211; TM2) that probably make it resistant to alkali extraction (Fig. 2A). The N-terminus of s-Msp1p, determined by Edman degradation (TOPLAB) begins with the SVDSV sequence, indicating that the short form of Msp1p is generated by proteolytic cleavage at the end of TM2 after residue L209 (see Fig. 2A). The weaker association of s-Msp1p to membranes could thus relate to its lack of TM1 and TM2.

The two transmembrane segments of Msp1p contribute to the anchoring of the dynamin to mitochondrial membranes

To investigate the specific roles of TM1 and TM2 in the association of Msp1p with mitochondrial membranes in vivo, we deleted each segment and expressed the corresponding variants in *S. pombe*. Immunoblot analyses were performed using anti-Msp1p antibodies on total protein extracts (Fig. 2B). The precursor (p-Msp1p) and the long and short isoforms of Msp1p were detected in cells expressing either the wild-type (Msp1p) or the TM1-deleted dynamin (Msp1p$^{\Delta TM1}$). In the latter case, a diminution of the amount of l-Msp1p$^{\Delta TM1}$ was reproducibly noticed. Deletion of TM2 (Msp1p$^{\Delta TM2}$) yielded a correspondingly shorter form l-Msp1p$^{\Delta TM2}$ and prevented formation of s-Msp1p because TM2, which contains

the protease maturation site, was absent (Fig. 2A). No signals were detected in non-transfected cells because of the low abundance of endogenous Msp1p.

C-terminally HA-tagged forms of Msp1p, Msp1p$^{\Delta TM1}$ and Msp1p$^{\Delta TM2}$ were used to monitor their association with mitochondrial membranes. HA-tagged Msp1p maintained its function, as assessed by its ability to complement the deletion of the *msp1*$^{+}$ gene (Guillou et al., 2005). Both l-Msp1p$^{\Delta TM1}$ and l-Msp1p$^{\Delta TM2}$ remained associated with mitochondrial membranes after treatment with NaCl, or basic pH (Fig. 1B,C). In cells expressing Msp1p$^{\Delta TM1}$, the s-Msp1p isoform was identical to the wild-type s-Msp1p and thus behaved similarly (Fig. 1B). These experiments led us to conclude that each of the two hydrophobic domains participate equally in anchoring l-Msp1p to membranes. Removal of these two domains is necessary to produce the peripherally membrane-associated isoform s-Msp1p. Other region(s) of the molecule might also be involved, because a percentage of s-Msp1p was resistant to salt and alkali extraction.

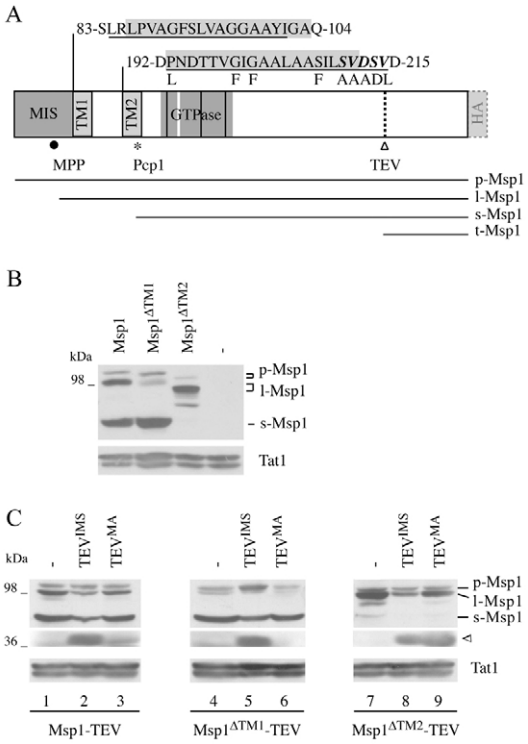

Fig. 2. Deletion of TM2 but not TM1 alters the maturation of Msp1p and its sub-mitochondrial localization. (A) Schematic representation of the structure of Msp1p showing the mitochondrial import sequence (MIS), the GTPase domain containing the three consensus GTP-binding sequences (black bars), and the dynamin signature (white bar). The sequences surrounding the two predicted hydrophobic transmembrane segments (TM1 and TM2, grey boxes) are shown with the ***SVDSV*** N-terminal residues determined for s-Msp1p in bold italics. Residues deleted in Msp1p$^{\Delta TM1}$ and Msp1p$^{\Delta TM2}$ are underlined and substitutions introduced in Msp1p^{mutTM2} (cleavage region and α-helix-breaking residues important for the activity of Pcp1p) are indicated below the wild-type TM2 sequence. The location of the TEV cleavage sequence introduced in the Msp1p ORF between S665 and P666, and the C-terminal HA tag are indicated by a dotted line and box, respectively. Cleavage sites for the mitochondrial matrix peptidase (•, MPP), the rhomboid protease (*, Pcp1) and the TEV protease (Δ, TEV) are shown. The full-length precursor Msp1p (p-Msp1p), mitochondrial matrix peptidase-matured Msp1p (l-Msp1p), mitochondrial rhomboid protease-matured Msp1p (s-Msp1p) and TEV cleavage product of Msp1p (t-Msp1p) are shown. (B) An *msp1*$^{+}$ strain was transfected or not (–) with wild-type Msp1p, or Msp1p$^{\Delta TM1}$, or Msp1p$^{\Delta TM2}$ plasmids under the control of the moderate *nmt1*$^{+}$ promoter. Total protein extracts obtained from cells grown for 24 hours without thiamine, were analyzed by SDS-PAGE and western blotting with anti-Msp1p or anti-tubulin (Tat1) antibodies as a loading control. (C) An *msp1*$^{+}$ strain was transfected with plasmids containing an Msp1p open reading frame with a C-terminal HA-tag and a TEV protease cleavage site – that specified wild-type Msp1p (Msp1p-TEV), Msp1p$^{\Delta TM1}$ (Msp1p$^{\Delta TM1}$-TEV) or Msp1p$^{\Delta TM2}$ (Msp1p$^{\Delta TM2}$-TEV) – either alone (–) or in combination with a TEV protease targeted to the IMS (TEV^{IMS}) or to the matrix (TEV^{MA}), all under the control of the moderate *nmt1*$^{+}$ promoter. Total protein extracts of cells grown for 24 hours without thiamine were analyzed by SDS-PAGE and western blotting with anti-HA antibodies. The arrowhead indicates the t-Msp1p TEV cleavage product.

Deletion of TM2, but not TM1, alters the sub-mitochondrial localization of Msp1p

To investigate the role of TM1 and TM2 in the sub-mitochondrial localization of Msp1p we used a method previously applied to Mgm1p (Wong et al., 2003) and Msp1p (Guillou et al., 2005). A TEV protease cleavage site was introduced (Fig. 2A) into the C-terminally HA tagged forms of wild-type (Msp1p-TEV), TM1-deleted (Msp1p$^{\Delta TM1}$-TEV) or TM2-deleted (Msp1p$^{\Delta TM2}$-TEV) Msp1p. These constructs were co-transfected into *S. pombe* together with the TEV protease targeted either to the inter-membrane space (IMS) (TEV^{IMS}) or to the matrix (TEV^{MA}). The expected 32 kDa peptide, corresponding to the C-terminal fragment containing the HA tag (Fig. 2A, t-Msp1p), was observed for both Msp1p-TEV and Msp1p$^{\Delta TM1}$-TEV when co-expressed with TEV^{IMS} (Fig. 2C, lanes 2 and 5), but not with TEV^{MA} (Fig. 2C, lanes 3 and 6), or without the protease (Fig. 2C, lanes 1 and 4). The TEV cleavage product of Msp1p$^{\Delta TM2}$-TEV was observed in cells co-expressing either TEV^{MA} or TEV^{IM} (Fig. 2C, lanes 8 and 9). These results indicate that the C-terminus of wild-type or TM1-deleted Msp1p was accessible only to the IMS-targeted protease, whereas the TM2-deleted dynamin exposed its C-terminal region to the IMS and matrix in equal proportions. Thus, the deletion of TM1 did not alter the sub-mitochondrial localization of Msp1p, and removal of TM2 led to a partial delocalization within the matrix. This delocalization was due to the loss of TM2 hydrophobicity because Msp1p^{mutTM2} that only produced a long isoform (as did Msp1p$^{\Delta TM2}$) but maintained an hydrophobic TM2 (Fig. 2A), also preserved a correct orientation to the IMS (supplementary material Fig. S1).

TM1 and TM2 are essential for the function of Msp1p

As it is a member of the dynamin family, the relationship of Msp1p with the mitochondrial membrane is expected to be crucial for its function (Praefcke and McMahon, 2004). To study this issue, we examined the capacity of Msp1p deleted for either of its two transmembrane segments to complement the deletion of the *msp1*$^{+}$ gene, which is essential for viability in *S. pombe* (Pelloquin et al., 1998). Each HA-tagged mutant Msp1p$^{\Delta TM1}$ and Msp1p$^{\Delta TM2}$ was transfected into a diploid strain containing a wild-type and a disrupted allele of *msp1*$^{+}$. After sporulation complementation of haploid Δ*msp1*$^{+}$ cells was assessed by their ability to grow on minimal medium (Fig. 3A). Full complementation was observed upon expression of Msp1p, whereas cells that expressed the bacterial CAT died. TM1 and TM2 deletion mutants failed to complement the *msp1*$^{+}$ deletion and thus did not support the function of Msp1p. The lack of complementation of Msp1p$^{\Delta TM2}$ was not due to its mislocalization because Msp1p^{mutTM2} that was correctly exposed to the IMS (supplementary material Fig. S1) also failed to rescue *msp1*$^{+}$ gene deletion (not shown). Complementation was, however, obtained when both deletion mutants were co-expressed

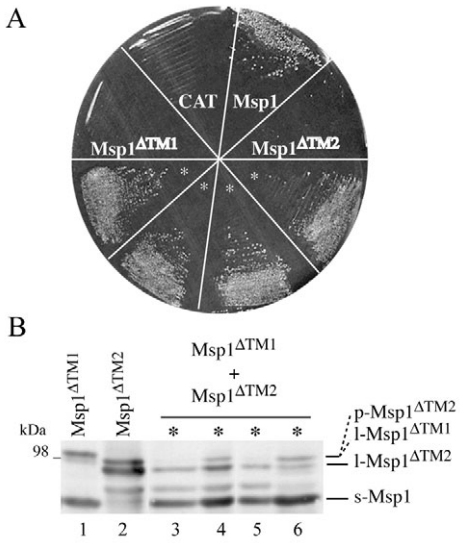

Fig. 3. TM1 and TM2 are essential for the function of Msp1p. (A) An *msp1*$^{+}$/*Δmsp1* diploid strain was transfected with CAT, or C-terminally HA-tagged versions of wild-type Msp1p, Msp1p$^{\Delta TM1}$ or Msp1p$^{\Delta TM2}$ plasmids under the control of the moderate *nmt1*$^{+}$ promoter. The ability of these constructs to rescue the *msp1*$^{+}$ gene deletion was verified by plating spores obtained from the transfected diploids on minimal medium lacking thiamine. Growth of spores obtained from four clones co-transfected with both Msp1p$^{\Delta TM1}$ and Msp1p$^{\Delta TM2}$ is shown in the lower half (asterisks). (B) Total protein extracts from four haploid Δ*msp1*$^{+}$ strains co-expressing Msp1p$^{\Delta TM1}$ and Msp1p$^{\Delta TM2}$ (asterisks, lanes 3-6), and from haploid *msp1*$^{+}$ strains expressing either Msp1p$^{\Delta TM1}$ (lane 1) or Msp1p$^{\Delta TM2}$ (lane 2) were analyzed by SDS-PAGE and western blotting with anti-HA antibodies.

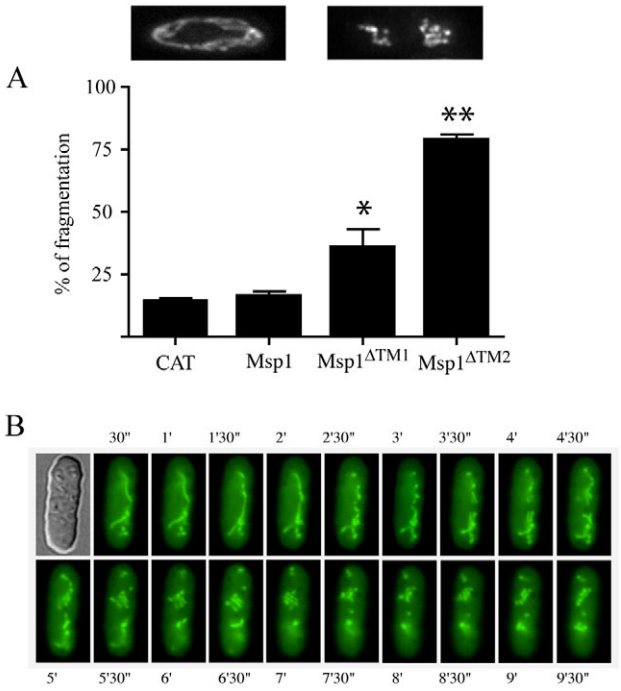

Fig. 4. Mitochondrial morphology requires the integrity of the two transmembrane segments of Msp1p. (A) An *msp1*$^{+}$ strain expressing a genome-tagged mCherry-Arg11 gene was transfected with CAT, wild-type Msp1p, Msp1p$^{\Delta TM1}$ or Msp1p$^{\Delta TM2}$ plasmids under the control of the *nmt1*$^{+}$ promoter. Cells were cultivated in medium without thiamine for 24 hours, and observed by fluorescence microscopy. Typical tubular or fragmented mitochondrial phenotypes (upper panels) were quantified after formaldehyde fixation. The lower graph shows the percentage of cells with fragmented mitochondria (mean ± s.e.m. of four independent experiments; n>300 cells per condition; *P<0.05, Msp1p$^{\Delta TM1}$ versus Msp1p and CAT, and **P<0.001, Msp1p$^{\Delta TM2}$ versus Msp1p, CAT and Msp1p$^{\Delta TM1}$; Tukey's ANOVA test). (B) Time-lapse fragmentation of the mitochondrial network in a single cell expressing a mitochondrial GFP and Msp1p$^{\Delta TM2}$.

(Fig. 3A). The presence of the two mutants in haploid rescued Δ*msp1*$^{+}$ strains was monitored by immunoblot analysis (Fig. 3B). Anti-HA antibodies revealed different isoforms of Msp1p: s-Msp1p, which was generated by cleavage of l-Msp1p$^{\Delta TM1}$; l-Msp1p$^{\Delta TM2}$; and a band that could correspond to either l-Msp1p$^{\Delta TM1}$ or p-Msp1p$^{\Delta TM2}$, which migrate at the same position within the gel. Thus neither Msp1p$^{\Delta TM1}$ nor Msp1p$^{\Delta TM2}$ are fully functional, but intermolecular complementation can restore viability of Δ*msp1*$^{+}$ cells.

Mitochondrial morphology requires the integrity of the two transmembrane segments of Msp1p

We have previously shown that Msp1p participates in the control of mitochondrial membrane dynamics (Guillou et al., 2005), as do its orthologues Mgm1p and OPA1 (Cipolat et al., 2004; Griparic et al., 2004; Olichon et al., 2003; Wong et al., 2003). The effects of TM1- or TM2-deleted Msp1p mutants on mitochondrial morphology were thus examined by fluorescence microscopy after transfection of *msp1*$^{+}$ strains harboring a genome-tagged mCherry-Arg11 gene (Snaith et al., 2005), which encodes a mitochondrial protein. As previously shown (Guillou et al., 2005), low-level expression of wild-type Msp1p had no effect on mitochondrial morphology; after 24 hours of induction a majority of transfected cells contained a tubular mitochondrial network that was similar to that of cells expressing the unrelated CAT gene (Fig. 4A). On the contrary, after a similar expression period of Msp1p$^{\Delta TM1}$ or Msp1p$^{\Delta TM2}$ (or Msp1p^{mutTM2}, supplementary material Fig. S1) 35% or 80% of the cells, respectively, had fragmented mitochondria that appeared as small individual dots more or less clustered (Fig. 4); expression periods longer than 24 hours led to mitochondrial fragmentation in about 95% of the cells, Msp1p$^{\Delta TM2}$ reaching this level faster than Msp1p$^{\Delta TM1}$ (not shown). Fig. 4B exemplifies the process of mitochondrial fission occurring in a single cell upon overexpression of Msp1p$^{\Delta TM2}$. The mitochondrial fragmentation induced by overexpression of TM-deleted Msp1p variants is reminiscent of that observed in *msp1*$^{+}$-deleted cells (Guillou et al., 2005), and suggests that these unfunctional mutants have a dominant-negative effect on mitochondrial morphology when overexpressed in wild-type cells.

Deletion of TM2 but not TM1 induces ρ^{0} lethality

Because the loss of function of Msp1p obtained by deletion of the *msp1*$^{+}$ gene, by overexpression of dominant-negative GTPase or of C-terminally deleted dynamin mutants, resulted in the depletion of mtDNA and cell death (Guillou et al., 2005; Pelloquin et al., 1998), we wondered whether this would also be the case with the present mutants. Cultures of *msp1*$^{+}$ strains expressing wild-type Msp1p, Msp1p$^{\Delta TM1}$, or Msp1p$^{\Delta TM2}$, or CAT were maintained in exponential growth by daily dilution into fresh medium. After 5 days of expression, mitochondrial nucleoids were visualized by fluorescence microscopy using DAPI staining (Fig. 5A), and the viability of each strain was monitored by drop dilution assays (Fig.

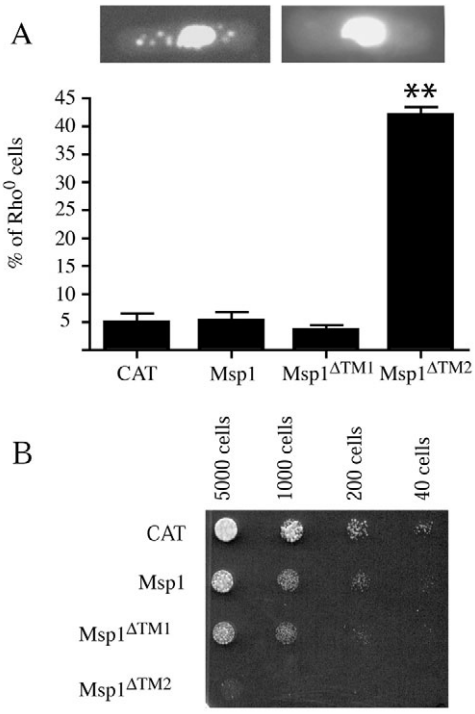

Fig. 5. Deletion of TM2 but not TM1 in Msp1p induces ρ^0 lethality. An *msp1*$^+$ strain was transfected with CAT, wild-type Msp1p, Msp1p$^{\Delta TM1}$ or Msp1p$^{\Delta TM2}$ plasmids under the control of the moderate *nmt1*$^+$ promoter, and cells were cultivated by daily dilution in fresh medium without thiamine for 5 days. (A) Cells were observed by fluorescence microscopy after formaldehyde fixation and DAPI staining. Upper panels exemplify typical wild-type (left) and ρ^0 (right) cells. The lower graph shows the percentage of ρ^0 cells (mean ± s.e.m. of four independent experiments; n>350 cells per condition; **P<0.001, Msp1p$^{\Delta TM2}$ versus CAT, Msp1p and Msp1p$^{\Delta TM1}$; Tukey's ANOVA test). (B) At day five, the indicated numbers of cells expressing the various constructs were spotted on minimal medium and grown for 8 days at 25°C.

5B). In 40% of the cells transfected with Msp1p$^{\Delta TM2}$, mitochondrial genomes were absent (Fig. 5A). This was not due to delocalization of Msp1p$^{\Delta TM2}$ in the matrix because a similar situation was observed with Msp1p^{mutTM2} (supplementary material Fig. S1). By comparison, expression of Msp1p$^{\Delta TM1}$, wild-type Msp1p or CAT led to only 5% ρ^0 cells (Fig. 5A). The behavior of the mitochondrial genome could be correlated with cell viability. Although overexpression of wild-type Msp1p or Msp1p$^{\Delta TM1}$ had no significant effect on cell survival when compared with CAT, expression of Msp1p$^{\Delta TM2}$ (or Msp1p^{mutTM2}, not shown) induced a lethal dominant-negative effect (Fig. 5B). This indicates that deletion or gross modifications of the second transmembrane domain of Msp1p drastically affects the maintenance of the mitochondrial genome and thus the viability of the petite-negative yeast *S. pombe*, whereas deletion of TM1 does not provoke these effects.

Discussion

Although it is now accepted that the three orthologues of mitochondrial dynamin Mgm1p/Msp1p/OPA1 are localized to the IMS, some discrepancies still persist regarding their relationships with mitochondrial membranes. Mgm1p has been shown to be either peripherally or strongly associated with mitochondrial membranes (Herlan et al., 2003; Sesaki et al., 2003b; Wong et al., 2000), Msp1p is anchored to the inner membrane (Pelloquin et al., 1999), and OPA1 binds more or less tightly to both mitochondrial membranes (Griparic et al., 2004; Olichon et al., 2002; Satoh et al., 2003). In this study, we compared membrane association of the two Msp1p isoforms that contain either both hydrophobic TM1 and TM2 regions (l-Msp1p) or none (s-Msp1p), with that of mutants deleted for either TM1 or TM2. The l-Msp1p isoform behaved as an integral membrane protein tightly associated with the membranes whereas s-Msp1p was partly extracted together with peripheral membrane proteins. This weaker membrane association of s-Msp1p correlated with the absence of TM1 and TM2, which contributed equally to anchor the l-Msp1p isoform to the mitochondrial membranes. The existence of several isoforms of the Mgm1p/Msp1p/OPA1 dynamin, each containing different sets of TM1 and TM2 because of alternative proteolytic cleavage and mRNA splicing (Delettre et al., 2001; Duvezin-Caubet et al., 2007; Griparic et al., 2007; Guillery et al., 2008; Herlan et al., 2004; Ishihara et al., 2006; Song et al., 2007), might explain the initial discrepancies reported in their localization and membrane association.

Although deletion of TM1 did not modify the mitochondrial localization of Msp1p, the lack of TM2 altered both its biogenesis and its localization. According to the model of alternative topogenesis of Mgm1p (Herlan et al., 2004), deletion or decreased hydrophobicity of TM1 abolishes its stop transfer properties that allow lateral diffusion and membrane insertion of l-Mgm1p, and leads to the exclusive formation of s-Mgm1p after Pcp1p cleavage at the C-terminus of TM2. If this model holds true for the biogenesis of Msp1p, the production of both l-Msp1p$^{\Delta TM1}$ and s-Msp1p isoforms upon expression of Msp1p$^{\Delta TM1}$ could be explained by the greater hydrophobicity of TM2 in Msp1p compared with Mgm1p. TM2 could thus possibly act as an alternative stop-transfer signal before Pcp1p cleavage. However, the levels of l-Msp1p$^{\Delta TM1}$ remained below those of wild-type l-Msp1p because TM2 has a lower hydrophobicity than TM1, and hence a less-efficient stop-transfer activity. The ability of TM2 to constitute a stop-transfer signal was substantiated here by the observation that deletion of TM2 resulted in a partial mislocalization of l-Msp1p$^{\Delta TM2}$ in the matrix whereas modifications in Msp1p^{mutTM2} that preserved its hydrophobicity also preserved a normal localization. According to the alternative topogenesis model of Mgm1p, arrest of the ATP-driven import occurs upon cleavage by Pcp1p leading to the release of the short isoform from the import motor. Consequently, in both *S. cerevisiae* and *S. pombe*, deletion or gross modifications of TM2, and of the rhomboid recognition sequence normally present in this domain, prevented the formation of the short isoform. Since l-Msp1p$^{\Delta TM2}$ was partially mislocalized to the matrix, whereas the corresponding l-Mgm1p isoform was totally present in the IMS (Herlan et al., 2004), it is suggested that, in contrast to Mgm1p, the part of MPP-cleaved l-Msp1p$^{\Delta TM2}$ that does not translate laterally into the IM using TM1, is completely pulled into the matrix when it is not released from the import machinery by protease cleavage. Together, our results suggest that, as is the case for OPA1, additional and/or different mechanisms possibly involving proteases other than Pcp1p, might operate during s-Msp1p biogenesis.

The importance of the association between Msp1p and the mitochondrial membrane is highlighted by the demonstration that both TM1 and TM2 are essential for viability. Expression of Msp1p$^{\Delta TM1}$ does not complement the deletion of the *msp1*$^+$ gene, although a long isoform (l-Msp1p$^{\Delta TM1}$) and a wild-type s-Msp1p are generated. Since l-Msp1p$^{\Delta TM1}$ is correctly located in the IMS and anchored to the mitochondrial membrane, we conclude that

TM1 is not only involved in the biogenesis of the dynamin isoforms but also has an essential function; this might relate to the presence of the AXXXG sequence, which mediates dimerization within membranes (Russ and Engelman, 2000). In this respect, *cis*-acting Mgm1p-Mgm1p interactions have been proposed to be important for inner-membrane fusion (Meeusen et al., 2006). Since the presence of l-Mgm1p and s-Mgm1p was shown to be necessary to rescue the deletion of *MGM1* (Herlan et al., 2003), the lack of complementation of Δ*msp1*$^{+}$ cells by expression of Msp1p$^{\Delta TM2}$ might be due to the absence of s-Msp1p, even though a loss of function of l-Msp1p$^{\Delta TM2}$ cannot be excluded. Nevertheless, when Msp1p$^{\Delta TM1}$ is co-expressed with Msp1p$^{\Delta TM2}$, intermolecular complementation is observed that might result from Msp1p self-assembly (our unpublished results). In this regard, *trans*-acting Mgm1p-Mgm1p interactions between the GTPase domain and GED have been shown to be involved in inner-membrane fusion (Meeusen et al., 2006).

Expression of TM1- or TM2-deleted variants of Msp1p in wild-type yeast leads to a dominant-negative fragmentation of the mitochondrial network, whereas only overexpression of Msp1p$^{\Delta TM2}$ induces a dominant-negative loss of mtDNA and death of the petite-negative *S. pombe*. In cells transfected with Msp1p$^{\Delta TM1}$, both l-Msp1p$^{\Delta TM1}$ and s-Msp1p are overexpressed, but only the smaller form is functional. The l-Msp1p$^{\Delta TM1}$ form, which is anchored to the mitochondrial membrane, might thus exert a dominant-negative effect on endogenous l-Msp1p, which, as has been proposed for Mgm1p (Sesaki et al., 2003a) and OPA1 (Ishihara et al., 2006), could be the fusogenic isoform of this dynamin. Alternatively, increased levels of s-Msp1p, leading to an imbalanced ratio of the two isoforms, could result in mitochondrial fragmentation by decreasing the availability of fusogenic l-Msp1p. The equilibrium between the short and long isoforms has indeed been shown to be crucial in *S. cerevisiae* (Herlan et al., 2003). In cells transfected with Msp1p$^{\Delta TM2}$, titration of endogenous l-Msp1p or s-Msp1p by l-Msp1p$^{\Delta TM2}$, the sole isoform overexpressed, could induce mitochondrial fragmentation or loss of mtDNA and cell death, respectively. These dominant-negative effects of Msp1p$^{\Delta TM2}$ did not relate to its partial delocalization in the matrix, because Msp1p^{mutTM2} had exactly the same effects while being correctly targeted to the IMS. This situation, where high levels of l-Msp1p$^{\Delta TM2}$ titrate out endogenous s-Msp1p, is reminiscent of that reported in a *PCP1*-deleted *S. cerevisiae* strain, which does not produce the short form of the dynamin (Sesaki et al., 2003a). From these observations, we speculate that Msp1p functions require oligomerisation. Homo-oligomers of l-Msp1p, or hetero-oligomers with s-Msp1p where the long form predominates – since it has also been reported that both the long and short isoforms of Mgm1p and OPA1 are required for fusion (Herlan et al., 2003; Song et al., 2007) – might trigger membrane deformations leading to fusion. Other types of membrane modifications induced by hetero-oligomers where s-Msp1p predominates might be involved in mtDNA maintenance.

Our results provide the first demonstration that the role of the Msp1p dynamin in the control of mitochondrial morphology can be uncoupled from its function in genome maintenance, and show that this latter function is essential for cell viability. This conclusion was supported by analysis (not shown) of haploid strains derived from sporulation of Δ*msp1*$^{+}$ cells complemented by coexpression of Msp1p$^{\Delta TM1}$ and Msp1p$^{\Delta TM2}$ (Fig. 3A). The loss of mtDNA was rescued in eight independent clones, with proportions of ρ0 cells never exceeding 8%. Out of these, two had a wild-type, and six a fragmented, mitochondrial morphology with no obvious correlation with the expression profile of the long and short isoforms of the dynamin. Such uncoupling of genome maintenance from mitochondrial morphology might parallel other observations showing that the roles of OPA1 in mitochondrial dynamics, apoptosis and cristae organization can be separated (Frezza et al., 2006; Olichon et al., 2007). Recent studies have shown that missense mutations in *OPA1* cause the accumulation of multiple mtDNA deletions in skeletal muscle (Amati-Bonneau et al., 2008; Hudson et al., 2008). Although the majority of OPA1 mutations are associated with a non-syndromic autosomal dominant atrophy (ADOA) (Ferre et al., 2005), these newly characterized mutations lead to a more complex syndrome consisting of a combination of ADOA with progressive external ophtalmoplegia, peripheral neuropathy, ataxia and deafness (ADOA-plus). Demonstrating that the role of the dynamin in mitochondrial dynamics can be uncoupled from its role in mtDNA maintenance might help to understand the distinct pathological mechanisms that lead to ADOA or ADOA-plus syndrome.

Materials and Methods

Plasmid constructions

Plasmids for expression of Myc-tagged versions of the tobacco etch virus (TEV) protease in the IMS (pREP42 TEV^{IMS}) or in the matrix (pREP42 TEV^{MA}), HA-tagged Msp1p containing a TEV protease consensus sequence (pREP41 Msp1p-TEV-HA), and mito-GFP have been described (Guillou et al., 2005). Deletions of the predicted hydrophobic transmembrane domains (TM1 and TM2) were performed by iPCR using sense and antisense primers, for which the first base was immediately adjacent to the 5′ and 3′ positions respectively of the sequences to be deleted. After PCR, the parental plasmid was digested to completion with *Dpn*I and the amplified plasmid was gel-purified before ligation and transformation into *E. coli* TOP10f'. Modified fragments were then reintroduced into the *Msp1*$^{+}$ coding region. When indicated, this region contained a TEV protease consensus sequence and a C-terminal HA epitope, to yield pREP41 Msp1p$^{\Delta TM1}$-TEV or pREP41 Msp1p$^{\Delta TM2}$-TEV. In Msp1p$^{\Delta TM1}$, 18 residues were deleted between S83 and G102. In Msp1p$^{\Delta TM2}$, 22 residues were removed between D192 and D215 and replaced with L residue. Msp1^{mutTM2} was engineered by inserting cohesive synthetic oligonucleotides with the desired sequence into the *Bgl*II site created at the D-L deletion junction of this latter construct. Each modification was verified by two-strand sequencing.

Yeast strains and cultures

Fission yeast growth media (EMM, ME) were from Bio101 (La Jolla, CA). *S. pombe* strains carrying the indicated plasmids were transfected by electroporation using the Bio-Rad Gene Pulser. Transformants were selected by growth on medium lacking leucine or uracil. Cells carrying plasmids with the *nmt1*$^{+}$ promoter were grown in the presence of 4 μM thiamine to repress Msp1 expression, which was induced by three washes in minimal medium and further growth at 25°C in the absence of thiamine. Sporulation was obtained by transfer of diploids to ME medium.

Preparation of total protein extracts

Cells (5×10^{8}) were harvested and suspended at 4°C in 200 μl lysis buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 1% Triton X-100 and 1% NP40) containing a cocktail of protease inhibitors (Roche). After addition of an equal volume of 500 μm glass beads, samples were vortexed using the Fast-Prep (Bio101 Inc) until approximately 90% of the cells were disrupted. The soluble protein fraction was recovered by two centrifugations of 10 minutes at 20,000 × ***g*** at 4°C.

Preparation and fractionation of mitochondria

Isolated mitochondria (Pelloquin et al., 1999) were disrupted by sonication (Chiron et al., 2007) in hypo-osmotic MEBI buffer (0.06 M mannitol, 2 mM EGTA, 10 mM imidazole HCl pH 6.4 and 0.01% BSA). Intact mitochondria were eliminated by centrifugation (12,000 × ***g***, 4°C, 10 minutes) before sedimentation of mitochondrial membranes by ultracentrifugation (100,000 × ***g***, 30 minutes, 4°C). The pellet was suspended in the same buffer either alone or containing 1 M NaCl, 0.1 M Na_2CO_3 pH 11 or 0.5% SDS. Samples were incubated for 1 hour at 4°C, and centrifuged (100,000 × ***g***, 30 minutes, 4°C) to separate extracted proteins from the membrane pellets.

Analysis of total and mitochondrial protein extracts

Total cellular (150 μg) or mitochondrial proteins (100 μg) were boiled for 3 minutes in Laemmli sample buffer, separated by 7.5% SDS-PAGE and transferred onto Protran membrane (Schleicher and Schuell). Immunodetection was performed with the

chemiluminescence detection kit from NEN, using antibodies as follows: anti-HA (1:5000, Boehringer), purified anti-Msp1p 1:400 (Pelloquin et al., 1999), anti-Tat-1 (1:1000, provided by Sylvie Tournier), anti-F1-ATPase (1:20,000, a gift from Marc Boutry), anti-CoxIIp (1:5000, a gift from Nathalie Bonnefoy), anti-rabbit IgG-HRP and anti-mouse IgG-HRP (1:5000, New England Biolabs).

Cytological observations

S. pombe cells expressing either a genome-tagged mCherry-Arg11 gene or a mito-GFP were fixed, or not, in 3.7% formaldehyde for 10 minutes and observed using Leica DM5000B or TCS SP2 microscopes. DAPI staining was performed as described (Moreno et al., 1991).

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