

# Dnm1p GTPase-mediated Mitochondrial Fission Is a Multi-step Process Requiring the Novel Integral Membrane Component Fis1p

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**Abstract.** Yeast Dnm1p is a soluble, dynamin-related GTPase that assembles on the outer mitochondrial membrane at sites where organelle division occurs. Although these Dnm1p-containing complexes are thought to trigger constriction and fission, little is known about their composition and assembly, and molecules required for their membrane recruitment have not been isolated. Using a genetic approach, we identified two new genes in the fission pathway, *FIS1* and *FIS2*. *FIS1* encodes a novel, outer mitochondrial membrane protein with its amino terminus exposed to the cytoplasm. Fis1p is the first integral membrane protein shown to participate in a eukaryotic membrane fission event. In a related study (Tieu, Q., and J. Nunnari. 2000. *J. Cell*

*Biol.* 151:353–365), it was shown that the *FIS2* gene product (called Mdv1p) colocalizes with Dnm1p on mitochondria. Genetic and morphological evidence indicate that Fis1p, but not Mdv1p, function is required for the proper assembly and distribution of Dnm1p-containing fission complexes on mitochondrial tubules. We propose that mitochondrial fission in yeast is a multi-step process, and that membrane-bound Fis1p is required for the proper assembly, membrane distribution, and function of Dnm1p-containing complexes during fission.

**Key words:** Fis1p • Dnm1p GTPase • mitochondria • fission • fusion

## Introduction

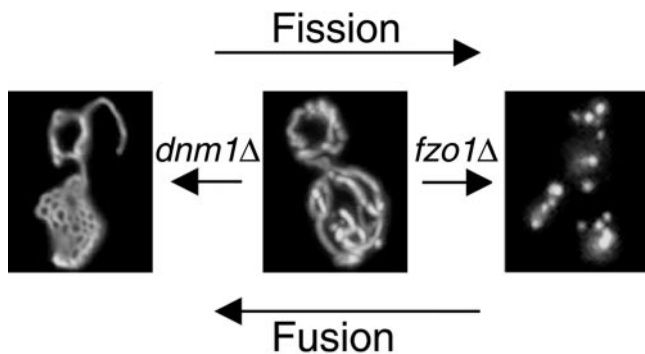
Maintenance of the tubular mitochondrial reticulum in budding yeast requires opposing fission and fusion events that regulate organelle copy number and morphology (Hoffman and Avers, 1973; Stevens, 1977; Nunnari et al., 1997). Time-lapse studies showed that these mitochondrial fusion and fission events occur at equal rates, with one event taking place on average every 2 min (Nunnari et al., 1997). The fusion reaction always occurs between the tips of two mitochondrial tubules or between a tip and the side of another mitochondrial tubule. In contrast, mitochondrial fission appears to initiate within a tubule, creating new mitochondrial tips.

In *Saccharomyces cerevisiae*, two evolutionarily conserved GTPases act on the outer mitochondrial membrane to regulate opposing fission and fusion reactions (Fig. 1) (Hales and Fuller, 1997; Hermann and Shaw, 1998; Otsuga et al., 1998; Bleazard et al., 1999; Labrousse et al., 1999; Sesaki and Jensen, 1999). Fission is regulated by the dynamin-related GTPase Dnm1p, which assembles on mitochondrial tubules at sites of future division (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999). *dnm1* mutations block mitochondrial fission and lead to formation of interconnected nets due to ongoing tip fusion (Fig.

1, left). This net formation does not affect mitochondrial function, mitochondrial DNA (mtDNA) maintenance, or mitochondrial transport into buds during division (Otsuga et al., 1998; Bleazard et al., 1999). Conversely, fusion is regulated by the Fzo1p transmembrane GTPase (Hermann et al., 1998; Rapaport et al., 1998). In *fzo1* mutant strains, mitochondrial fusion is blocked and tubules fragment due to unopposed mitochondrial fission (Fig. 1, right). As a secondary consequence of this fragmentation, mtDNA is lost and the resulting respiratory-deficient *fzo1* cells fail to grow on the nonfermentable carbon source glycerol (Hermann et al., 1998).

In a previous study (Bleazard et al., 1999), we showed that *dnm1* mutations prevent mitochondrial fission, fragmentation, and mtDNA loss at 37°C in a conditional *fzo1-1* mutant. To identify new genes required for fission, we selected for mutations that, like *dnm1*, blocked mitochondrial fragmentation and restored glycerol growth in *fzo1-1*. Using this approach, we identified two new genes in the fission pathway, called *FIS1* and *FIS2* (*FIS*, fission). Here we show that *FIS1* encodes an outer mitochondrial membrane protein required for the proper assembly and function of Dnm1p-containing fission complexes. In a related study, it was reported that the *FIS2* gene product (named Mdv1p for mitochondrial division) forms a complex with Dnm1p on the outer mitochondrial membrane (Tieu and Nunnari, 2000). We propose that mitochondrial fission in yeast is a multi-step

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**Figure 1.** Dnm1p and Fzo1p act in opposing fission and fusion pathways to maintain the yeast mitochondrial network. Ongoing fission and fusion events maintain a tubular mitochondrial network in wild-type yeast cells (middle). Loss of the Dnm1p GTPase (*dnm1Δ*) leads to net formation due to unopposed mitochondrial tip fusion (left); loss of the Fzo1p GTPase (*fzo1Δ*) leads to fragmentation due to unopposed mitochondrial fission (right).

process, and that membrane-bound Fis1p is directly involved in the assembly, membrane distribution, and function of Dnm1p-containing complexes during fission. Fis1p defines a novel protein family in eukaryotes and is the first integral membrane protein known to play a role in a membrane fission event regulated by a dynamin-like GTPase.

## Materials and Methods

### Strain and Plasmid Construction

Table I lists the strains used in this study. All strains are derivatives of the FY10 strain (Winston et al., 1995). Standard genetic methods were used to grow, transform, and manipulate yeast (Sherman et al., 1986; Guthrie and Fink, 1991) and bacterial (Maniatis et al., 1982) strains. All mutations, disruptions, tag insertions, and replacements were confirmed by PCR, DNA sequencing, and, where appropriate, Western blotting. The sequences of primers used in this study are available upon request.

For pRS415-MET25 + 9xMYC-FIS1 (Myc-Fis1p), a *FIS1* PCR fragment flanked by HindIII/XhoI sites was cloned into pRS415-MET25 + 9xMYC (J. Thatcher and J.M. Shaw, manuscript in preparation). For pRS415-MET25 + rsGFP-FIS1<sup>aa1-155</sup> (GFP-Fis1p), an XbaI/HindIII digest was first used to remove 9xMYC from the pRS415-MET25 + 9xMYC-FIS1 plasmid described above. The 9xMyc was then replaced by a red-shifted green fluorescent protein (GFP)<sup>1</sup> variant (rsGFP) PCR amplified from pQBI25-fc1 (Quantum Biotechnologies Inc.) and flanked by XbaI/HindIII sites. An ATG initiation codon was engineered into the 5' primer used to amplify rsGFP. For pRS416 + *FIS1*, a *FIS1* PCR fragment flanked by XhoI sites was cloned into pRS416. For pGEX-4T-3 + *FIS1*<sup>aa1-127</sup>, a PCR fragment encoding amino acids 1–127 of *FIS1* and flanked by BamHI/XhoI sites was cloned into pGEX-4T-3 (Amersham Pharmacia Biotech) to generate an in-frame GST-Fis1p fusion. For pRS414 + *DNM1*-rsGFP and pRS416 + *DNM1*-rsGFP, first a *DNM1*-containing PCR fragment flanked by SacII/SpeI sites was cloned into the SacII/NheI sites of pQBI25 (Quantum Biotechnologies Inc.), creating pQBI25 + *DNM1*-rsGFP. Second, an XhoI/BamHI fragment containing the *DNM1*-rsGFP sequence was cloned into pRS414 and pRS416. For pRS415-MET25 + rsGFP-FIS1<sup>aa1-127</sup>, a HindIII/XhoI digest was used to remove *FIS1*<sup>aa1-155</sup> from pRS415-MET25 + rsGFP-FIS1<sup>aa1-155</sup>, which was then replaced by a HindIII/XhoI fragment containing *FIS1*<sup>aa1-127</sup>. For pRS416-GAL1 + *PrF<sub>0</sub>ATP9*-RFP (mito-RFP), a BamHI/XhoI fragment containing RFP (the red fluorescent protein from the pDsRed vector; CLONTECH Laboratories, Inc.) was cloned into pRS416-GAL1 + *PrF<sub>0</sub>ATP9* (J. Thatcher and J.M. Shaw, manuscript in preparation). For pRS424-ADHI + *PrF<sub>0</sub>ATP9*-RFP (mito-RFP), a SpeI/

XhoI fragment containing *PrF<sub>0</sub>ATP9*-RFP from pRS416-GAL1 + *PrF<sub>0</sub>ATP9*-RFP was cloned into pRS424-ADHI.

GFP-Fis1p and Myc-Fis1p were expressed from *CEN* plasmids under control of the *MET25* promoter. In the presence of methionine, this promoter is leaky and GFP-Fis1p and Myc-Fis1p are expressed at levels comparable with that of endogenous Fis1p in wild-type cells (verified by quantitative Western blotting). Under these conditions, GFP-Fis1p and Myc-Fis1p rescued the mitochondrial morphology defect in the *fis1Δ* mutant (83 and 55% wild type, respectively).

### Identification of *fis1* and *fis2* and Cloning and Disruption of *FIS1*

14 spontaneous, second-site suppressors of the *fzo1-1* temperature-sensitive (37°C) glycerol growth defect were isolated in JSY2788 (*MATA ura3-52 his3Δ200 leu2Δ1 trp1Δ63 fzo1::HIS3* + pRS414-*fzo1-1*). Standard genetic methods were used to show that suppression in each case was due to a single recessive mutation. Complementation and meiotic segregation analyses revealed that the 14 *fzo1-1* suppressors defined three linkage groups. Seven suppressors comprising one linkage group failed to complement the *dnm1Δ* mutation in diploid cells and a *DNM1*-containing plasmid reversed the *fzo1-1* suppression phenotype of the mutants in this group. Sequence analysis indicated that these suppressors contained mutant *dnm1* alleles. The remaining seven suppressor strains fell into two linkage groups (later named *fis1* and *fis2*).

*FIS1* was cloned by complementation of a *fis1* allele (restoration of the *fzo1-1* no-growth-on-glycerol-at-37°C phenotype) using a yeast genomic library in YEP213 (Lagosky et al., 1987). Standard procedures were used to identify the rescuing open reading frame as YIL065C in the *Saccharomyces* Genome Database, which we named *FIS1*. The predicted Fis1 protein sequence was compared with all available databases using the BLAST and Prosite programs.

A *fis1::HIS3* disruption that precisely replaced the *FIS1* coding region was generated by gene replacement in a diploid strain as described (Baudin et al., 1993). After sporulation and dissection to obtain a haploid *fis1::HIS3* strain, integrative mapping studies confirmed that the original *fis1* mutations were linked to *fis1Δ::HIS3*. Sequence analysis indicated that the *fis1* suppressors contained mutations in *FIS1*.

### Quantification of Mitochondrial Morphology and Fusion Phenotypes

Mitochondrial morphology was scored in wild-type and mutant cells expressing a mitochondrial-targeted form of GFP (mito-GFP) either from the plasmid pVT100UGFP (provided by B. Westermann and W. Neupert, Universitaet Muenchen, Muenchen, Germany) or the plasmid pRS416 + preCox4-GFP (Otsuga et al., 1998). In some cases, mitochondria were labeled with a matrix-targeted form of the red fluorescent protein, mito-RFP (A.D. Mozdy and J.M. Shaw) or MitoTracker Red CMXRos (Molecular Probes, Inc.). Growth conditions were essentially as described (Bleazard et al., 1999). DAPI (4',6-diamidino-2-phenylindole) staining was used to assess the presence/absence of mtDNA (Pringle et al., 1991). Mitochondrial fusion was examined essentially as described previously (Nunnari et al., 1997), except that mito-RFP was used in place of the vital dye MitoTracker Red CMXRos (Molecular Probes, Inc.). Digital microscopic images of cells were acquired using a Axioplan microscope or a Confocal microscope (Carl Zeiss, Inc.), as described previously (Hermann et al., 1998; Otsuga et al., 1998).

### Subcellular Localization of Fis1p and Dnm1p-GFP

To generate polyclonal serum specific for Fis1p, the soluble GST-Fis1p<sup>aa1-127</sup> fusion protein was expressed in *Escherichia coli* BL21-(DE3) cells from pGEX-4T-3 + *FIS1*<sup>aa1-127</sup> and batch purified on Glutathione Sepharose 4B beads (Amersham Pharmacia Biotech). After separation by SDS-PAGE, the fusion protein was excised and used to immunize rabbits (Covance Research Products, Inc.).

For the protease protection experiments, a *fis1Δ* strain (ADM549) expressing GFP-Fis1p was grown in S-galactose medium lacking leucine to select for the pRS415-MET25 + rsGFP-FIS1 plasmid. Differential sedimentation experiments and protease protection experiments were performed using either wild-type cells (ADM548) or the ADM549 strain, as described previously (Hermann et al., 1998).

To localize Dnm1p in wild-type and mutant strains, Dnm1p-rsGFP expressed from either pRS414 + *DNM1*-rsGFP, pRS416 + *DNM1*-rsGFP, or pHS20 (Sesaki and Jensen, 1999) was localized in strains expressing

<sup>1</sup>Abbreviations used in this paper: GFP, green fluorescent protein; KDH, alpha-ketoglutarate dehydrogenase; RFP, red fluorescent protein.

Table I. Yeast Strains Used in this Study

Strain	Genotype
ADM33	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 fzo1::HIS3 FIS1 fis2-5 with pRS416 + FZO1</i>
ADM176	<i>MAT<math>\alpha</math> ura3-52 LEU2 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 fzo1-1 FIS1 fis2-5</i>
ADM179	<i>MAT<math>\alpha</math> ura3-52 LEU2 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 fzo1-1 FIS1 fis2-5</i>
ADM203	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 DNM1 fzo1-1 fis1-21 FIS2</i>
ADM378	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 dnm1::HIS3 fzo1-1 FIS1 FIS2</i>
ADM379	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 dnm1::HIS3 FZO1 FIS1 FIS2</i>
ADM415	<i>MAT<math>\alpha</math> ura3-52 TRP1 his3<math>\Delta</math>200 dnm1::HIS3 FZO1 FIS1 FIS2</i>
ADM416	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 dnm1::HIS3 fzo1-1 FIS1 FIS2</i>
ADM547	<i>MAT<math>\alpha</math> ura3-52 leu2 <math>\Delta</math> 1 TRP1 his3 <math>\Delta</math> 200 DNM1 fzo1-1 FIS1 FIS2</i>
ADM548	<i>MAT<math>\alpha</math> ura3-52 LEU2 trp1 <math>\Delta</math> 63 his3 <math>\Delta</math> 200 DNM1 FZO1 FIS1 FIS2</i>
ADM549	<i>MAT<math>\alpha</math> ura3-52 leu2 <math>\Delta</math> 1 TRP1 his3 <math>\Delta</math> 200 DNM1 FZO1 fis1::HIS3 FIS2</i>
ADM550	<i>MAT<math>\alpha</math> ura3-52 LEU2 trp1 <math>\Delta</math> 63 his3 <math>\Delta</math> 200 DNM1 fzo1-1 fis1::HIS3 FIS2</i>
ADM551	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 FZO1 FIS1 FIS2</i>
ADM552	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 FZO1 fis1::HIS3 FIS2</i>
ADM554	<i>MAT<math>\alpha</math> ura3-52 LEU2 TRP1 his3<math>\Delta</math>200 DNM1 fzo1-1 fis1::HIS3 FIS2</i>
ADM561	<i>MAT<math>\alpha</math> ura3-52 LEU2 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 FZO1 FIS1 FIS2</i>
ADM562	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 DNM1 fzo1-1 FIS1 FIS2</i>
ADM574	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 lys2<math>\Delta</math>-202 DNM1 fzo1::HIS3 fis1::HIS3 FIS2 MGM1</i>
ADM575	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 LYS2 DNM1 fzo1::HIS3 FIS1 FIS2 MGM1</i>
ADM742	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 DNM1 FZO1 fis1::HIS3 fis2-5</i>
ADM750	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 DNM1 fzo1-1 FIS1 fis2-5</i>
ADM752	<i>MAT<math>\alpha</math> ura3-52 LEU2 TRP1 his3<math>\Delta</math>200 DNM1 FZO1 FIS1 fis2-5</i>
ADM763	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 TRP1 his3<math>\Delta</math>200 DNM1 FZO1 fis1::HIS3 FIS2 mgm1::HIS3</i>
ADM764	<i>MAT<math>\alpha</math> ura3-52 leu2<math>\Delta</math>1 trp1<math>\Delta</math>63 his3<math>\Delta</math>200 DNM1 FZO1 FIS1 FIS2 mgm1::HIS3</i>

mito-RFP from pRS416-*GALI* + *PrF<sub>o</sub>ATP9*-RFP to visualize the mitochondrial membranes.

Immunoelectron microscopy was performed as described by Rieder et al. (1996) and Bleazard et al. (1999).

## Results

### Mutations in *FIS1* and *FIS2* Suppress mtDNA Loss and Glycerol Growth Defects in *fzo1-1*

To identify yeast genes required for mitochondrial fission, we selected spontaneous, second-site suppressor mutations that restored glycerol growth at 37°C to *fzo1-1* cells. The 14 suppressor strains isolated were recessive in heterozygous diploids and segregated 2:2 in backcrosses with the *fzo1-1* mutant. Complementation analysis revealed that seven of these strains represented new alleles of the previously characterized *DNM1* gene (Gammie et al., 1995; Otsuga et al., 1998; Bleazard et al., 1999). The remaining suppressors defined two new genes, initially designated *SFZ1* (four *sfz1* alleles) and *SFZ2* (three *sfz2* alleles), for suppressor of *fzo1-1*. As described below, several lines of evidence indicated that *SFZ1* and *SFZ2* act together with *DNM1* to regulate mitochondrial fission in yeast. Accordingly, we named them *FIS1* and *FIS2*, respectively.

Like *dnm1* mutations, *fis1* and *fis2* mutations rescued the *fzo1-1* temperature-sensitive mtDNA loss and glycerol growth defects. Mitochondrial networks in wild-type cells retained their mtDNA nucleoids (DAPI staining, not shown) and these cells grew well on medium containing the nonfermentable carbon source glycerol at both 25° and 37°C (Fig. 2 A, WT). In contrast, the *fzo1-1* strain failed to grow on glycerol at 37°C where the mitochondrial reticulum fragments and mtDNA is lost (Fig. 2 A, *fzo1-1*) (Hermann et al., 1998). As reported previously, this mtDNA loss was prevented when mitochondrial fission and fragmentation were blocked by introducing a *dnm1 $\Delta$*  mutation

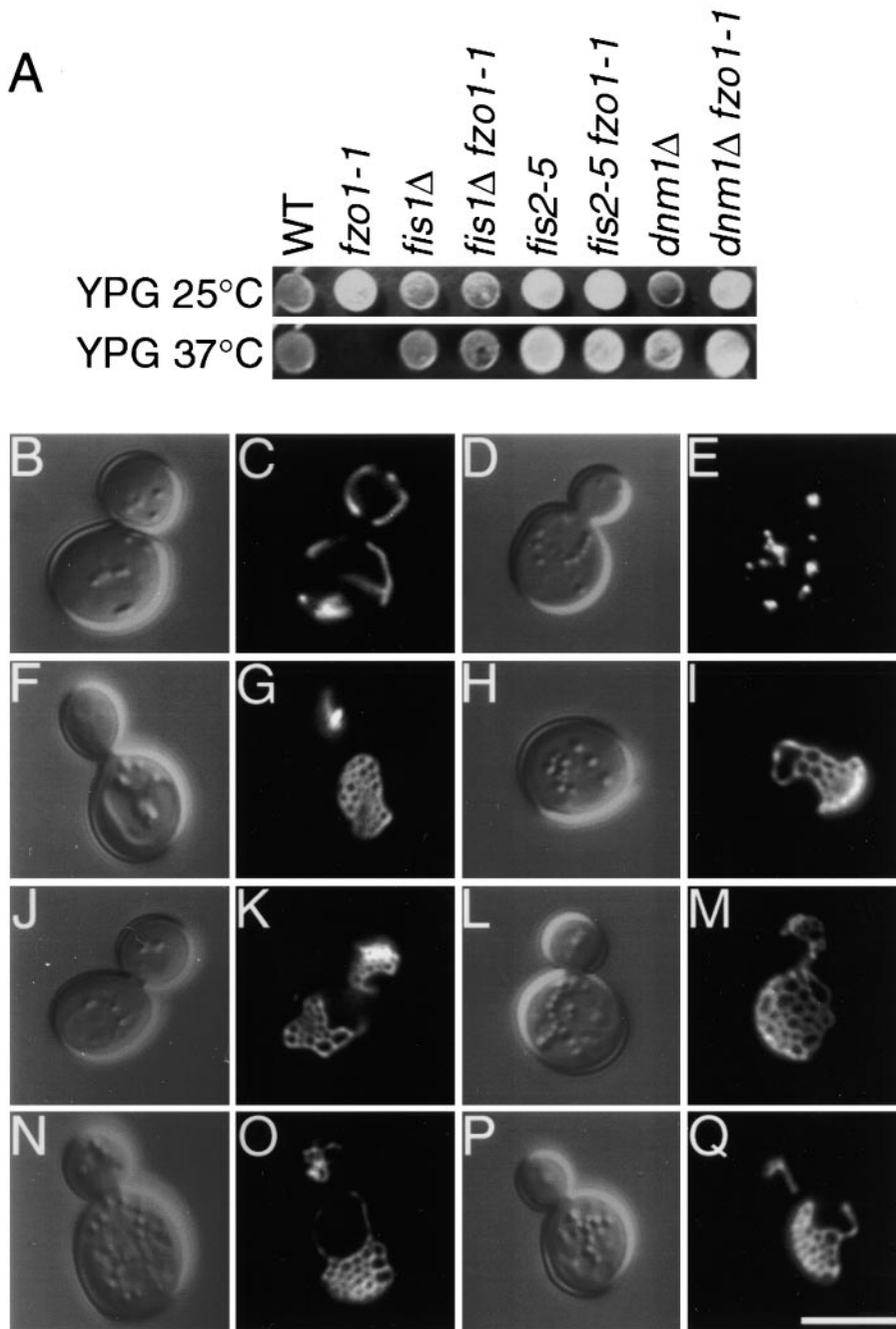
into *fzo1-1* (Fig. 2 A, *dnm1 $\Delta$  fzo1-1*) or *fzo1 $\Delta$*  cells (Bleazard et al., 1999). Although the single *fis1* and *fis2* mutations did not affect glycerol growth in an otherwise wild-type strain (Fig. 2 A, *fis1 $\Delta$* , *fis2-5*, both mutations suppressed the temperature-sensitive glycerol growth defect (Fig. 2 A, *fis1 $\Delta$  fzo1-1*, *fis2-5 fzo1-1*) and mtDNA loss defect (not shown) of *fzo1-1*. (Note that *fis2-5* is truncated after 191 of 714 amino acids and behaves like a null allele.)

### Mitochondrial Membranes Form Nets in *fis1* and *fis2* Mutant Strains

The morphology of mitochondrial membranes in *fis1* and *fis2* mutant strains was consistent with a role for these genes in fission. As shown previously (Bleazard et al., 1999), the wild-type mitochondrial reticulum (Fig. 2 C; Table II, 100% branched tubules) is converted into an interconnected “net” in *dnm1* mutant cells due to unopposed tip fusion (Fig. 2 O; Table II, 67% mitochondrial nets). Analysis of GFP-labeled mitochondria revealed that 85% of *fis1* (Fig. 2 G; Table II) and 77% of *fis2* (Fig. 2 K; Table II) mutant cells contained mitochondrial nets strikingly like those in *dnm1 $\Delta$* . These nets appeared identical when visualized with matrix-targeted, and inner and outer membrane-targeted forms of the red and green fluorescent proteins, indicating that fission of both the inner and outer membranes was abolished in these mutants (not shown). Mitochondrial nets were also observed in all combinations of *dnm1*, *fis1*, and *fis2* double and triple mutant strains (Table II and not shown), suggesting that *FIS1* and *FIS2* act in the same pathway as *DNM1* to regulate mitochondrial fission.

### *fis1* and *fis2* Mutations Block Mitochondrial Fission and Fragmentation in *fzo1-1*

We previously reported (Bleazard et al., 1999) that *dnm1* mutations blocked mitochondrial fission, fragmentation,



**Figure 2.** *fis1* and *fis2* mutations cause mitochondrial net formation and suppress glycerol growth defects and mitochondrial fragmentation in *fzo1-1*. (A) Wild-type (ADM548), *fzo1-1* (ADM547), *fis1Δ* (ADM549), *fis1Δ fzo1-1* (ADM550), *fis2-5* (ADM752), *fis2-5 fzo1-1* (ADM176), *dnm1Δ* (ADM379), and *dnm1Δ fzo1-1* (ADM378) cells were spotted on YPG-glycerol and grown at 25° or 37°C for 5 d. The presence/absence of mtDNA nucleoids in each strain was evaluated by DAPI staining (not shown). (B–Q) Morphology of mito-GFP-labeled (pVT100UGFP) mitochondria in (C) wild-type, (E) *fzo1-1*, (G) *fis1Δ*, (I) *fis1Δ fzo1-1*, (K) *fis2-5*, (M) *fis2-5 fzo1-1*, (O) *dnm1Δ*, and (Q) *dnm1Δ fzo1-1* cells grown at 37°C. The corresponding interference differential contrast images are shown in B, D, F, H, J, L, N, and P. Bar, 5 μm.

and mtDNA loss in *fzo1-1* cells at 37°C (compare *fzo1-1* in Fig. 2 E and *dnm1Δ fzo1-1* in Q; Table II). To determine whether *fis1* and *fis2* mutations also prevented mitochondrial fragmentation in *fzo1-1*, we examined mitochondrial morphology in *fis1Δ fzo1-1* and *fis2-5 fzo1-1* strains after a shift to the nonpermissive temperature. At 25°C, 80% of *fis1Δ fzo1-1* and 44% of *fis2-5 fzo1-1* cells contain mitochondrial nets similar to those observed in the single *fis1Δ* and *fis2-5* mutants at 25°, 30°, and 37°C (Table II). Upon shifting *fis1Δ fzo1-1* (Fig. 2 I; Table II) and *fis2-5 fzo1-1* (Fig. 2 M; Table II) cells to 37°C, mitochondrial fragmentation was blocked despite the absence of *FZO1* function and mitochondrial membranes remained net-like. These results were similar to those observed for the *fzo1-1 dnm1* double mutant (Bleazard et al., 1999), and indicated that loss of Fis1p or Fis2p function in the *fis1Δ fzo1-1* and *fis2-5*

*fzo1-1* double mutants blocked mitochondrial fission and fragmentation in *fzo1-1* cells at the nonpermissive temperature.

Because both *fis1Δ* and *fis2-5* are null alleles, suppression of the *fzo1-1* mitochondrial fragmentation, mtDNA loss and glycerol growth phenotypes cannot occur via direct interactions between Fzo1-1p and the *FIS1* and *FIS2* gene products. Indeed, the genetic interactions described above did not require expression of the Fzo1-1 protein, ruling out the possibility that *fis1* and *fis2* restore an Fzo1p-dependent fusion pathway. In *fzo1Δ fis1Δ* (Fig. 3, A, second row, and E) and *fzo1Δ fis2-5* (not shown), double mutant strains, the *fzo1Δ* glycerol growth defect was suppressed and mitochondrial membranes remained tubular, although the tubules sometimes appeared ragged or frayed (Table II). Interestingly, mitochondrial nets were

Table II. Mitochondrial-Membrane Morphology in *fis1* and *fis2* Mutant Cells

Strain	°C	No. cells scored	Branched tubules	Frayed/clumpy tubules	Collapsed tubules*	Mitochondrial nets	Fragmented mitochondria
		%	%	%	%	%	%
WT	30	300	100				
<i>fis1</i> Δ <sup>‡</sup>	30	500			15	85	
<i>fis2-5</i> <sup>‡</sup>	30	100	1		22	77	
<i>dnm1</i> Δ <sup>‡</sup>	30	200			33	67	
<i>fis1</i> Δ <i>fis2-5</i>	30	100			26	74	
<i>fis1</i> Δ <i>dnm1</i> Δ	30	200			20	80	
<i>fis2-5</i> Δ <i>dnm1</i> Δ	30	100			6	94	
<i>fis1</i> Δ <i>fis2-5</i> <i>dnm1</i> Δ	30	100			33	67	
WT	25	100	96	4			
WT	37	100	100				
<i>fzo1-1</i>	25	100	51	46			3
<i>fzo1-1</i> <sup>§</sup>	37	100		24			76
<i>fis1</i> Δ <i>fzo1-1</i>	25	100			20	80	
<i>fis1</i> Δ <i>fzo1-1</i> <sup>§</sup>	37	100			14	86	
<i>fis2-5</i> <i>fzo1-1</i>	25	100	15		41	44	
<i>fis2-5</i> <i>fzo1-1</i> <sup>§</sup>	37	100	9		47	41	3
<i>dnm1</i> Δ <i>fzo1-1</i>	25	100	7		29	64	
<i>dnm1</i> Δ <i>fzo1-1</i> <sup>§</sup>	37	100	5		29	66	
<i>fzo1</i> Δ	30	200		3			97
<i>fis1</i> Δ <i>fzo1</i> Δ	30	300	24	62	9	3	2
<i>mgm1</i> Δ	30	100		1			99
<i>fis1</i> Δ <i>mgm1</i> Δ	30	100	46	15			38

\*Collapsed tubules may contain nets that cannot be resolved microscopically (Otsuga et al., 1998).

<sup>‡</sup>Mitochondrial-membrane morphology is similar at 25°, 30°, and 37°C.

<sup>§</sup>Scored mitochondrial morphology in *fzo1-1* cells after 30 min at 37°C. WT, wild type.

largely absent when double mutants contained the *fzo1*Δ allele (Table II). This observation indicates that formation of mitochondrial nets in *fis1* and *fis2* mutant strains requires *FZO1*-mediated fusion.

### *dnm1*, *fis1* and *fis2* Mutations Are General Suppressors of Mitochondrial Fragmentation Defects

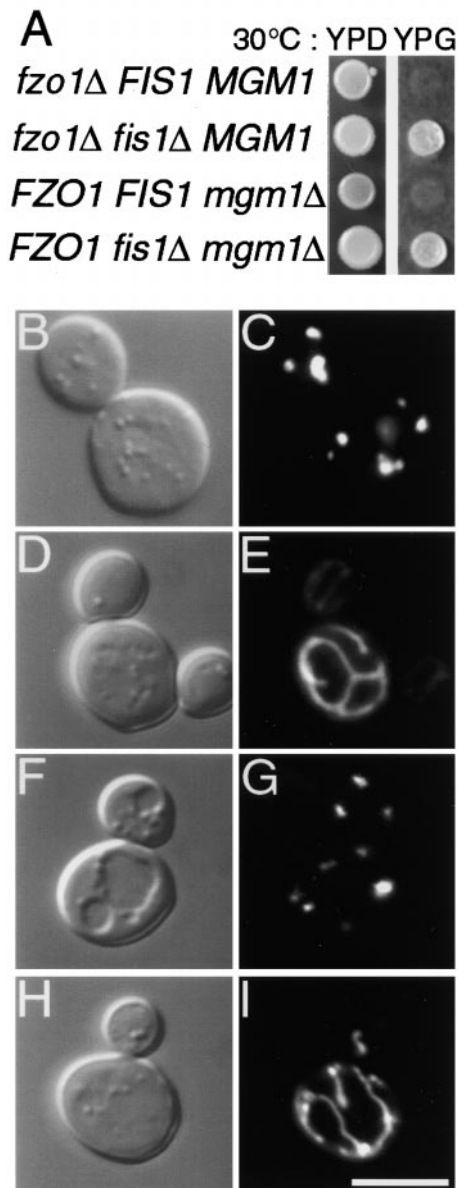
To determine whether the *dnm1*, *fis1*, and *fis2* mutations were specific suppressors of the *fzo1* fusion defect or were general suppressors of mitochondrial fragmentation defects, we examined the effect of *dnm1*, *fis1*, and *fis2* mutations in an *mgm1*Δ mutant (Jones and Fangman, 1992; Guan et al., 1993; Shepard and Yaffe, 1999). Cells with mutations in *MGM1* have fragmented mitochondrial membranes (Fig. 3 G), although the *mgm1* mutant does not block *FZO1*-mediated mitochondrial fusion (Wong et al., 2000). The results of these studies with the *fis1*Δ mutant are shown in Fig. 3 and Table II. As observed for the *fis1* Δ *fzo1* Δ double mutant, *fis1*Δ is able to suppress the *mgm1*Δ mtDNA loss (not shown), glycerol growth defect (Fig. 3 A, bottom two rows) and mitochondrial fragmentation defect (Fig. 3 I; Table II) in the *fis1*Δ *mgm1*Δ double mutant strain. Similar results were observed for the *mgm1*Δ *fis2-5* double mutant strains (not shown) and *mgm1*Δ *dnm1*Δ (Gorsich, S., and J.M. Shaw, unpublished data), consistent with the idea that *dnm1*, *fis1*, and *fis2* mutations prevent fragmentation of mitochondrial membranes by blocking the fission pathway.

### Mutations in *fis1* and *fis2* Do Not Restore Mitochondrial Fusion in *fzo1-1* Cells

The observation that *fis1* and *fis2* mutations block mitochondrial fragmentation in *fzo1-1* cells is consistent with a

role for Fis1p and Fis2p in mitochondrial fission. However, similar results could be obtained if mutations in *fis1* and *fis2* activated an *FZO1*-independent fusion pathway. To determine whether *fis1* and *fis2* mutations restored fusion in *fzo1-1*, we assayed mitochondrial fusion in zygotes formed from either *fis1*Δ *fzo1-1* or *fis2-5* *fzo1-1* double mutants. Fusion was assayed essentially as described by Nunnari et al. (1997) by labeling mitochondria in haploid cells of opposite mating type with either mito-GFP or -RFP (instead of MitoTracker Red CMXRos), and then examining the distribution of the two fluorescent markers in zygotes formed at the permissive and nonpermissive temperatures.

Both mito-GFP and -RFP colocalized in 100% of wild-type zygotes formed at 25° and 37°C, indicating that mitochondria from each haploid parent had fused and their contents had mixed (Table III) (Nunnari et al., 1997). Mitochondrial content mixing also occurred efficiently in *fis1* X *fis1*, *fis2* X *fis2*, and *dnm1* X *dnm1* zygotes formed at both temperatures, indicating that these three genes are not required for fusion (Table III). As reported previously, mitochondrial fusion occurred in *fzo1-1* X *fzo1-1* zygotes at 25°C, but fragmented and failed to fuse at 37°C, consistent with Fzo1p's role in fusion (Table III) (Hermann et al., 1998). Although disruption of *FIS1* or *FIS2* prevented mitochondrial fragmentation in *fzo1-1* zygotes at the nonpermissive temperature, fusion was not restored (Table III). These findings are similar to those reported for *DNM1* (Table III) (Bleazard et al., 1999) and indicate that the maintenance of mitochondrial tubules in *fis1*Δ *fzo1-1* and *fis2-5* *fzo1-1* cells results from a block in mitochondrial fission rather than the activation of an *FZO1*-independent fusion pathway. Together, the results presented here indicate that *FIS1* and *FIS2* act in the same pathway as *DNM1* to regulate mitochondrial fission.



**Figure 3.** *fis1Δ* acts as a bypass suppressor of mitochondrial fragmentation defects. (A) *fzo1Δ FIS1 MGM1* (ADM575), *fzo1Δ fis1Δ MGM1* (ADM574), *FZO1 FIS1 mgm1Δ* (ADM764), and *FZO1 fis1Δ mgm1Δ* (ADM763) strains were spotted on YPDextrose or YPGlycerol and grown at 30°C for 5 d, respectively. Morphology of mito-GFP-labeled (pVT100UGFP) mitochondria in (C) *fzo1Δ FIS1 MGM1*, (E) *fzo1Δ fis1Δ MGM1*, (G) *FZO1 FIS1 mgm1Δ*, and (I) *FZO1 fis1Δ mgm1Δ* cells grown at 30°C. The corresponding differential interference contrast images are shown in B, D, F, and H. Bar, 5 μm.

### *FIS1* Defines a Novel Gene Family

The *FIS1* gene (YIL065C in the *Saccharomyces* Genome Database) was cloned by library complementation of the glycerol growth suppression phenotype in *fis1 fzo1-1* strains. A fragment of the rescuing plasmid containing *FIS1*/YIL065C also rescued the mitochondrial morphology defects in a *fis1* mutant strain. Integrative mapping studies and DNA sequencing were used to show that the original *fis1* mutations were allelic to the YIL065C cloned

**Table III.** Mitochondrial Fusion in Budded Zygotes

Strains crossed	°C	No. Fused	Mitochondria %
<i>FIS1 FIS2 DNM1 FZO1 X</i>	25	35/35	100
<i>FIS1 FIS2 DNM1 FZO1</i>	37	76/76	100
<i>FIS1 FIS2 DNM1 fzo1-1 X</i>	25	24/30	80
<i>FIS1 FIS2 DNM1 fzo1-1</i>	37	0/55	0
<i>fis1Δ FIS2 DNM1 FZO1 X</i>	25	50/50	100
<i>fis1Δ FIS2 DNM1 FZO1</i>	37	75/75	100
<i>fis1Δ FIS2 DNM1 fzo1-1 X</i>	25	40/40	100
<i>fis1Δ FIS2 DNM1 fzo1-1</i>	37	0/77	0
<i>FIS1 fis2-5 DNM1 FZO1 X</i>	25	29/30	97
<i>FIS1 fis2-5 DNM1 fzo1 X</i>	37	29/30	97
with <i>pRS416 + FZO1*</i>			
<i>FIS1 fis2-5 DNM1 fzo1-1 X</i>	25	48/50	96
<i>FIS1 fis2-5 DNM1 fzo1-1</i>	37	0/50	0
<i>FIS1 FIS2 dnm1 Δ FZO1 X</i>	25	40/40	100
<i>FIS1 FIS2 dnm1 Δ FZO1</i>	37	50/50	100
<i>FIS1 FIS2 dnm1 Δ fzo1-1 X</i>	25	30/30	100
<i>FIS1 FIS2 dnm1 Δ fzo1-1</i>	37	0/35	0

\**pRS416 + FZO1* rescues *fzo1Δ* mitochondrial defects (Hermann et al., 1998).

open reading frame. *FIS1* encodes a novel protein of 155 amino acids with an estimated molecular mass of 17,000 D (Fig. 4). The only identifiable protein motif in the open reading frame is a single, predicted, COOH-terminal transmembrane domain (Fig. 4, TM). A database search identified Fis1p orthologs in fission yeast, humans, plants, worms, and flies, suggesting that Fis1p function has been conserved during evolution (Fig. 4). Whether these orthologs participate in membrane division events mediated by Dnm1p-like GTPases remains to be seen.

### *FIS1* Encodes an Integral, Outer Mitochondrial Membrane Protein with Its NH<sub>2</sub> Terminus Facing the Cytoplasm

The subcellular localization of Fis1p was investigated by fluorescence microscopy and cryoimmunoelectron microscopy using functional, amino-terminal GFP-tagged (GFP-Fis1p) and Myc-tagged (Myc-Fis1p) forms of the protein (see Materials and Methods; GFP-Fis1p and Myc-Fis1p were expressed at levels comparable with that of endogenous Fis1p in these experiments). When expressed in yeast, GFP-Fis1p and Myc-Fis1p rescued the mitochondrial net morphology defect in *fis1Δ* and the glycerol growth suppression phenotype in the *fis1Δ fzo1-1* double mutant.

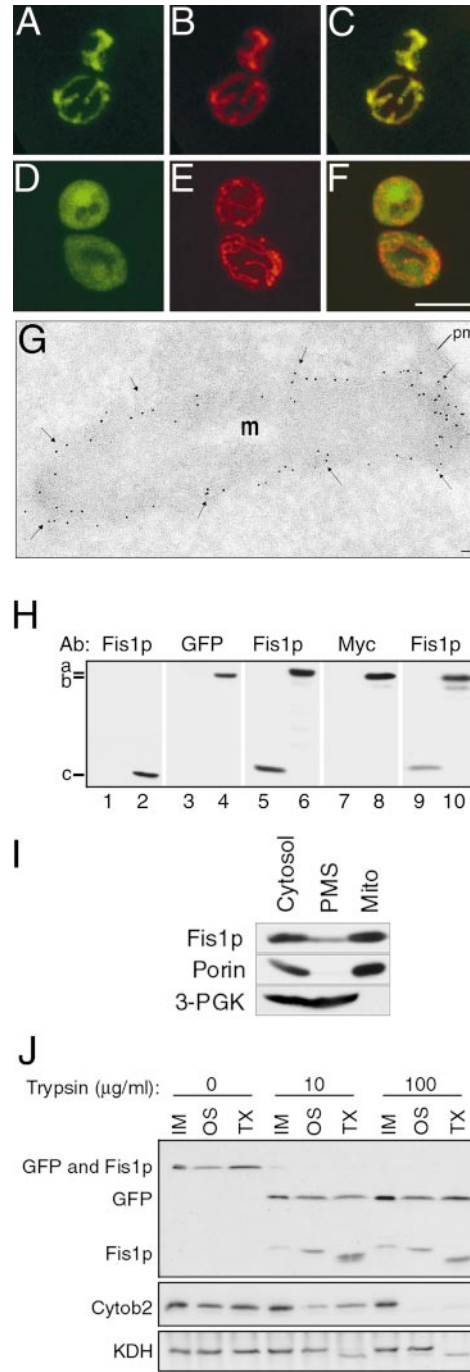
In dividing yeast cells, GFP-Fis1p colocalized completely with the MitoTracker red CMXRos-labeled mitochondrial reticulum (Fig. 5, A–C), indicating that Fis1p is a mitochondrial protein. The identical Fis1p localization pattern was observed in *dnm1Δ*, *fis2-5*, and *fis2-5 dnm1Δ* mutants, suggesting that Fis1p's localization is not determined or affected by other known components of the fission machinery (not shown). In addition, the predicted COOH-terminal transmembrane domain (Fig. 4) was required for Fis1p's mitochondrial localization and function. A GFP-Fis1p fusion protein lacking the transmembrane domain (GFP-Fis1p<sup>aa1-127</sup>) failed to colocalize with MitoTracker red CMXRos-labeled mitochondria (Fig. 5, D–F) and did not rescue *fis1* mitochondrial morphology defects or the glycerol growth suppression phenotype in a

Organism	Length	%ID	%SIM	
<i>S. cerevisiae</i>	155 aa	100	100	N ————— C
<i>S. pombe</i>	160 aa	30	61	————— ■
<i>H. sapiens</i>	152 aa	26	61	————— ■
<i>A. thaliana</i>	170 aa	25	50	————— ■
<i>C. elegans</i>	138 aa	28	52	————— ■
<i>C. elegans</i>	143 aa	20	50	————— ■
<i>D. melanogaster</i>	85 aa	20	36	————— ■
<i>S. mansoni</i>	142 aa	14	37	————— ■

**Figure 4.** Domain structure of the Fis1p Family. Domain structure of eight Fis1p family members with the predicted carboxy-terminal transmembrane domain shown in black (TM). The length in amino acids, percent sequence identity, and percent sequence similarity between the *Saccharomyces cerevisiae* predicted protein (NCBI RefSeq NP012199) and the predicted *Schizosaccharomyces pombe* (PIR T39328), *Caenorhabditis elegans* (T16053 and T16332), *Homo sapiens* (NP057152), *Arabidopsis thaliana* (CAB72179.1), *Schistosoma mansoni* (AAA99799), and *Drosophila melanogaster* (AAF45343) orthologs are indicated. The *D. melanogaster* and *S. mansoni* amino acid sequences may be incomplete. All sequences can be accessed through the NCBI database (<http://www.ncbi.nlm.nih.gov>). The Jotun Hein program (MegAlign, DNA\*) was used to construct pairwise alignments between the *S. cerevisiae* protein sequence and each Fis1p ortholog.

*fis1Δ fz01-1* strain (not shown). Carbonate and detergent extraction studies confirmed that Fis1p was an integral mitochondrial membrane protein (not shown). Interestingly, overexpression of cytoplasmic GFP-Fis1p<sup>aa1-127</sup> from a *MET25*-inducible promoter did not cause dominant mitochondrial morphology defects in wild-type cells, suggesting that this form of Fis1p is not competent to titrate essential components of the fission reaction (data not shown). Cryo-immunoelectron microscopy revealed that, unlike Dnm1p, which was found in discrete spots associated with mitochondrial constriction sites (Bleazard et al., 1999), Myc-Fis1p appeared to be distributed evenly on the surface of the mitochondrial compartment (Fig. 5 G; see H for anti-Myc detection of Myc-Fis1p). These localization studies were consistent with biochemical studies (de-

**Figure 5.** Fis1p is a mitochondrial membrane protein with its amino terminus exposed to the cytoplasm. (A–F) Wild-type (ADM551) cells expressing GFP-Fis1p (A–C; pRS415MET25 + rsGFP-*FIS1*<sup>aa1-155</sup>) or GFP-Fis1p lacking the COOH-terminal transmembrane domain (D–F; pRS415MET25 + rsGFP-*FIS1*<sup>aa1-127</sup>) were stained with MitoTracker red CMXRos (B, C, E, and F). Confocal microscopy was used to evaluate the distribution of the GFP fusion proteins (green) and mitochondrial compartments (red) in individual (A, B, D, and E) and merged (C and F) images. Bar, 5µm. (G) The distribution of Myc-Fis1p (black arrows) (ADM551; pRS415MET25 + 9xMYC-*FIS1*) was determined by immunogold labeling of ultrathin cryosections. 5-nm gold particles were found concentrated at the periphery of the mitochondrial compartment. Percentage distribution of 1,807 gold particles counted: mitochondrial tips 8.5%, mitochondrial sides (excluding constriction sites) 77.6%, mitochondrial constriction sites 12.5%, and cytoplasm 1.4%. m, mitochondria; pm, plasma membrane. Bar, 0.1 µm. (H) Total cell extracts from *fis1Δ* (lane 1, ADM549), wild-type (lane 2, ADM548), or *fis1Δ*



cells expressing either Fis1p (lanes 3, 5, 7, and 9, ADM549; pRS416 + *FIS1*), GFP-Fis1p (lanes 4 and 6, ADM549; pRS415MET25 + rsGFP-*FIS1*) or Myc-Fis1p proteins (lanes 8 and 10, ADM549; pRS415MET25 + 9xMYC-*FIS1*) were analyzed by Western blotting with anti-Fis1p, -GFP, and -Myc serum as indicated. a, the position of GFP-Fis1p (47 kD); b, Myc-Fis1p (35 kD); and c, native Fis1p (17 kD). (I) Wild-type protein extract was fractionated by differential centrifugation, separated by SDS-PAGE, and analyzed by Western blotting with anti-Fis1p, -porin, and -3-PGK serum. Cytosol, postnuclear cytoplasmic extract; PMS, supernatant depleted of mitochondria; Mito, mitochondrial pellet. (J) Untreated (IM), osmotically shocked (OS), and Triton X-100 solubilized (TX) mitochondrial fractions were treated with 0, 10, or 100 µg/ml Trypsin and analyzed by SDS-PAGE and Western blotting with anti-GFP, -Fis1p, -cytochrome b2, and -KDH serum. \*Protease-clipped form of KDH.

Table IV. Number of Dnm1p-GFP Complexes in Wild-Type and Mutant Strains

Strain	No. of cells scored	>8	4-8	1-3
		%	%	%
WT	100	97	3	0
<i>fis1Δ</i>	100	3	16	81
<i>fis2-5</i>	100	86	14	0
<i>fis1Δ fis2-5</i>	100	0	7	93
<i>dnm1Δ</i>	100	92	5	3
<i>fis1-21* fzo1-1</i>	100	61	32	7

\**fis1-21* is not a temperature-sensitive allele; mitochondrial morphology in *fis1-21* is identical at 25° and 37°C. WT, wild type.

scribed below) demonstrating that Fis1p was an outer mitochondrial membrane protein.

Subcellular fractionation experiments using anti-Fis1p and anti-GFP antibodies confirmed that Fis1p is a mitochondrial protein. Anti-Fis1p antibodies recognized a 17-kD protein in extracts prepared from *FIS1* wild-type but not *fis1Δ* cells (Fig. 5 H, compare 1 and 2, band c). Similarly, anti-GFP antibodies detected a 47-kD fusion protein in extracts prepared from *fis1Δ* cells expressing GFP-Fis1p but not in extracts prepared from *fis1Δ* cells expressing untagged Fis1p (Fig. 5 H, compare 3 and 4, band a). As shown in Fig. 4 I, native Fis1p cofractionated predominantly with

the mitochondrial pellet, along with the outer mitochondrial membrane protein porin, during differential centrifugation of a postnuclear cell extract. In extracts prepared from *fis1Δ* cells expressing GFP-Fis1p, GFP-Fis1p also cofractionated with porin, and not the cytoplasmic marker, 3-phosphoglycerate kinase (3-PGK; data not shown).

Protease protection experiments revealed that Fis1p is an outer mitochondrial membrane protein with its NH<sub>2</sub> terminus exposed to the cytoplasm. Anti-GFP and anti-Fis1p antibodies detected the full-length GFP-Fis1p fusion protein in untreated mitochondrial fractions (Fig. 5 J, 0 μg/ml Trypsin). Treatment of intact mitochondria with 10 or 100 μg/ml trypsin resulted in the clipping and release of the NH<sub>2</sub>-terminal, 29-kD GFP tag from Fis1p (Fig. 5 J, 10 and 100 μg/ml trypsin, IM; the linker region between GFP and Fis1p contains a predicted trypsin cleavage site). In the presence of 100 μg/ml trypsin, the intermembrane space protein cytochrome b<sub>2</sub> was not digested, indicating that the outer mitochondrial membrane remained intact (Fig. 5 J, 100 μg/ml trypsin, IM). When the outer membrane was disrupted by osmotic shock, however, cytochrome b<sub>2</sub> was degraded by trypsin, although the matrix marker alpha-ketoglutarate dehydrogenase (KDH) was still protected (Fig. 5 J, 100 μg/ml trypsin, OS). Protease clipping of KDH (\*) only occurred when mitochondria were solubilized with Triton X-100 in the presence of

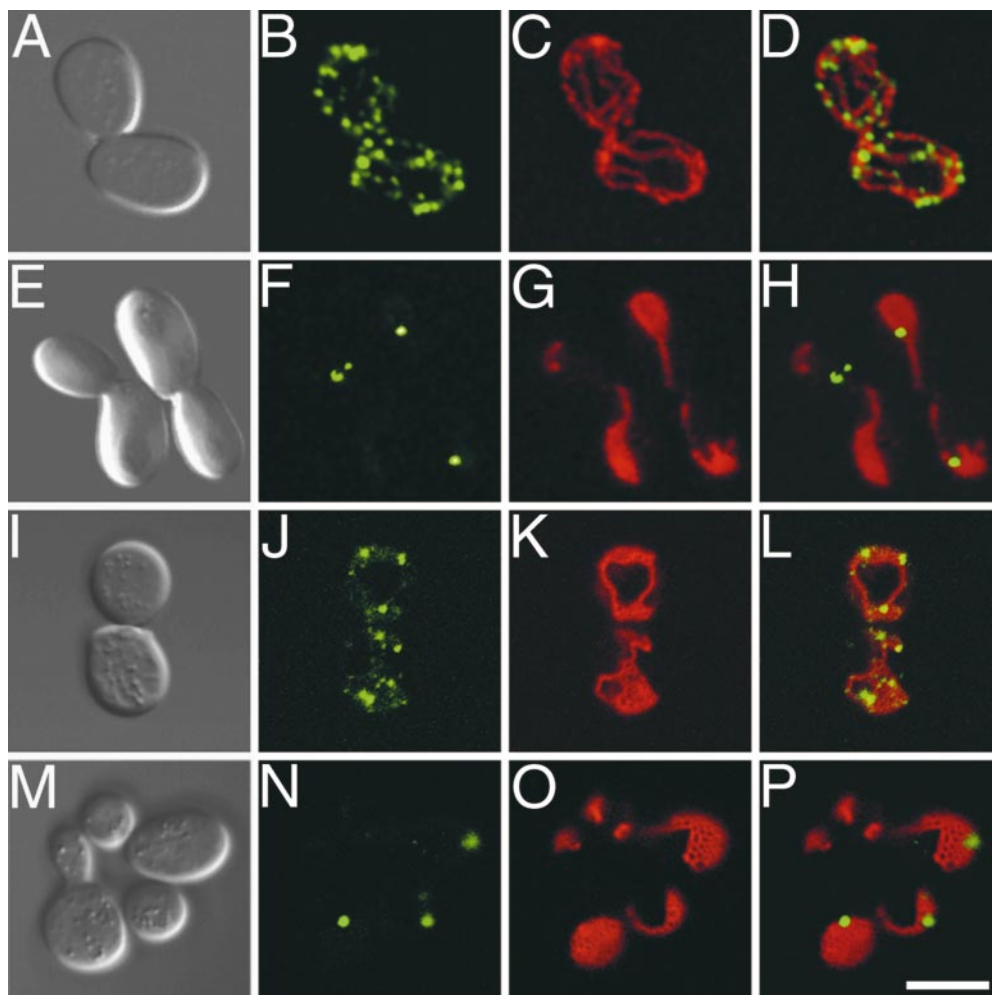


Figure 6. Dnm1-GFP localization requires *FIS1* but not *FIS2* function. (A, E, I, and M) differential interference contrast, (B, F, J, and N) Dnm1-GFP (pRS414-*DNM1*-rsGFP, pRS416-*DNM1*-rsGFP, or pHS20), (C, G, K, and O) mito-RFP (pRS416-*GAL1* + *PrF<sub>0</sub>ATP9*-RFP), and (D, H, L, and P) merged Dnm1-GFP/mito-RFP images of (A–D) wild-type (ADM548), (E–H) *fis1Δ* (ADM552), (I–L) *fis2-5* (ADM752), and (M–P) *fis1Δ fis2-5* (ADM742) cells grown at 30°C. Bar, 5 μm.



trypsin (Fig. 5 J, 100  $\mu$ g/ml trypsin, TX). Interestingly, significant degradation of Fis1p was only observed after treatment of mitochondria with detergent, suggesting that Fis1p's small size and outer membrane association affects its protease susceptibility (Fig. 5 J, note shifted and smeared Fis1p bands in TX lanes treated with trypsin). The fact that (a) the GFP tag is at the NH<sub>2</sub> terminus of Fis1p, (b) Fis1p has only one predicted transmembrane domain, and (c) there are only nine amino acids COOH-terminal of the transmembrane domain suggests that the COOH-terminal nine amino acids of Fis1p face the mitochondrial intermembrane space.

### Proper Assembly and Distribution of Dnm1p on the Outer Mitochondrial Membrane Requires Fis1p, but Not Fis2p Function

Two-hybrid, coimmunoprecipitation (Fukushima, N.H., B.R. Keegan, and J.M. Shaw. 1999. *Mol. Biol. Cell.* 10: 315a. [Abstr.]), and genetic studies (Mozdy and Shaw, unpublished observations) indicate that Dnm1p interacts with itself and probably functions as a multimer. Our previous work demonstrates that these Dnm1p multimer-containing complexes assemble on the cytoplasmic face of the outer mitochondrial membrane at sites of future constriction and fission (Otsuga et al., 1998; Bleazard et al., 1999). Both the *fis1* mitochondrial fission defect and the topology of Fis1p on the outer mitochondrial membrane raised the possibility that Fis1p was required to localize or activate the Dnm1p GTPase. To test this idea, we examined the localization of a Dnm1p-GFP fusion protein in the presence and absence of Fis1p. We also labeled mitochondria in the same cells with mito-RFP to evaluate colocalization of Dnm1p-GFP with mitochondrial membranes.

In 97% of wild-type cells and 92% of *dnm1* $\Delta$  cells examined, Dnm1p-GFP was organized in eight or more punctate structures that colocalized completely with wild-type mito-RFP-labeled networks (Fig. 6, A–D; Table IV). This pattern was consistent with the Dnm1p localization pattern determined previously by indirect immunofluorescence studies (Otsuga et al., 1998), immunogold labeling studies (Bleazard et al., 1999), and Dnm1p-GFP studies (Sesaki and Jensen, 1999). In contrast, 81% of cells lacking the *Fis1* protein contained only one to three of the Dnm1p-GFP structures and these structures often appeared larger than normal (Fig. 6, E–H, Table IV). This change in Dnm1p-GFP localization is not a secondary consequence of net formation since Dnm1p-GFP distribution

appears wild type in *fis2* mutants, which also contain mitochondrial nets (see below). Interestingly, the Dnm1p-GFP spots in *fis1* $\Delta$  cells colocalized with mitochondrial membranes in this mutant. However, these structures were not able to catalyze fission, as evidenced by the mitochondrial nets formed in the *fis1* $\Delta$  strain (Table II).

Although Dnm1p-GFP complexes colocalized with mitochondria in the absence of Fis1p, Fis1p was clearly required to properly distribute these complexes into punctate structures along mitochondrial tubules. Analysis of one *FIS1* allele, *fis1-21*, suggested that Fis1p may also be required for the function of these punctate Dnm1p-containing structures. The *fis1-21* allele blocked mitochondrial fission and fragmentation in a *fis1-21 fzo1-1* double mutant at 37°C (mitochondrial membranes remained tubular in 97% of the cells). However, the number and distribution of punctate Dnm1p-GFP structures on *fis1-21 fzo1-1* mitochondria appeared more wild type (Table IV). These data suggested that the rate or extent of fission by properly distributed Dnm1p complexes was impaired in the *fis1-21 fzo1-1* strain. Thus, while Fis1p plays a role in determining the punctate distribution of Dnm1p complexes on mitochondrial tubules, it is also required for Dnm1p complex function once that distribution is achieved.

Similar experiments demonstrated that wild-type Dnm1p-GFP distribution did not require *FIS2* gene function. In the majority (86%) of *fis2-5* cells, the number and distribution of Dnm1p-GFP structures was indistinguishable from wild type (Fig. 6, I–L; Table IV). Once again, these structures colocalized with *fis2-5* mitochondrial tubules and nets, suggesting that the *Fis2* protein was required for the function but not the localization of Dnm1p-containing complexes (see Discussion for more information about *Fis2p*). Finally, the number and distribution of Dnm1p-GFP structures in a *fis1* $\Delta$  *fis2-5* double mutant was similar to that in a *fis1* $\Delta$  single mutant, indicating that *fis1* is epistatic to *fis2* with respect to Dnm1p-GFP localization (Fig. 6, M–P). Thus, *Fis1p* apparently functions upstream of *Fis2p* to localize Dnm1p.

Immunogold labeling of ultrathin cryosections confirmed that Dnm1p localization was altered in the *fis1* mutant. In wild-type cells expressing the tagged Dnm1p-HA<sub>c</sub> protein, 89.3% of the 5-nm gold particles were associated with the tips and sides of mitochondrial tubules and with constriction sites in these tubules (Table V). These results are similar to those reported previously for Dnm1p-HA<sub>c</sub> localization (Bleazard et al., 1999). In contrast, in *fis1* $\Delta$

Table V. Distribution of Dnm1-HA<sub>c</sub> Protein in *FIS1* and *fis1* $\Delta$  Cells

Compartment	Dnm1p-HA in wild-type cells	Dnm1p-HA in <i>fis1</i> $\Delta$ cells
	% total gold particles, n = 531	% total gold particles, n = 121
Mitochondrial tips	38.4	6.2
Mitochondrial sides*	15.4	1.8
Mitochondrial constriction sites	35.5	0
Mitochondrial other <sup>‡</sup>	1.1	0
Mitochondrial total	90.4	8.0
Plasma membrane	2.2	0
Cytoplasm	7.5	92.0
Vacuole	0	0

\*Excluding constriction sites.

<sup>‡</sup>Mitochondrial boundary unclear.

cells, only 8% of gold particles were found on the tips and sides of mitochondrial tubules, while the majority (92%) were found in the cytoplasm (Table V). Quantitative Western blot analysis indicated that this difference was not due to reduced Dnm1p expression in *fis1Δ* relative to *FIS1* wild type (not shown). Rather, in *fis1Δ* cells, the total amount of Dnm1p associated with mitochondria appeared to be reduced, and the distribution of this mitochondrial-associated Dnm1p appeared to be altered.

## Discussion

Our previous studies established that the *S. cerevisiae* dynamin-related GTPase, Dnm1p, associates with the outer mitochondrial membrane at sites of future constriction and fission (Otsuga et al., 1998; Bleazard et al., 1999). Here we provide genetic and morphological evidence that *FIS1* and *FIS2* encode two additional components of the Dnm1p fission apparatus. Like *dnm1* (Bleazard et al., 1999), *fis1* and *fis2* mutations abolish fission and suppress fragmentation in the *fzo1-1* strain. This suppression occurs via a bypass mechanism since *fis1Δ* and *fis2-5* (null) alleles prevent fragmentation in an *fzo1Δ* strain and in *mgm1Δ* cells (Fig. 3). Fis1p is a novel outer mitochondrial membrane protein essential for the proper distribution and function of the Dnm1p GTPase during the fission reaction. Fis1p is, to our knowledge, the first integral membrane protein known to function with a dynamin-related GTPase in a membrane fission event. In contrast, the *FIS2* gene product is required for the function, but not the mitochondrial association and distribution, of Dnm1p.

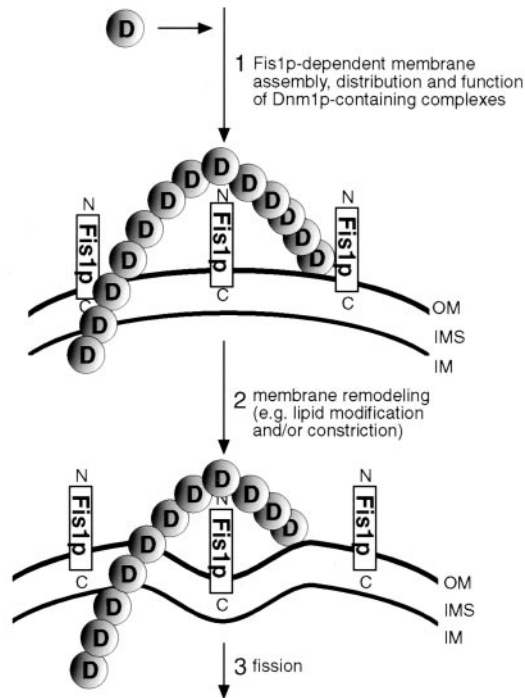
The results described here raised the possibility that Fis1p functions early in the fission pathway to recruit Dnm1p or Dnm1p-containing complexes to the mitochondrial membrane. Although we do not exclude such a function for Fis1p, several lines of evidence argue against this model. First, we have been unable to detect interactions of Fis1p with Dnm1p using coimmunoprecipitation and yeast two-hybrid studies (not shown). It is possible that such interactions are simply too transient to detect by these methods. Alternatively, one or more additional molecules may serve as a bridge between Fis1p and its binding partner(s). It is also possible that the methods we used failed to mimic a guanine nucleotide-bound state of Dnm1p required for Fis1p binding interactions. Second, we found that one to three large structures containing Dnm1p-GFP continued to colocalize with mitochondria in the absence of Fis1p (Fig. 6). This result suggests that Dnm1p contains Fis1p-independent signals for protein or lipid binding that mediate its mitochondrial attachment. A number of previously identified Dnm1p structural domains might function in this manner, including the middle domain, insert B, or the alpha-helical domain (van der Blik, 1999). Third, one of our *FIS1* alleles, *fis1-21*, interferes with fission and suppresses fragmentation in *fzo1-1* but does not completely disrupt the mitochondrial distribution of Dnm1p-GFP (Table IV). This last observation is consistent with the idea that Fis1p plays a role in the activation or function of Dnm1p complexes after they have assembled on the outer mitochondrial membrane. Interestingly, we recently detected Fis1p self-interactions using the two-hybrid assay. We are currently exploring the possibility that Fis1p self-

interactions are required for Dnm1p mitochondrial distribution or function.

Even though punctate Dnm1p-GFP complexes are properly distributed in *fis1-21 fzo1-1* cells (Table IV), mitochondrial fragmentation is still blocked in this double mutant at 37°C (97% tubular mitochondria), suggesting that the ability of these complexes to catalyze fission is impaired. Thus, Fis1p may be required (a) to distribute Dnm1p complexes on mitochondrial tubules and (b) to activate the fission activity of Dnm1p complexes. One intriguing possibility is that Fis1p functions late in the fission pathway (after mitochondrial recruitment of Dnm1p complexes) as an effector molecule to regulate some aspect of the Dnm1p GTPase cycle. Preliminary studies of wild-type Dnm1p-GFP localization in the presence of a dominant *dnm1-T62A* allele (Otsuga et al., 1998) are consistent with this idea. The *dnm1-T62A* mutation is equivalent to a mutation in the ras GTPase that is predicted to abolish effector binding (Vojtek et al., 1993; Adari et al., 1988; Cales et al., 1988). In *FIS1* wild-type cells, over-expressed Dnm1-T62A protein interacts with wild-type Dnm1p-GFP, causing the wild-type protein to redistribute into one to three large structures on the mitochondrial compartment (not shown). This altered Dnm1p-GFP localization pattern is similar to that observed in the *fis1Δ* mutant, suggesting that the *fis1Δ* and *dnm1-T62A* mutations affect the same step in Dnm1p assembly, distribution, or activation.

A related study reported the cloning and characterization of the *FIS2* gene, which they named *MDV1* for mitochondrial division (Tieu and Nunnari, 2000). *MDV1* encodes an 80-kD protein containing seven COOH-terminal WD-40 repeats. Together, these WD-40 repeats are predicted to form a tertiary structure called the  $\beta$  propeller that has the potential to interact with multiple protein-binding partners (Smith et al., 1999). Several lines of evidence suggest that Mdv1p and the Dnm1p GTPase interact (Tieu and Nunnari, 2000). In genetic crosses, nonallelic noncomplementation is observed between specific *MDV1* and *DNM1* alleles. In addition, fluorescence microscopy studies reveal that Mdv1p and Dnm1p colocalize in punctate structures on the cytoplasmic face of mitochondrial tubules. Finally, Mdv1p and Dnm1p have been shown to interact in a yeast two-hybrid assay (Tieu and Nunnari, 2000; Uetz et al., 2000). Although Mdv1p is not necessary for the proper localization of Dnm1p, Dnm1p is required for the proper mitochondrial distribution of Mdv1p (Tieu and Nunnari, 2000). In cells lacking Dnm1p, Mdv1p is uniformly dispersed on the outer mitochondrial membrane (Tieu and Nunnari, 2000), similar to the localization pattern we observe for Fis1p (Fig. 5 A). This observation suggests that Mdv1p contains signals that allow targeting to the mitochondrial compartment in the absence of Dnm1p. In cells lacking Fis1p, Mdv1p continues to colocalize with Dnm1p in one to three large structures associated with mitochondria (see Results, and Tieu and Nunnari, 2000). Thus, while Fis1p is required for proper mitochondrial distribution of Dnm1p, Mdv1p's association with Dnm1p is unaffected by Fis1p. In the absence of both Dnm1p and Fis1p, Mdv1p is cytoplasmic, indicating that Fis1p is also required for the mitochondrial recruitment of Mdv1p. These combined results suggest that Mdv1p and Dnm1p bind to the mitochondrial outer membrane as a complex,

**D** = Dnm1p-containing complexes



**Figure 7.** Model for mitochondrial constriction and fission mediated by Dnm1p-containing complexes. In response to an unknown signal(s), fission complexes containing the Dnm1p GTPase and probably Mdv1p are assembled in a Fis1p-dependent manner on the outer mitochondrial membrane (step 1). Dnm1p-containing fission complexes may assemble into higher-order structures, possibly forming rings or collars around the outer surface of mitochondrial tubules, similar to structures formed by dynamin during endocytosis (rings/collars are visualized as punctate structures in intact yeast cells). Dnm1p-containing complexes could play a role in the mechanical constriction and fission of mitochondrial tubules, or, alternatively, these complexes might recruit or activate additional components that mediate constriction and fission (steps 2 and 3). Although both the inner and outer mitochondrial membranes are shown in cross section, Dnm1p-containing fission complexes are predicted to contact only the outer membrane. OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

or that Dnm1p binds to mitochondria first and Mdv1p is a late recruit to Dnm1p-containing structures. In both cases, association of Mdv1p with Dnm1p appears to be required for the function of Dnm1p-containing complexes during fission.

The data presented here and in the accompanying study by Tieu and Nunnari (2000) suggest that mitochondrial fission is a multi-step process requiring functional Dnm1p GTPase, its putative binding partner Mdv1p, and the integral, outer membrane protein, Fis1p. The results of our studies indicate that Fis1p is involved in the assembly, spatial distribution, and function of Dnm1p-containing fission complexes on the outer mitochondrial membrane (Fig. 7, step 1). Based on the results of Tieu and Nunnari (2000), these Dnm1p-containing complexes also contain Mdv1p, although the stoichiometry of this interaction is not

known. We are currently determining whether Dnm1p or Mdv1p bind directly or indirectly to Fis1p in the mitochondrial membrane. The Dnm1p-containing fission complexes may polymerize to form rings or collars surrounding the mitochondrial outer membrane (visualized as punctate structures on mitochondria; Fig. 7, step 1), similar to the collars formed at the base of clathrin-coated pits by the dynamin GTPase (Takei et al., 1995). Formation of such a structure could coincide with, or subsequently result in, membrane constriction and fission. Alternatively, Dnm1p might be required to recruit or activate additional components that mediate constriction and fission (Fig. 7, steps 2 and 3).

Whether or not this model is correct, it seems likely that Dnm1p's GTPase cycle will play an important role in regulating one or more steps in the division process. For example, GTP binding or hydrolysis by Dnm1p might regulate: (a) self assembly into a complex with itself or other proteins, (b) its association with the outer mitochondrial membrane, (c) the assembly of Dnm1p complexes into higher order structures, or (d) the recruitment of additional components to the site of fission. A model in which GTP hydrolysis by Dnm1p provides mechanical energy for membrane constriction and fission cannot yet be excluded. It is likely that lipid modification activities are also required to remodel the outer mitochondrial membrane during constriction and fission, similar to endophilin's proposed activity during endocytosis (Ringstad et al., 1999; Schmidt et al., 1999). Future studies should identify any additional activities of Dnm1p, Fis1p, and Mdv1p as well as new proteins required for outer membrane division.

Although molecules required for outer mitochondrial membrane fission have been identified in a number of organisms (Otsuga et al., 1998; Smirnova et al., 1998; Bleazard et al., 1999; Labrousse et al., 1999; Sesaki and Jensen, 1999), essentially nothing is known about molecules that mediate inner mitochondrial membrane fission. Recently, however, another dynamin-related GTPase in budding yeast, called Mgm1p, was shown to function in mitochondrial membrane dynamics (Jones and Fangman, 1992; Guan et al., 1993; Shepard and Yaffe, 1999; Wong et al., 2000). The Mgm1p GTPase localizes to the mitochondrial intermembrane space and associates with the inner membrane, suggesting that it may play a role analogous to Dnm1p in inner membrane fission (Wong et al., 2000). If this model is correct, there may be structural or functional homologues of Mdv1p and Fis1p that work together with Mgm1p to regulate fission of the mitochondrial inner membrane. It seems likely that at least some of the molecules required for outer and inner membrane fission will also play a role in coordinating the behavior of both membranes during division. The integral outer membrane protein, Fis1p, is one potential candidate, since its COOH terminus probably extends into the intermembrane space and is exposed to the inner mitochondrial membrane. We are currently determining the submitochondrial topology of the Fis1p COOH terminus and its role in mitochondrial fission.

Fis1p and Mdv1p are new additions to the list of players that regulate division of prokaryotic cells and eukaryotic organelles of prokaryotic origin, including mitochondria and chloroplasts. *E. coli* cell division is mediated by the

FtsZ GTPase that forms a ring on the cytoplasmic face of the inner membrane and constricts during cytokinesis (Rothfield et al., 1991; Bramhill, 1997; Lutkenhaus and Addinall, 1997; Nanninga, 1998; Erickson, 2000). In this case, an integral inner membrane protein called ZipA binds directly to FtsZ and is thought to be required for the function of the FtsZ ring (Hale and de Boer, 1997). In the chloroplast, two different FtsZ homologues, FtsZ1 and FtsZ2, form rings at the organelle midpoint and coordinate division of the inner and outer membranes, respectively (Osteryoung and Vierling, 1995; Osteryoung and Pyke, 1998; Osteryoung et al., 1998). FtsZ homologues have not been identified in higher eukaryotes, although FtsZ homologues that localize to mitochondria have been identified in unicellular alga (Beech and Gilson, 2000; Beech et al., 2000). During mitochondrial division in higher eukaryotes, the role of the FtsZ GTPase appears to have been replaced by dynamin-related GTPases like *S. cerevisiae* Dnm1p. Homologues of the Dnm1p GTPase have been identified in a wide range of organisms including humans (Smirnova et al., 1998) and worms (Labrousse et al., 1999), and a role for the *Caenorhabditis elegans* Dnm1p counterpart, DRP-1, in outer mitochondrial membrane fission has been established (Labrousse et al., 1999). Although homologues of Mdv1p have not yet been identified (Tieu and Nunnari, 2000), the fact that *S. cerevisiae* Fis1p defines a novel family of outer mitochondrial membrane proteins conserved in humans, plants, worms, and flies (Fig. 4) strongly suggests that the molecular machinery regulating the Dnm1p-mediated type of mitochondrial fission has been conserved during eukaryotic evolution.

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## References

- Adari, H., D.R. Lowy, B.M. Willumsen, C.J. Der, and F. McCormick. 1988. Guanosine triphosphatase activating protein (GAP) interacts with the p21 ras effector binding domain. *Science*. 240:518–521.
- Baudin, A., O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, and C. Cullin. 1993. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 21:3329–3330.
- Beech, P.L., and P.R. Gilson. 2000. FtsZ and organelle division in Protists. *Protist*. 151:11–16.
- Beech, P.L., T. Nheu, T. Schultz, S. Herbert, T. Lithgow, P.R. Gilson, and G.I. McFadden. 2000. Mitochondrial FtsZ in a chromophyte alga. *Science*. 287:1276–1279.
- Bleazard, W., J.M. McCaffery, E.J. King, S. Bale, A. Mozdy, Q. Tieu, J. Nunnari, and J.M. Shaw. 1999. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat. Cell Biol.* 1:298–304.
- Bramhill, D. 1997. Bacterial cell division. *Annu. Rev. Cell Dev. Biol.* 13:395–424.
- Cales, C., J.F. Hancock, C.J. Marshall, and A. Hall. 1988. The cytoplasmic protein GAP is implicated as the target for regulation by the ras gene product.

- Nature*. 332:548–551.
- Erickson, H.P. 2000. Dynamin and FtsZ. Missing links in mitochondrial and bacterial division. *J. Cell Biol.* 148:1103–1105.
- Gammie, A.E., L.J. Kurihara, R.B. Vallee, and M.D. Rose. 1995. DNMI1, a dynamin-related gene, participates in endosomal trafficking in yeast. *J. Cell Biol.* 130:553–566.
- Guan, K., L. Farh, T.K. Marshall, and R.J. Deschenes. 1993. Normal mitochondrial structure and genome maintenance in yeast requires the dynamin-like product of the MGM1 gene. *Curr. Genet.* 24:141–148.
- Guthrie, C., and G.R. Fink. 1991. Guide to yeast genetics and molecular biology. In *Methods in Enzymology*. Vol. 194. J.N. Abelson and M.I. Simon, editors. Academic Press, Inc., San Diego, CA. pp. 1–933.
- Hale, C.A., and P.A. de Boer. 1997. Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*. *Cell*. 88:175–185.
- Hales, K.G., and M.T. Fuller. 1997. Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell*. 90:121–129.
- Hermann, G.J., and J.M. Shaw. 1998. Mitochondrial dynamics in yeast. *Annu. Rev. Cell Dev. Biol.* 14:265–303.
- Hermann, G.J., J.W. Thatcher, J.P. Mills, K.G. Hales, M.T. Fuller, J. Nunnari, and J.M. Shaw. 1998. Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. *J. Cell Biol.* 143:359–373.
- Hoffman, H., and C.J. Avers. 1973. Mitochondrion of yeast: ultrastructural evidence for one giant, branched organelle per cell. *Science*. 181:749–751.
- Jones, B.A., and W.L. Fangman. 1992. Mitochondrial DNA maintenance in yeast requires a protein containing a region related to the GTP-binding domain of dynamin. *Genes Dev.* 6:380–389.
- Labrousse, A.M., M.D. Zappaterra, D.A. Ruble, and A.M. van der Bliek. 1999. *C. elegans* dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. *Mol. Cell*. 4:815–826.
- Lagosky, P.A., G.R. Taylor, and R.H. Haynes. 1987. Molecular characterization of the *Saccharomyces cerevisiae* dihydrofolate reductase gene (DFR1). *Nucleic Acids Res.* 15:10355–10371.
- Lutkenhaus, J., and S.G. Addinall. 1997. Bacterial cell division and the Z ring. *Annu. Rev. Biochem.* 66:93–116.
- Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
- Nanninga, N. 1998. Morphogenesis of *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* 62:110–129.
- Nunnari, J., W.F. Marshall, A. Straight, A. Murray, J.W. Sedat, and P. Walter. 1997. Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol. Biol. Cell*. 8:1233–1242.
- Osteryoung, K., and K. Pyke. 1998. Plastid division: evidence for a prokaryotically derived mechanism. *Curr. Opin. Plant Biol.* 1:475–479.
- Osteryoung, K.W., K.D. Stokes, S.M. Rutherford, A.L. Percival, and W.Y. Lee. 1998. Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial ftsZ. *Plant Cell*. 10:1991–2004.
- Osteryoung, K.W., and E. Vierling. 1995. Conserved cell and organelle division. *Nature*. 376:473–474.
- Otsuga, D., B.R. Keegan, E. Brisch, J.W. Thatcher, G.J. Hermann, W. Bleazard, and J.M. Shaw. 1998. The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. *J. Cell Biol.* 143:333–349.
- Pringle, J.R., A.E.M. Adams, D.G. Drubin, and B.K. Haarer. 1991. Immunofluorescence methods for yeast. *Methods Enzymol.* 194:565–602.
- Rapaport, D., M. Brunner, W. Neupert, and B. Westermann. 1998. Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273:20150–20155.
- Rieder, S.E., L.M. Banta, K. Köhrer, J.M. McCaffery, and S.E. Emr. 1996. Multilamellar endosome-like compartment accumulates in the yeast vps28 vacuolar protein sorting mutant. *Mol. Biol. Cell*. 7:985–999.
- Ringstad, N., H. Gad, P. Low, G. Di Paolo, L. Brodin, O. Shupliakov, and P. De Camilli. 1999. Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. *Neuron*. 24:143–154.
- Rothfield, L.I., W.R. Cook, and P.A. de Boer. 1991. Biogenesis of the *Escherichia coli* cell division system. *Cold Spring Harbor Symp. Quant. Biol.* 56:751–756.
- Schmidt, A., M. Wolde, C. Thiele, W. Fest, H. Kratzin, A.V. Podtelejnikov, W. Witke, W.B. Huttner, and H.D. Soling. 1999. Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. *Nature*. 401:133–141.
- Sesaki, H., and R.E. Jensen. 1999. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J. Cell Biol.* 147:699–706.
- Shepard, K.A., and M.P. Yaffe. 1999. The yeast dynamin-like protein, Mgm1p, functions on the mitochondrial outer membrane to mediate mitochondrial inheritance. *J. Cell Biol.* 144:711–720.
- Sherman, F., G.R. Fink, and J.B. Hicks. 1986. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 186 pp.
- Smirnova, E., D.-L. Shurland, S.M. Ryazantsev, and A.M. van der Bliek. 1998. A human dynamin-related protein controls the distribution of mitochondria. *J. Cell Biol.* 143:351–358.

- Smith, T.F., C. Gaitatzes, K. Saxena, and E.J. Neer. 1999. The WD repeat: a common architecture for diverse functions. *Trends Biochem. Sci.* 24:181–185.
- Stevens, B.J. 1977. Variation in number and volume of mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphics reconstruction. *Biol. Cell.* 28:37–56.
- Takei, K., P.S. McPherson, S.L. Schmid, and P. De Camilli. 1995. Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. *Nature.* 374:186–190.
- Tieu, Q., and J. Nunnari. 2000. A novel WD protein, Mdv1p, interacts with the dynamin-related GTPase, Dnm1p, to trigger mitochondrial fission in yeast. *J. Cell Biol.* 151:353–365.
- Uetz, P., L. Giot, G. Cagney, T.A. Mansfield, R.S. Judson, J.R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, et al. 2000. A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature.* 403:623–627.
- van der Blik, A.M. 1999. Functional diversity in the dynamin family. *Trends Cell Biol.* 9:96–102.
- Vojtek, A.B., S.M. Hollenberg, and J.A. Cooper. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell.* 74:205–214.
- Winston, F., C. Dollard, and S.L. Ricupero-Hovasse. 1995. Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S228C. *Yeast.* 11:53–55.
- Wong, E.D., J.A. Wagner, S. Gorsich, J.M. McCaffery, J.M. Shaw, and J. Nunnari. 2000. The dynamin-related GTPase, Mgm1p, is a mitochondrial intermembrane space protein required for maintenance of fusion competent mitochondria. *J. Cell Biol.* 151:341–352.