UGO1 Encodes an Outer Membrane Protein Required for Mitochondrial Fusion

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Abstract. Membrane fusion plays an important role in controlling the shape, number, and distribution of mitochondria. In the yeast *Saccharomyces cerevisiae*, the outer membrane protein Fzo1p has been shown to mediate mitochondrial fusion. Using a novel genetic screen, we have isolated new mutants defective in the fusion of their mitochondria. One of these mutants, *ugo1*, shows several similarities to *fzo1* mutants. *ugo1* cells contain numerous mitochondrial fragments instead of the few long, tubular organelles seen in wild-type cells. *ugo1* mutants lose mitochondrial DNA (mtDNA). In zygotes formed by mating two *ugo1* cells, mitochondria do not fuse and mix their matrix contents. Fragmentation of mitochondria and loss of mtDNA in *ugo1* mutants are rescued by disrupting *DNM1*, a gene required for mitochondrial division. We find that *UGO1* encodes a 58-kD protein located in the mitochondrial outer membrane. Ugo1p appears to contain a single transmembrane segment, with its NH_2 terminus facing the cytosol and its COOH terminus in the intermembrane space. Our results suggest that Ugo1p is a new outer membrane component of the mitochondrial fusion machinery.

Key words: mitochondria • organelle dynamics • membrane fusion • outer membrane • yeast

Introduction

Mitochondrial fusion is a fundamental process required for establishing and maintaining the specialized shapes and numbers of mitochondria in many cell types (Tyler, 1992; Bereiter-Hahn and Voth, 1994). In the yeast *Saccharomyces cerevisiae*, mitochondrial fusion and its opposite activity, division, are highly regulated during growth, mating, and sporulation (Hermann and Shaw, 1998; Yaffe, 1999; Jensen et al., 2000). During vegetative growth, mitochondria constitutively fuse, divide (Nunnari et al., 1997), and change their number depending on growth conditions (Stevens, 1977). For instance, exponentially growing cells contain several branched, tubular mitochondria. When cells enter stationary phase, the mitochondrial tubules fragment into numerous small organelles (Hoffman and Avers, 1973; Stevens, 1977). When yeast cells mate, mitochondria fuse immediately after cell fusion, mixing their contents, including mitochondrial DNA (mtDNA)[1] and proteins (Thomas and Wilkie, 1968; Dujon, 1981; Nunnari et al., 1997; Okamoto et al., 1998). When diploids go through meiosis and sporulation, mitochondria undergo several fusion and division events, eventually encircling each of the four haploid nuclei (Miyakawa et al., 1984).

Mitochondrial fusion in yeast requires the Fzo1 protein (Hermann et al., 1998; Rapaport et al., 1998). Fzo1p was identified as a homologue to the *Drosophila* fuzzy onions protein, which is required for mitochondrial fusion during fly spermatogenesis (Hales and Fuller, 1997). Fzo1p is a mitochondrial outer membrane protein with a cytosolic GTPase domain at its NH_2 terminus (Hermann et al., 1998; Rapaport et al., 1998). In *FZO1* disruption mutants, cells contain many small mitochondrial fragments instead of the few tubular mitochondria seen in wild-type cells (Hermann et al., 1998; Rapaport et al., 1998), and a defect in mitochondrial fusion in *fzo1* mutants has been directly demonstrated using a mating assay (Hermann et al., 1998). In addition to fusion, Fzo1p is also important for maintenance of mtDNA. *fzo1* mutants lack mtDNA, but the mechanism by which mtDNA is lost in *fzo1* cells is not understood (Hermann et al., 1998; Rapaport et al., 1998).

The fragmentation of mitochondria in *fzo1* mutants depends on mitochondrial division. Dnm1p is a dynamin-related GTPase (Gammie et al., 1995; Otsuga et al., 1998) and *dnm1* mutants are defective in mitochondrial division (Bleazard et al., 1999; Sesaki and Jensen, 1999). Cells disrupted for *DNM1* contain a single mitochondrion consisting of a network of interconnected tubules. In *dnm1 fzo1*

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[1]*Abbreviations used in this paper:* CFP, cyan fluorescent protein; DIC, differential interference contrast; 5FOA, 5-fluoro-orotic acid; GFP, green fluorescent protein; HA, hemagglutinin; IMS, intermembrane space; mtDNA, mitochondrial DNA; RFP, red fluorescent protein.

double mutants, normal tubular-shaped mitochondria are seen, suggesting that mitochondrial shape and number is normally controlled, at least in part, by a balance between division and fusion mediated by Dnm1p and Fzo1p, respectively (Sesaki and Jensen, 1999). Like fragmentation, the loss of mtDNA in *fzo1* mutants requires Dnm1p and *fzo1 dnm1* double mutants to maintain mtDNA (Bleazard et al., 1999; Sesaki and Jensen, 1999; Jensen et al., 2000).

In this report, we have used a novel genetic screen to isolate new yeast mutants defective in mitochondrial fusion. We show that one of these mutants, *ugo1*, identifies a new mitochondrial outer membrane protein required for the fusion of mitochondria.

Materials and Methods

Strains, Media, and Genetic Methods

Yeast strains used in this study are listed in Table I. Yeast media, including YEPD (yeast-extract peptone [YEP] medium containing 2% glucose), YEPGE (YEP medium containing 2% glycerol and 2% ethanol), YEPGal (YEP medium containing 2% galactose), SD (synthetic medium containing 2% glucose), SRaf (synthetic medium containing 2% raffinose), SGal (synthetic medium containing 2% galactose), SGalSuc (synthetic medium containing 2% galactose and 2% sucrose), 5FOAD (synthetic medium containing 0.1% 5-fluoro-orotic acid [5FOA] and 2% glucose), and 5FOAGE (synthetic medium containing 0.1% 5FOA, 2% glycerol, and 2% ethanol) are as described (Boeke et al., 1984). Standard molecular genetic techniques were used (Adams et al., 1997).

Plasmid Construction

pHS29, a *CEN-LEU2* plasmid containing *DNM1-111* (Jensen et al., 2000) tagged with the triple influenza hemagglutinin (HA) epitope (Field et al., 1988) at the COOH terminus (Dnm1p-111-HA), was constructed as follows. The *DNM1-111* gene, which contains two mutations in the GTPase domain of Dnm1p (Jensen et al., 2000), was PCR amplified from yeast genomic DNA (Hoffman and Winston, 1987) prepared from *DNM1-111* strain, YHS15 (Jensen et al., 2000), using oligos 268 (5′-CCGCTCGAGGAATACGATACAGAGGAAG-3′) and 269 (5′-ATAAGAATGCGGCCGCCCAGAATATTACTAATAAG-3′). PCR product was digested with XhoI and NotI, and subcloned into XhoI-NotI–digested pAA3 (Sesaki and Jensen, 1999).

pHS50, a *2μ-URA3* plasmid expressing Dnm1p-111–HA, was constructed by cotransforming a PvuII fragment from pHS29 with PvuII- and XhoI-HindIII–digested pRS426 (Sikorski and Hieter, 1989) into yeast cells. Homologous recombination between the *DNM1-111–HA* fragment and the linearized plasmid (Oldenburg et al., 1997) created plasmid pHS50.

pHS58, a *CEN-LEU2* plasmid containing the *UGO1* gene, was constructed by PCR amplifying *UGO1* from yeast genomic DNA using oligos 446 (5′-GGGCTCGAGTGATTTCTTTGAGCGAC-3′) and 448 (5′-GAAGCGGCCGCTAGACAAGTGGTGGAGG-3′). The PCR product was subcloned into XhoI-NotI–digested pRS314 (Sikorski and Hieter, 1989).

pHS51, a *CEN-URA3* plasmid expressing red fluorescent protein (RFP) fused to the presequence of Cox4p under the control of the *GAL1* promoter was constructed as follows. RFP was PCR amplified from plasmid ST10 (a gift from B. Glick, University of Chicago, Chicago, IL) using oligos 461 (5′-GGGTCTAGACCACCGGTCGCCACCATG-3′) and 462 (5′-ATCTAGAGTCGCGGCCG-3′). The PCR fragment was digested with XbaI and NotI and subcloned into XbaI-NotI–digested pHS12 (Sesaki and Jensen, 1999), forming pHS12-RFP. COX4-RFP was PCR amplified from pHS12-RFP using oligos 473 (5′-GGGCTCGAGATGCTTTCACTACGTCAATC-3′) and 474 (5′-GGGGGATCCCTACAGGAACAGGTGGTG-3′). The PCR fragment was digested with XhoI and BamHI, and subcloned downstream of the *GAL1* promoter in XhoI-BamHI–digested pRS316GU (Nigro et al., 1992).

pHS52, a *CEN-URA3* plasmid expressing cyan fluorescent protein (CFP) fused to the presequence of Cox4 from the *GAL1* promoter, was constructed by PCR amplifying CFP from pECFP (CLONTECH Laboratories, Inc.) using oligos 355 (5′-TGCTCTAGAATGGTGAGCAAGGGC-3′) and 498 (5′-CCGGATCCTTACTTGTACAGCTCGTC-3′). The PCR fragment was digested with XbaI and BamHI and subcloned into XbaI-BamHI–digested pHS51.

Table I. Yeast Strains

Strain	Genotype (plasmid)	Source
W303-1A	*MATa ade2 his3 leu2 trp1 ura3 rho*$^+$	Thomas and Rothstein, 1989
W303-1B	*MATα ade2 his3 leu2 trp1 ura3 rho*$^+$	Thomas and Rothstein, 1989
YHS60	*MATa ade2 his3 leu2 trp1 ura3 rho*$^+$ *(pURA3-DNM1-111)*	This study
YHS61	*MATα ade2 his3 leu2 trp1 ura3 rho*$^+$ *(pURA3-DNM1-111)*	This study
YHS62	*MATa ade2 his3 leu2 trp1 ura3 ugo1-1 rho*$^+$ *(pURA3-DNM1-111)*	This study
YHS63	*MATa ade2 his3 leu2 trp1 ura3 ugo2-1 rho*$^+$ *(pURA3-DNM1-111)*	This study
YHS64	*MATa ade2 his3 leu2 trp1 ura3 ugo1-1 rho*^{0}	This study
YHS65	*MATa ade2 his3 leu2 trp1 ura3 ugo2-1 rho*^{0}	This study
YHS1	*MATa his4 leu2 trp1 ura3 mfa2::ADH1-COX4-GFP rho*$^+$	Sesaki and Jensen, 1999
YHS2	*MATα his4 leu2 trp1 ura3 mfa2::ADH1-COX4-GFP rho*$^+$	Sesaki and Jensen, 1999
YHS21	*MATa his3 leu2 met15 trp1 ura3 fzo1::kanMX4 rho*^{0}	Sesaki and Jensen, 1999
YHS22	*MATα his3 leu2 met15 trp1 ura3 fzo1::kanMX4 rho*^{0}	Sesaki and Jensen, 1999
2467	*MATa his3 leu2 ura3 mgm1::kanMX4 rho*^{0}	Research Genetics, Inc.
12467	*MATα his3 leu2 ura3 mgm1::kanMX4 rho*^{0}	Research Genetics, Inc.
FY833	*MATa his3 leu2 lys2 trp1 ura3 rho*$^+$	Winston et al., 1995
FY844	*MATα his3 leu2 lys2 trp1 ura3 rho*$^+$	Winston et al., 1995
FY833/FY834	*MATa/MATa his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 rho*$^+$	Winston et al., 1995
YHS92	*MATa his3 leu2 lys2 trp1 ura3 rho*^{0}	This study
YHS72	*MATa his3 leu2 lys2 trp1 ura3 ugo1::HIS3 rho*^{0}	This study
YHS73	*MATα his3 leu2 lys2 trp1 ura3 ugo1::HIS3 rho*^{0}	This study
YHS74	*MATa his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 rho*^{0}	This study
YHS75	*MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 rho*^{0}	This study
YHS83	*MATa his3 leu2 lys2 trp1 ura3 dnm1::kanMX4 rho*$^+$	This study
YHS84	*MATα his3 leu2 lys2 trp1 ura3 dnm1::kanMX4 rho*$^+$	This study
YHS85	*MATa his3 leu2 lys2 trp1 ura3 ugo1::HIS3 dnm1::kanMX4 rho*$^+$	This study
YHS86	*MATα his3 leu2 lys2 trp1 ura3 ugo1::HIS3 dnm1::kanMX4 rho*$^+$	This study
YHS87	*MATa his3 leu2 lys2 trp1 ura3 ugo1::HIS3 rho*$^+$ *(pLEU2-UGO1-HA)*	This study
YHS88	*MATa his3 leu2 lys2 trp1 ura3 ugo1::HIS3 rho*$^+$ *(pTRP1-myc-UGO1)*	This study
YHS91	*MATa/MATa his3/his3 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 ugo1::HIS3/UGO1 rho*$^+$	This study

pHS57, a *CEN-TRP1* plasmid expressing Ugo1p with the triple myc epitope (Munro and Pelham, 1986) at its NH_2 terminus, was constructed by amplifying the promoter region of *UGO1* from yeast genomic DNA (Hoffman and Winston, 1987) using oligos 446 and 517 (5′-GGGATCGATTGGGGGGTTGAGTTAAAC-3′). The PCR product was digested with XhoI and ClaI and subcloned into XhoI-ClaI–digested pRS314 (Sikorski and Hieter, 1989), forming pHS57-1. The *UGO1* open reading frame was PCR amplified using oligos 518 (5′-GGGATCGATATGAACAACAATAATGTTAC-3′) and 448 (5′-GAAGCGGCCGCTAGACAAGTGGTGGAGG-3′), digested with ClaI and NotI, and subcloned into ClaI-NotI–digested pHS57-1, forming pHS57-2. The triple myc epitope was PCR amplified from KB241 (a gift from D. Kornitzer, S. Kron, and G. Fink, Whitehead Institute, Cambridge, MA) using oligos 519 (5′-GGGATCGATGAGCTCGGTACCCGGGG-3′) and 520 (5′-GGGATCGATGCATGCCTGCAGGTCGAC-3′), digested with ClaI, and subcloned into ClaI-digested pHS57-2, forming pHS57.

pHS55, a *CEN-LEU2* plasmid containing Ugo1p with the triple HA epitope at its COOH terminus, was constructed by amplifying *UGO1* as from yeast DNA using oligos 446 and 447 (5′-GAAGCGGCCGCCGAACTTCTCTTGTTCCATG-3′). The PCR product was digested with XhoI and NotI, and subcloned into XhoI-NotI–digested pAA3 (Sesaki and Jensen, 1999).

Mutant Isolation

Strains YHS60 and YHS61 were constructed by transforming *MAT*a and *MAT*α W303 strains (Thomas and Rothstein, 1989) with pHS50, a *2μ-URA3* plasmid which expresses Dnm1p-111–HA. These parental strains were mutagenized with 3% ethane methylsulfonate (Sigma-Aldrich) in 50 mM potassium phosphate, pH 7.0, for 1.5 h at 30°C to ~30% survival (Lawrence, 1991). Cells were plated onto SD medium lacking uracil at 30°C and formed 16,000 colonies after 7 d. Cells were then replica-plated onto 5FOAD medium. We isolated 11 mutants that gave rise to red colonies on a synthetic medium containing 2% glucose (SD-Ura), but formed white colonies on 5FOAD medium. These mutants were also replica-plated from SD-Ura medium onto YEPGE and 5FOAGE media. All 11 mutants were viable on YEPGE, but failed to grow on 5FOAGE. Of those, eight mutants were found to be defective in a single gene in crosses to wild-type strains YHS1 and YHS2 (Sesaki and Jensen, 1999) and were further analyzed. For complementation analysis, mutants were crossed to *fzo1*Δ cells (YHS21 and YHS22) and to *mgm1*Δ cells (strains 2467 and 12467).

Identification of the UGO1 Gene

ugo1-1 leu2 strain YHS62 was transformed with a library of yeast genomic DNA carried in the *CEN-LEU2* plasmid p366 (a gift from F. Spencer, Johns Hopkins University). 5,000 Leu^+ transformants were selected and tested for growth on 5FOAGE medium. Eight colonies that grew on 5FOAGE medium were isolated and plasmid DNA was obtained from each (Hoffman and Winston, 1987). Plasmids were partially sequenced and were found to contain overlapping fragments of chromosome IV. To localize the region of the complementing activity, we digested one of the complementing plasmids, pa3-1/1A, with XbaI and religated, forming pa3-1/1A/XbaI. pa3-1/1A/XbaI complemented the *ugo1-1* growth defect and carried two open reading frames, *YDR469w* and *YDR470c*. To test which open reading frame contains *UGO1*, pa3-1/1A/XbaI was digested using NcoI and religated to remove *YDR470c*. The resulting plasmid failed to complement the *ugo1-1* growth defect. To confirm that *YDR470c* carries the complementing activity, we constructed pHS58, which carries only *YDR470c*, and showed that this plasmid rescued the growth defect on 5FOAGE of *ugo1-1*. Furthermore, we constructed cells disrupted for *YDR470c* (YHS73; see below) and crossed them to *ugo1-1* cells. The resulting diploids failed to grow on 5FOAGE.

Gene Disruption

Complete disruptions of the *UGO1*, *FZO1*, and *DNM1* genes were constructed by PCR-mediated gene replacement as described (Lawrence, 1991) into diploid strain FY833/844 (Winston et al., 1995). For *ugo1::HIS3*, we used the *HIS3* gene from plasmid pRS303 (Sikorski and Hieter, 1989) and for *fzo1::KAN* and *dnm1::KAN*, we used *kanMX4* plasmid pRS400 (Brachmann et al., 1998). Heterozygous diploids were sporulated and dissected to obtain *MAT*a *ugo1*Δ strain YHS72, *MAT*α *ugo1*Δ strain YHS73, *MAT*a *fzo1*Δ strain YHS74, *MAT*α *fzo1*Δ strain YHS75, *MAT*a *dnm1*Δ strain YHS83, and *MAT*α *dnm1*Δ strain YHS84. *MAT*a *ugo1*Δ *dnm1*Δ strain YHS85 and *MAT*α *ugo1*Δ *dnm1*Δ strain YHS86 were constructed by crossing *MAT*a *ugo1*Δ strain YHS72 and *MAT*α *dnm1*Δ strain YHS84.

Mitochondrial Fusion Assay

Mitochondrial fusion during mating was observed as described (Nunnari et al., 1997; Okamoto et al., 1998), but with the following modifications. *MAT*a strains that carry pGAL1-COX4–RFP (pHS51) were grown to log phase in SRaf medium overnight, pelleted by centrifugation, and resuspended in S medium with 2% galactose and 2% sucrose (SgalSuc) to an OD_{600} of 0.2 for 3–5 h to induce COX4-RFP expression. *MAT*α strains carrying pGAL-COX4-CFP were grown to log phase in SGalSuc medium overnight. Cells were collected, washed, and resuspended in 2 ml of YEPD medium at an OD_{600} of 0.2. Two strains were mixed and collected by centrifugation. Cells were resuspended in 5 μl of YEPD medium and placed on a nitrocellulose membrane and excess solution was removed by placing the membrane on filter papers. The nitrocellulose membrane was then incubated on YEPD medium at 30°C for 3.5 h. Zygotes were examined by fluorescence microscopy.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde, converted to spheroplasts, attached to poly-L-lysine coated coverslips, and permeabilized as described (Harlow and Lane, 1988). Samples were incubated with a 1:100 dilution of antibodies to the myc epitope (9E10; Covance) in PBS containing 1% BSA and 0.05% Tween 20), and with 1:100 dilution of antiserum to the β subunit of the F_1-ATPase (a gift from M. Yaffe, University of California, San Diego, CA) for 1 h, washed three times in PBS containing 0.05% Tween 20, and then stained a 1:200 dilution of FITC-conjugated goat anti–mouse IgG (Boehringer) and a 1:500 dilution of rhodamine-conjugated goat anti–rabbit IgG (Boehringer) for 1 h. Samples were washed and mounted in 95% glycerol containing 0.1% *p*-phenylenediamine and observed.

Subcellular and Submitochondrial Fractionation

Yeast cells were grown to an OD_{600} of ~2 in SGal medium. Cells were converted to spheroplasts, homogenized, and separated into a mitochondrial pellet and a postmitochondrial supernatant by centrifugation at 9,600 *g* for 10 min as described (Daum et al., 1982). Separation of outer membrane and inner membrane vesicles on sucrose gradients was performed as described (Ryan et al., 1994). For protease digestion, mitochondria were resuspended at 1 mg/ml in 250 mM sucrose, 20 mM Hepes-HCl, pH 7.5, and treated with 200 μg/ml trypsin (Sigma-Aldrich) for 20 min on ice, followed by the addition of 2 mg/ml soybean trypsin inhibitor (Sigma-Aldrich). To disrupt the mitochondrial outer membrane, mitochondria were resuspended at 1 mg/ml in 20 mM Hepes-HCl, pH 7.5, and incubated on ice for 30 min.

For analysis, proteins were separated on SDS-PAGE (Laemmli, 1970) and transferred to Immobilon filters (Millipore; Haid and Suissa, 1983). Filters were probed with antibodies to the myc epitope (9E10), the HA epitope (12CA5; Niman et al., 1983), F_1β ATPase, Tim23p (Emtage and Jensen, 1993), and OM45p (Yaffe et al., 1989), all at 1:10,000 dilution, or hexokinase (Kerscher et al., 2000) at 1:20,000 dilution. Immune complexes were visualized using 1:10,000 dilution of HRP-conjugated secondary antibodies (Amersham Pharmacia Biotech) followed by chemiluminescence (SuperSignal; Pierce Chemical Co.).

Fluorescence Microscopy

Cells were observed using a Axioskop microscope (ZEISS) with a 100× Plan-Neofluar objective. Fluorescence and differential interference contrast (DIC) images were captured with a MicroMax CCD camera (Princeton Instruments) using IP Lab software v3.2.0 (Signal Analytics Co.).

Results

Isolation of Mutants That Lose Mitochondrial DNA in a Dnm1p-dependent Manner

fzo1 mutants are defective in mitochondrial fusion and also lose mtDNA. The loss of mtDNA in *fzo1* cells can be suppressed by inactivation of Dnm1p function (Bleazard et al., 1999; Sesaki and Jensen, 1999; Jensen et al., 2000). To identify new components required for fusion, we screened for mutants that maintain mtDNA when Dnm1p activity is absent, but lose mtDNA in the presence of func-

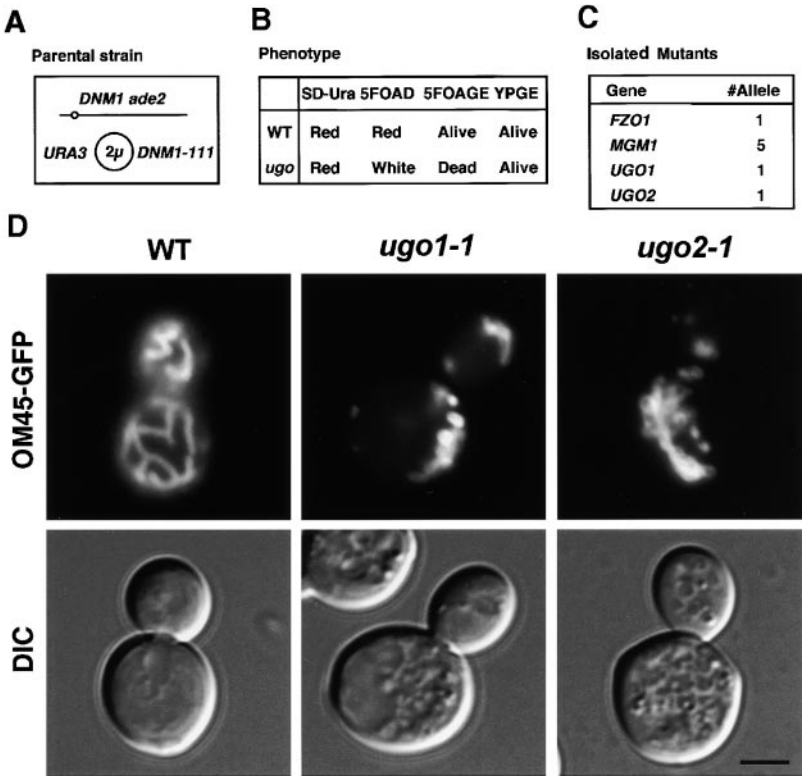

Figure 1. Isolation of *ugo* mutants. (A) Strain used to isolate *ugo1* and *ugo2*. *ade2 DNM1* strains that carry plasmid pHS50, which expresses the dominant negative Dnm1-111 protein, lack Dnm1p activity. Cells that lose pHS50 contain functional Dnm1p. (B) Mutant isolation scheme. The parental strain (WT) maintains mtDNA on glucose-containing medium in the presence (SD-Ura) or absence (5FOAD) of the *URA3-DNM1-111* plasmid and form red colonies due to the *ade2* mutation (Reaume and Tatum, 1949). *ugo* mutants maintain mtDNA only in the presence of the pHS50, forming red colonies on SD-Ura, but become white on 5FOAD medium, which selects for loss of the *URA3-DNM1-111* plasmid (Boeke et al., 1984). Growth on glycerol and ethanol medium confirms that *ugo* mutants contain mtDNA in the presence of the *URA3-DNM1-111* plasmid (YPGE), but that *ugo* mutants are inviable on 5FOA medium containing glycerol and ethanol (5FOAGE). (C) Four different genes were identified in the *ugo* screen. Complementation tests showed one *fzo1* mutant, five *mgm1* mutants, and two new mutants, *ugo1* and *ugo2*, were isolated. (D) *ugo* mutants contain highly fragmented mitochondria. Wild-type (W303), *ugo1-1* (YHS64), and *ugo2-1* (YHS65) were grown on 5FOAD medium to select for cells that lost the *URA3-DNM1-111* plasmid, pHS50, and then transformed with pKC2, which expresses GFP fused to the COOH terminus of the mitochondrial outer membrane protein, OM45p (OM45-GFP; Cerveny et al., 2001). Cells were grown to log phase in SRaf medium and examined by fluorescence microscopy. Fluorescence (OM45-GFP) and DIC images are shown. Bar, 3 μm.

tional Dnm1p. We controlled Dnm1p activity using the *URA3* plasmid pHS50, which carries a dominant negative version of Dnm1p, Dnm1p-111 (Fig. 1 A; Sesaki and Jensen, 1999; Jensen et al., 2000). Cells that contain pHS50 lack Dnm1p function, which is restored upon loss of the plasmid. To monitor the presence of mtDNA, we took advantage of the *ade2* mutation. *ade2* cells that contain mtDNA are competent for respiration, producing a red pigment and forming red colonies (Reaume and Tatum, 1949). Cells that lose mtDNA and the ability to respire form white colonies.

Strains YHS60 and YHS61, which contain the plasmid pHS50, were mutagenized and plated on SD-Ura. Colonies were then replica-plated to 5FOAD to select for cells that had lost the *URA3-DNM1-111* plasmid (Boeke et al., 1984). Out of 16,000 colonies screened, 11 formed red colonies on SD-Ura and then formed white colonies when replica-plated onto 5FOAD medium (Fig. 1 B), suggesting that the 11 mutants contained mtDNA when the *DNM1-111*–containing plasmid was present, but lost mtDNA when pHS50 was absent. Since mtDNA is required for cell growth on nonfermentable carbon sources, we tested the 11 mutants for their ability to grow on glycerol- and ethanol-containing medium. When cells contained pHS50 (YPGE medium), all 11 mutants were able to grow, but in the absence of pHS50 (5FOADE medium), all 11 mutants failed to grow (Fig. 1 B). We crossed the 11 mutants to wild-type cells and found that all were recessive. After meiotic analysis, we found that 8 of the 11 mutants were defective in single genes and these 8 were further analyzed.

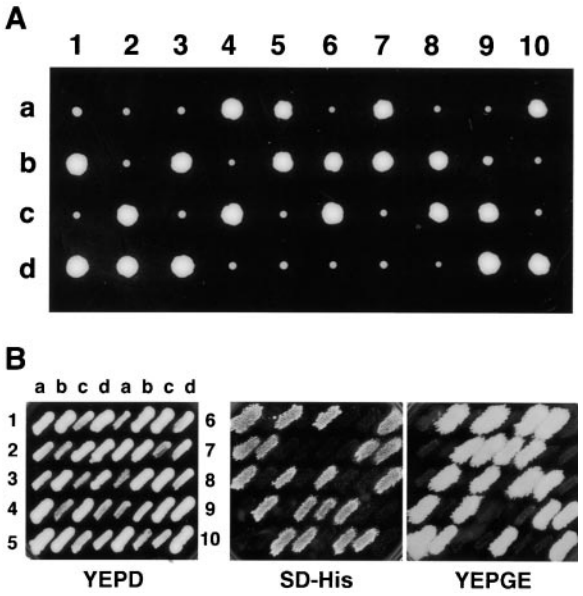

Figure 2. *ugo1*Δ cells grow slowly on glucose-containing medium and are inviable on nonfermentable carbon sources. (A) 10 meiotic products from the *ugo1::HIS3/UGO1* diploid strain, YHS91, were separated by micromanipulation and allowed to grow at 30°C for 5 d on YEPD. (B) 10 tetrads from the sporulated *ugo1::HIS3/UGO1* diploid strain, YHS91, were patched onto YEPD medium and then replica-plated to SD medium lacking histidine (SD-His) and YEPGE. Cells were incubated at 30°C for 6 d.

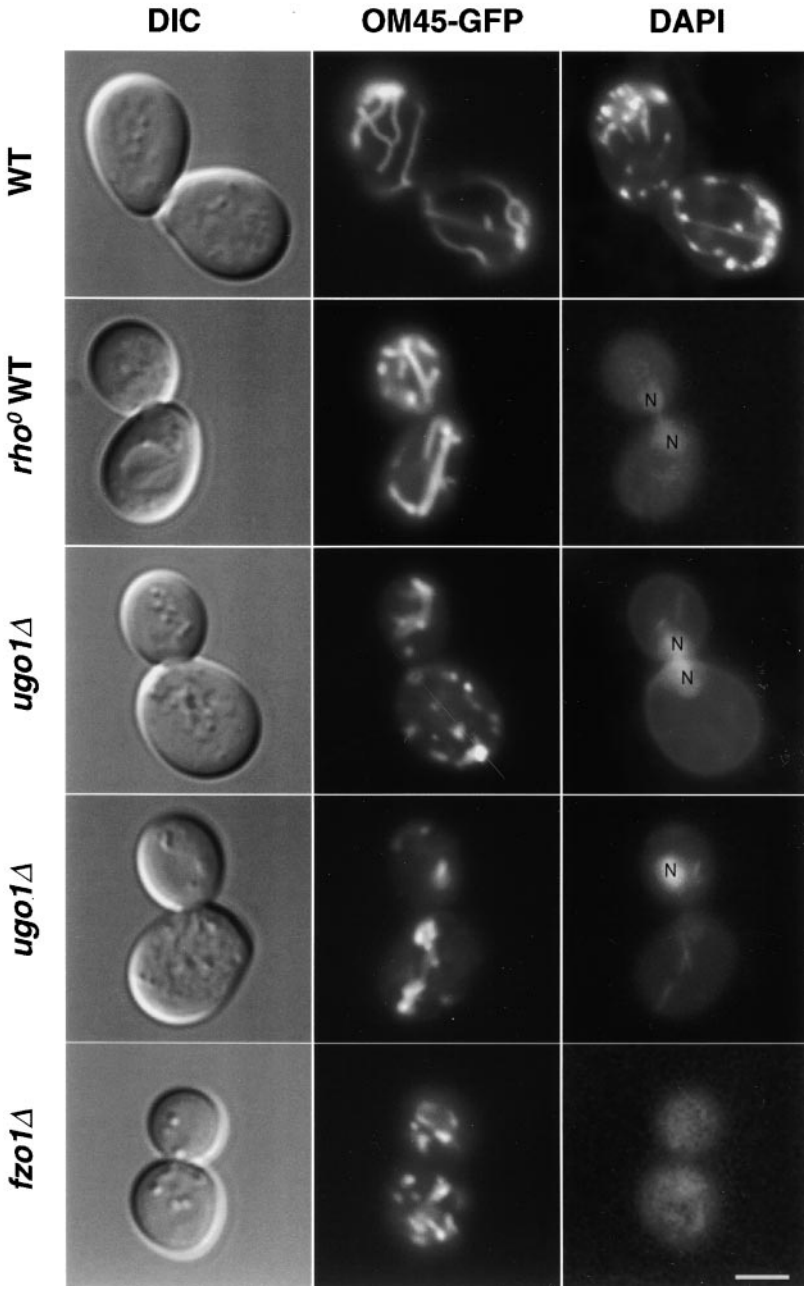

Figure 3. *ugo1*Δ cells contain fragmented mitochondria and lack mtDNA. Wild-type (FY833), *rho*^{0} WT (YHS92), and *ugo1*Δ (YHS72) and *fzo1*Δ (YHS74) cells were transformed with OM45-GFP–expressing plasmid, pKC2 (Cerveny et al., 2001). Cells were grown to log phase in SRaf medium. Cells were stained using 1 μg/ml DAPI, and viewed by DIC and fluorescence (OM45-GFP or DAPI) microscopy. *rho*^{0} cells (YHS92) were generated by treating wild-type cells (FY833) with 25 μg/ml ethidium bromide as described (Fox et al., 1991). N, nuclear DNA staining. Bar, 3 μm.

Our genetic screen identified four different genes. In crosses to *fzo1* cells, we found that one of our mutants carried an *fzo1* allele (Fig. 1 C). Since our genetic screen was based on the behavior of *fzo1* mutants, this result was expected. Furthermore, since *mgm1* mutants lose mtDNA in a Dnm1p-dependent manner (Fekkes et al., 2000), we anticipated that we would find *mgm1* mutants. Five of our mutants carried *mgm1* alleles. The two remaining mutants formed two new complementation groups, which we have called *ugo1* and *ugo2* (ugo is Japanese for fusion). We examined mitochondrial shape in our *ugo1* and *ugo2* mutants (Fig. 1 D). Wild-type, *ugo1-1*, and *ugo2-1* cells were transformed with a plasmid expressing green fluorescent protein (GFP) fused to the mitochondrial outer membrane protein, OM45 (Cerveny et al., 2001). In wild-type cells, mitochondria were seen as long, tubular structures with occasional branches. In contrast, in both *ugo1-1* and *ugo2-1* mutants, fragmented mitochondria were found, similar to those seen in *fzo1* cells (Hermann et al., 1998; Rapaport et al., 1998; see below). In this report, we focus on the characterization of the *ugo1* mutant and the description of *ugo2* is the subject of another study.

UGO1 Encodes a Novel 58-kD Protein

Since *ugo1* cells lose mtDNA in the presence of functional Dnm1p, we isolated *UGO1* by screening a genomic library for clones that allow *ugo1-1* to maintain mtDNA. *ugo1-1* cells containing the *URA3-DNM1-111* plasmid pHS50 were transformed with a yeast genomic DNA library. We then selected for loss of pHS50 and asked if cells could retain their mtDNA by replica-plating transformants to 5FOA medium with glycerol and ethanol as the sole car-

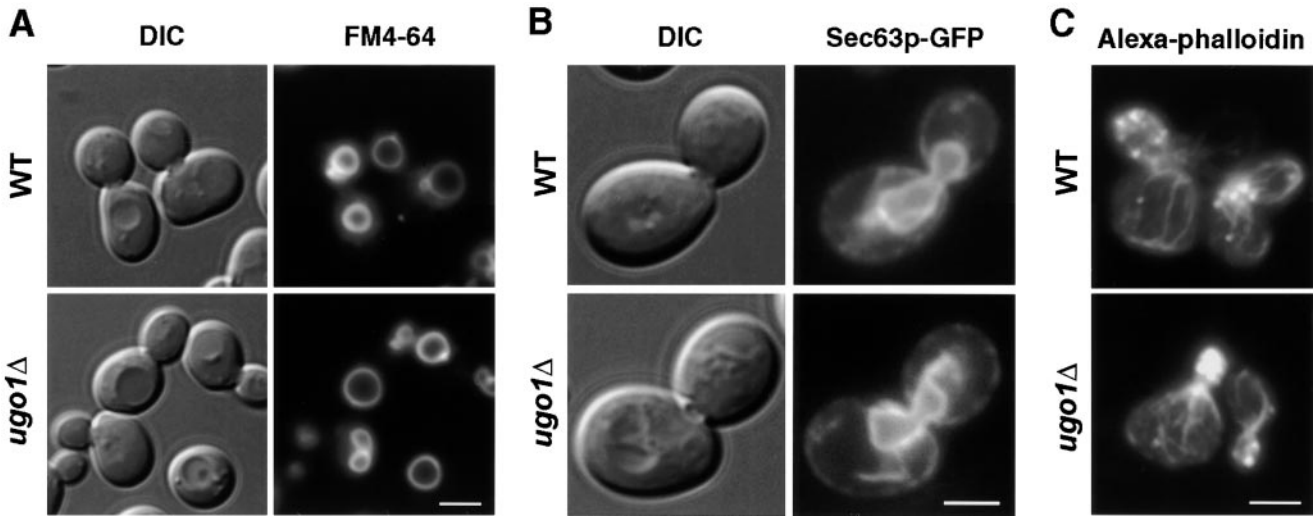

Figure 4. Morphology and distribution of vacuoles, the endoplasmic reticulum, and the actin cytoskeleton are normal in *ugo1*Δ cells. (A) Wild-type (FY833, WT) and *ugo1*Δ (YHS72) cells were grown to log phase in YEPD medium. Cells were then stained with 12 μM FM4-64 (Molecular Probes) to label vacuoles (Vida and Emr, 1995) and examined by fluorescence (FM4-64) and DIC microscopy. (B) Wild-type and *ugo1*Δ cells expressing GFP fused to the COOH terminus of Sec63p from pPS1530 (Prinz et al., 2000) were grown to log phase in SD medium and examined by fluorescence microscopy. (C) Wild-type and *ugo1*Δ cells were grown to log phase in YEPGal medium. Cells were then fixed in 3.7% formaldehyde and stained with 1 μM Alexa 594–phalloidin (Molecular Probes) to visualize actin filaments (Adams and Pringle, 1991). Bars, 3 μm.

bon source. Eight plasmids with overlapping inserts were identified that allowed *ugo1-1* cells to maintain mtDNA in the absence of pHS50. Subcloning studies localized the *UGO1*-complementing activity to open reading frame *YDR470c*, a previously uncharacterized protein.

UGO1 encodes a 503–amino acid protein (~58 kD), and hydropathy analysis (Kyte and Doolittle, 1982) predicts that Ugo1p contains a single transmembrane domain in the middle of the protein, between residues 295 and 311. Interestingly, we found that Ugo1p carries two mitochondrial energy transfer protein signatures (Nelson et al., 1998) at residues 132–144 and 310–319. This motif consists of 10 loosely conserved amino acids and is found in many mitochondrial carrier proteins such as *AAC2*, *CTP1*, and *DIC1* (Nelson et al., 1998). We also found that Ugo1p is ~22% identical to a *Schizosaccharomyces pombe* protein encoded by the SPAC1B2.02c gene.

Ugo1p Is Essential for Growth on Nonfermentable Carbon Sources and for Maintenance of mtDNA

To further investigate the function of Ugo1p, we created a null allele by replacing the *UGO1* open reading frame with the yeast *HIS3* gene (*ugo1*Δ). *UGO1/ugo1*Δ diploid cells were sporulated and the haploid segregants were allowed to grow on YEPD. We found that all four spores in each tetrad were viable, but that two spores formed small colonies (Fig. 2 A). The slower growing cells were shown to carry the *ugo1*Δ gene, whereas cells from larger-sized colonies contained *UGO1*. Ugo1p appears to be required for normal cell growth. As shown in Fig. 2 B, cells carrying the *ugo1*Δ disruption failed to grow on YEPGE. Since mtDNA is essential for growth on glycerol and ethanol, we asked if *ugo1*Δ cells lack mtDNA by staining them with the DNA-specific dye, DAPI. DAPI staining showed that mtDNA nucleoids were present in wild-type cells (Fig. 3). In contrast, *ugo1*Δ cells contained little or no mtDNA and only faint staining of nuclear DNA was seen, similar to wild-type cells lacking mtDNA (*rho*^{0} WT). Our results indicate that *UGO1* is required for growth on nonfermentable carbon sources and to maintain mtDNA.

ugo1Δ Cells Contain Fragmented Mitochondria

To further probe the function of *UGO1*, we examined mitochondria in wild-type, *fzo1*Δ, and *ugo1*Δ cells. Mitochondria were visualized using an outer membrane–targeted GFP fusion protein, OM45-GFP (Fig. 3). Although wild-type cells contained a few elongated mitochondrial tubules with occasional branches, *ugo1*Δ and *fzo1*Δ cells showed many small mitochondrial fragments. In most *ugo1*Δ cells (64%, $n = 102$) mitochondria were distributed uniformly at the cell periphery (Fig. 3, left panel of *ugo1*Δ image). In the remaining *ugo1*Δ cells, mitochondria were somewhat aggregated (Fig. 3, right panel of *ugo1*Δ image). Although the morphology of mitochondria in *ugo1*Δ cells was dramatically altered, mitochondrial transmission was not altered. Mitochondrial fragments were always seen in both mother and daughter cells. The altered mitochondrial shape in *ugo1*Δ cells was not due to lack of mtDNA. Wild-type cells which lack mtDNA showed normal tubular-shaped mitochondria (Fig. 3, *rho*^{0} WT).

Although the mitochondrial fragments seen in *ugo1*Δ cells are similar to those seen in *fzo1*Δ cells, we found noticeable differences between *ugo1*Δ and *fzo1*Δ mutants (Fig. 3). For example, *ugo1*Δ cells contained slightly larger fragments (0.47 μm in average diameter, $n = 50$) than *fzo1*Δ cells (0.40 μm, $n = 50$). Mitochondria in *ugo1*Δ cells showed markedly different sizes (ranging from 0.21 to 1.06 μm), whereas the organelles in *fzo1*Δ cells displayed relatively uniform sizes (ranging from 0.28 to 0.69 μm). We also noticed that mitochondria in *ugo1*Δ cells were aggregated

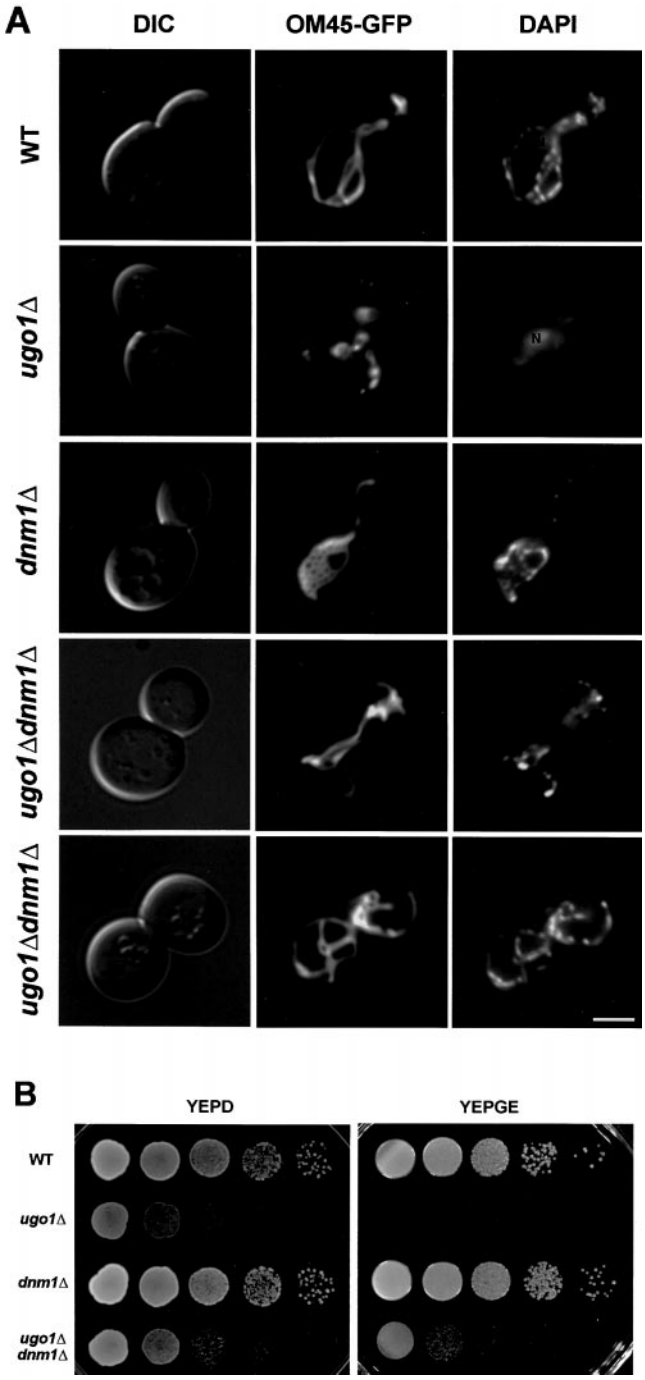

Figure 5. *ugo1*Δ *dnm1*Δ cells contain mitochondrial tubules and maintain mtDNA. Wild-type (FY833, WT), *ugo1*Δ (YHS72), *dnm1*Δ (YHS83), and *ugo1*Δ *dnm1*Δ (YHS85) cells expressing OM45-GFP (pKC2) were grown to log phase in SRaf medium, stained with 1 μg/ml DAPI, and viewed by DIC and fluorescence (OM45-GFP) microscopy. (B) Wild-type, *ugo1*Δ, *dnm1*Δ, and *ugo1*Δ *dnm1*Δ cells were grown to log phase in YEPD medium. Cells were collected and resuspended in YEPD medium to an OD_{600} of 2. Cells were then diluted in 10-fold increments, and 10 μl of each dilution was spotted onto YEPD and YEPGE media and incubated at 30°C for 2 and 6 d, respectively. N, nuclear DNA staining. Bar, 3 μm.

more often than mitochondria in *fzo1*Δ cells. 36% (n = 102) of *ugo1*Δ cells contained aggregated mitochondria, whereas only 10% (n = 110) of *fzo1*Δ mitochondria were clumped.

The morphology of intracellular structures other than mitochondria was not defective in *ugo1*Δ cells. When we stained vacuoles using FM4-64 (Vida and Emr, 1995), their morphology in *ugo1*Δ cells was indistinguishable from vacuoles seen in wild-type cells (Fig. 4 A). The endoplasmic reticulum, visualized with Sec63p-GFP (Prinz et al., 2000), also displayed normal shape in *ugo1*Δ cells (Fig. 4 B). Since the actin cytoskeleton is important for the shape of mitochondria in budding yeast (Drubin et al., 1993; Boldogh et al., 1998), we examined the organization of the actin in wild-type and *ugo1*Δ cells using Alexa 594–phalloidin. We found that the distribution of actin cables and patches in *ugo1*Δ cells was the same as that in wild-type cells (Fig. 4 C). Thus, our results suggest that the defect in *ugo1*Δ cells is limited to mitochondria.

Disruption of DNM1 Rescues Fragmentation of Mitochondria and Loss of mtDNA in ugo1Δ Cells

Mitochondrial fusion and division are normally balanced in cells, leading to the few tubular-shaped mitochondria seen in wild-type cells. The fragmentation of mitochondria in *fzo1* results from continued division in the absence of fusion, and disruption of a gene required for division, *DNM1*, in *fzo1* cells restores normal mitochondrial shape and number (Sesaki and Jensen, 1999). To test if *ugo1* shows a similar interplay with *dnm1* as that seen with *fzo1* and *dnm1*, we compared mitochondrial shape in either *ugo1*Δ mutants or *dnm1*Δ mutants to those in *ugo1*Δ *dnm1*Δ double mutants. In *dnm1*Δ mutants, a single mitochondria consisting of a network of interconnected tubules is seen, resulting from ongoing fusion in the absence of mitochondrial division (Fig. 5 A; Bleazard et al., 1999; Sesaki and Jensen, 1999). As noted previously, *ugo1*Δ cells contain many small mitochondrial fragments (Figs. 3 and 5). In contrast, in the majority of *ugo1*Δ *dnm1*Δ cells (90%, n = 100), mitochondria appeared as elongated tubules, similar to those in wild-type cells (Fig. 5 A) or in *fzo1 dnm1* double mutants (Sesaki and Jensen, 1999). In ~56% of *ugo1*Δ *dnm1*Δ cells mitochondrial tubules were often collapsed to one side of the cell and appeared to be bundled (Fig. 5 A, left panel of *ugo1*Δ *dnm1*Δ images). In 34% of *ugo1*Δ *dnm1*Δ cells individual mitochondrial tubules were clearly separated from other tubules (Fig. 5 A, right panel of *ugo1*Δ *dnm1*Δ images). Only a small fraction of *ugo1*Δ *dnm1*Δ cells (~10%) showed mitochondria that appeared to be fragmented and aggregated. Thus, our results demonstrate that the fragmentation of mitochondria in *ugo1*Δ cells can be suppressed by *dnm1* disruption. Ugo1p, like Fzo1p, appears to function in mitochondrial fusion, an activity antagonistic to the Dnm1p-mediated division of mitochondria.

Disruption of *DNM1* also rescued the loss of mtDNA in *ugo1*Δ cells. When wild-type cells, *ugo1*Δ mutants, *dnm1*Δ mutants, or *ugo1*Δ *dnm1*Δ double mutants were stained with DAPI, we found that mtDNA was absent from *ugo1*Δ cells (Fig. 5 A). However, similar amounts of mtDNA nucleoids were found in wild-type cells, *dnm1*Δ mutants, and *ugo1*Δ *dnm1*Δ mutants (Fig. 5 A). Furthermore, in con-

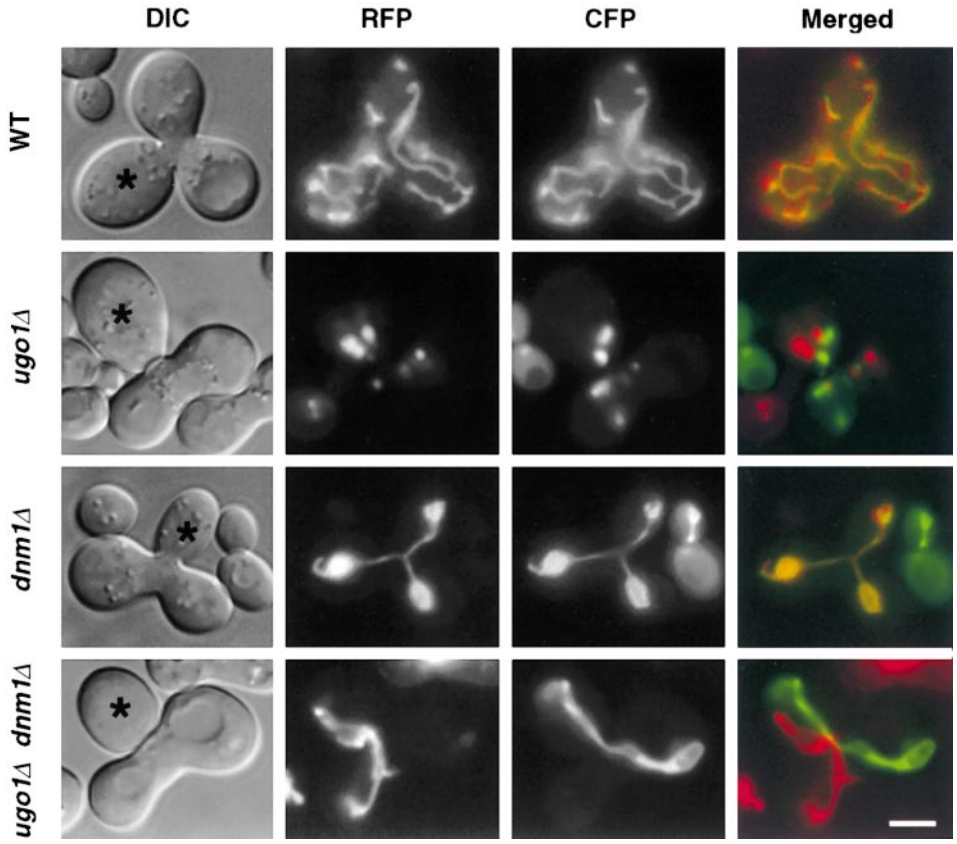

Figure 6. Mitochondrial fusion is defective in *ugo1*Δ and *ugo1*Δ *dnm1*Δ cells. *MAT*a cells containing matrix-targeted RFP under the control of the *GAL1* promoter (pHS51) were grown to log phase in SRaf medium and then transferred to SGalSuc medium for 3–5 h to induce the expression of the COX4-RFP fusion protein. *MAT*α cells containing matrix-targeted CFP under the control of the *GAL1* promoter (pHS52) were grown to log phase in SGalSuc medium overnight to induce COX-CFP. *MAT*a and α cells were mated for 3.5 h on YEPD medium. The distribution of COX4-RFP and COX4-CFP in representative zygotes containing a medial bud (asterisks) is shown. Zygotes formed by mating between wild-type cells (FY833 and FY834, WT), *ugo1*Δ mutants (YHS72 and YHS73), *dnm1*Δ mutants (YHS83 and YHS84), and *ugo1*Δ *dnm1*Δ mutants (YHS85 and YHS86) were examined. Bar, 3 μm.

trast to *ugo1*Δ mutants, we found that *ugo1*Δ *dnm1*Δ cells were able to grow on a glycerol and ethanol-containing medium, indicating that the double mutant contained mtDNA (Fig. 5 B). We note that *ugo1*Δ *dnm1*Δ cells grew more slowly than wild-type and *dnm1*Δ cells on both glucose and glycerol/ethanol media. This growth appears to result from lack of Ugo1p, since *ugo1*Δ and *ugo1*Δ *dnm1*Δ cells grew more slowly than wild-type or *dnm1*Δ on glucose-containing medium (Figs. 2 A and 5 B).

ugo1Δ and ugo1Δ dnm1Δ Cells Are Defective in Mitochondrial Fusion

To ask if Ugo1p plays a direct role in fusion, we examined the ability of *ugo1* cells to fuse their mitochondria after yeast cell mating (Nunnari et al., 1997; Okamoto et al., 1998). The mitochondria in *MAT*a cells were labeled using a matrix-targeted RFP (pGAL1-COX4–RFP) expressed from pHS51. In *MAT*α cells, mitochondria were visualized with a matrix-targeted CFP (pGAL1-COX4–CFP) carried on pHS52. Both plasmids express the fusion protein under control of the inducible *GAL1* promoter. *MAT*a and *MAT*α cells were pregrown in galactose-containing medium to induce the expression of the fusion proteins and transferred to glucose medium to inhibit their further synthesis. Cells were mixed and allowed to mate on glucose-containing medium. If mitochondrial fusion occurred in the resulting zygotes, RFP and CFP fluorescence should completely overlap due to the diffusion of the matrix COX4-RFP and COX4-CFP proteins. If no fusion occurs, RFP and CFP should be seen in separate organelles.

We found that *ugo1*Δ and *ugo1*Δ *dnm1*Δ mutants are defective in fusion. In Fig. 6, representative examples of zygotes containing a medial diploid bud from each mating mixture are shown, but >50 zygotes for each mating mixture were actually examined. When two wild-type cells were mated, mitochondria in the zygote efficiently fused and a complete overlap of the RFP and CFP fluorescence was seen. Consistent with previous studies (Bleazard et al., 1999; Sesaki and Jensen, 1999), mitochondrial fusion also occurred in *dnm1*Δ/*dnm1*Δ zygotes. Interestingly, *dnm1*Δ/*dnm1*Δ zygotes often contained a single tubule emerging from the mitochondrial network of each parent. Fusion appeared to occur at a discrete point near the middle of the zygote. In contrast to wild-type and *dnm1*Δ cells, fusion was defective in *ugo1*Δ mutants. *ugo1*Δ/*ugo1*Δ zygotes contained many mitochondrial fragments, but these organelles contained only RFP or CFP fluorescence. No organelles containing both fluorophores were seen. Although disruption of *DNM1* suppresses the fragmentation of mitochondria and the loss of mtDNA of *ugo1*Δ mutants, *ugo1*Δ *dnm1*Δ double mutants still failed to fuse their mitochondria. Although *ugo1*Δ *dnm1*Δ cells displayed tubular mitochondrial shape, each mitochondrial tubule contained only RFP or CFP. We found that mitochondria in *ugo1*Δ/*ugo1*Δ or *ugo1*Δ *dnm1*Δ/*ugo1*Δ *dnm1*Δ zygotes were often closely positioned to each other near the middle of zygotes, but nonetheless did not fuse. Our results thus indicate that *ugo1*Δ and *ugo1*Δ *dnm1*Δ cells are defective in mitochondrial fusion and argue that Ugo1p plays a direct role in the fusion pathway.

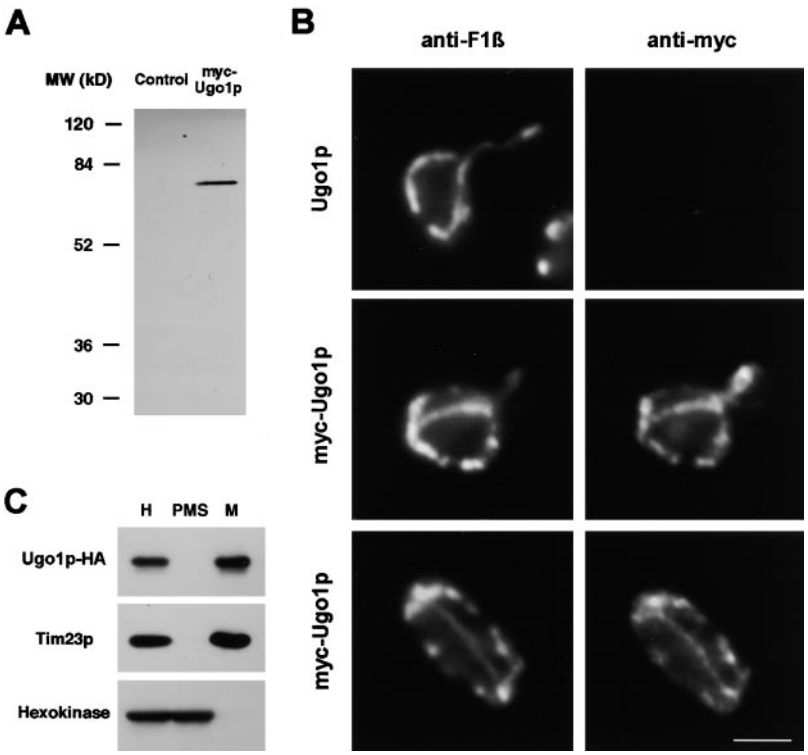

Figure 7. Ugo1p is a mitochondrial protein. (A) Expression of myc-Ugo1p. Wild-type cells (FY833) containing an empty vector, pRS314 (Control), and *ugo1*Δ cells (YHS88) containing the myc-Ugo1p plasmid (pHS57) were grown to log phase in SGal medium. Whole cell extracts were prepared (Yaffe and Schatz, 1984) and analyzed by immune blotting using antibodies to the myc epitope. (B) Ugo1p colocalizes with a mitochondrial protein. Wild-type cells containing pRS314 (Ugo1p) and *ugo1*Δ cells containing the myc-Ugo1p plasmid (pHS57) were grown to log phase in SGal medium. Cells were then fixed, spheroplasted, permeabilized (Harlow and Lane, 1988), and incubated with rabbit antibodies to the β subunit of F_1-ATPase (anti-F_1β) and mouse IgG to the myc epitope (anti-myc). Immune complexes were visualized by fluorescence microscopy using FITC-conjugated anti–mouse IgG and rhodamine-conjugated anti–rabbit IgG. (C) Ugo1p cofractionates with a mitochondrial marker. *ugo1*Δ cells (YHS87) expressing Ugo1p-HA (pHS55) were grown in SGal medium. Cells were homogenized and separated into a mitochondrial pellet and a postmitochondrial supernatant by centrifugation. Cell-equivalent amounts of homogenate (H), mitochondrial pellet (M), and postmitochondrial supernatant (PMS) were analyzed by immune blotting using antibodies to the HA epitope (Ugo1p-HA), Tim23p, and hexokinase. Bar, 3 μm.

Ugo1p Is a Mitochondrial Outer Membrane Protein, with its NH_2 Terminus Facing the Cytosol and COOH Terminus in the Intermembrane Space

To localize Ugo1p in yeast cells, we constructed two epitope-tagged versions of Ugo1p, myc-Ugo1p, and Ugo1p-HA. myc-Ugo1p carries the myc epitope (Munro and Pelham, 1986) fused to the NH_2 terminus of the Ugo1 protein, and Ugo1p-HA contains the influenza HA epitope (Field et al., 1988) at its COOH terminus. Cells that expressed either myc-Ugo1p (Fig. 7 A) or Ugo1p-HA (data not shown) contained a single protein of ~65 kD. We found that both fusion proteins were functional and *ugo1*Δ cells expressing either myc-Ugo1p (Fig. 7 B) or Ugo1p-HA (data not shown) maintained mtDNA and normal mitochondrial shape.

Immunofluorescence studies showed that Ugo1p is a mitochondrial protein (Fig. 7 B). *ugo1*Δ cells expressing the myc-Ugo1p fusion protein were fixed, permeabilized, and then incubated with antibodies to the myc-epitope and the mitochondrial ATPase β subunit (F_1β) protein. When immune complexes were visualized using fluorescence microscopy, we found that myc-Ugo1 protein colocalized with the mitochondrial F_1β protein. Cell fractionation experiments also confirmed the mitochondrial localization of Ugo1p (Fig. 7 C). Cells expressing Ugo1p-HA were homogenized and separated into a mitochondrial fraction and a postmitochondrial supernatant. We found that Ugo1p cofractionated with the mitochondrial Tim23 protein, whereas little or no Ugo1p was found in the supernatant along with cytosolic hexokinase protein.

Ugo1p is an integral membrane protein located in the outer membrane. When mitochondria isolated from cells expressing Ugo1p-HA were treated with 1.5 M sodium chloride or 0.1 M sodium carbonate (Fig. 8 A), Ugo1p was not extracted from the mitochondria like the peripheral membrane protein, the β subunit of the F_1-ATPase (F_1β). Instead, Ugo1p remained in the membrane pellet with the integral membrane protein, Tim23p. To determine which mitochondrial membrane contains Ugo1p, we prepared membrane vesicles from myc-Ugo1p mitochondria and separated them into outer membrane and inner membrane fractions on sucrose gradients. As shown in Fig. 8 B, myc-Ugo1 cofractionated with the outer membrane vesicle fraction, along with OM45. The F_1β protein, a marker for the inner membrane, was found in more dense fractions, separate from myc-Ugo1p and OM45.

The COOH terminus of Ugo1p faces the intermembrane space (IMS). When Ugo1p-HA mitochondria were treated with trypsin, the outer membrane protein, OM45, was completely digested, whereas the inner membrane proteins, Tim23p and F_1β, remained intact (Fig. 8 C). Trypsin digested the 65-kD Ugo1-HA protein, producing an ~25 kD fragment that reacted with our HA antibodies (Fig. 8 C, asterisk). The size of this fragment is consistent with the length of the COOH terminus of Ugo1p including its transmembrane segment. When the mitochondrial outer membrane was disrupted by osmotic shock to form mitoplasts, protease treatment now digested both Tim23p and Ugo1p-HA. Since our antiserum recognizes the NH_2 terminus of Tim23p, which faces the IMS, we conclude that the COOH-terminal HA epitope of Ugo1p-HA similarly faces the IMS. Trypsin treatment of mitoplasts did

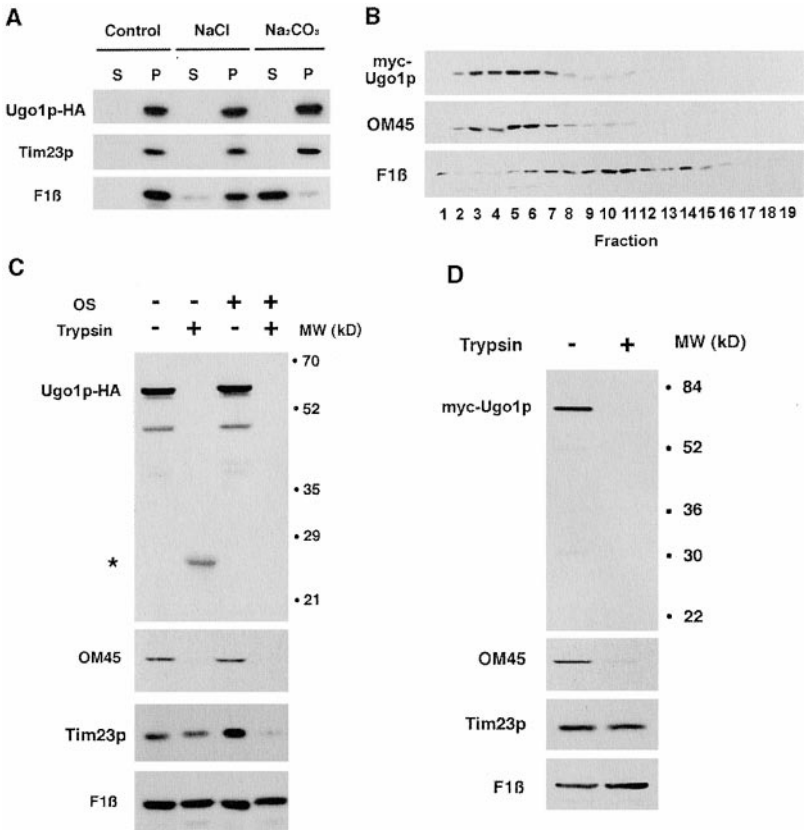

Figure 8. Ugo1p is located in the mitochondrial outer membrane, with its NH_2 terminus facing the cytosol and COOH terminus in the IMS. (A) Ugo1p is an integral membrane protein. Mitochondria isolated from *ugo1*Δ cells (YHS87) expressing Ugo1p-HA (pHS55) were treated with either 1.5 M sodium chloride, 0.1 M sodium carbonate, or untreated (Control). Mitochondrial membranes were then separated into supernatant (S) and pellet (P) fractions by centrifugation at 100,000 *g* for 60 min. Aliquots from each fraction were analyzed by immune blotting with antibodies to the HA epitope (Ugo1p-HA), Tim23p, an integral membrane protein, and the β subunit of the F_1-ATPase ($F_1\beta$), a peripheral membrane protein. (B) Ugo1p is located in the outer membrane. myc-Ugo1p mitochondria were sonicated and membrane vesicles were loaded on sucrose gradients. After centrifugation, fractions were collected and analyzed by immune blotting with antibodies to the myc epitope (myc-Ugo1p), the outer membrane protein OM45, and the inner membrane protein $F_1\beta$. Fraction 1 represents the top of the gradient. (C) The COOH terminus of Ugo1p faces the IMS. Ugo1p-HA mitochondria were digested with 200 μg/ml trypsin for 20 min on ice and analyzed by immune blotting with antibodies to the HA epitope (Ugo1p-HA), OM45, and the inner membrane proteins, $F_1\beta$ and Tim23p. To expose proteins located in the IMS, the mitochondrial outer membrane was disrupted by osmotic shock (OS) and then treated with protease. Asterisk, a proteolytic fragment of the COOH terminus of Ugo1p-HA. (D) The NH_2 terminus of Ugo1p faces the cytosol. Mitochondria were isolated from *ugo1*Δ cells (YHS72) expressing myc-Ugo1p (pHS57). Mitochondria were treated with trypsin and analyzed by immune blotting.

not digest the matrix-facing $F_1\beta$ protein. We also directly showed that NH_2 terminus of Ugo1p faces the cytosol. Mitochondria were isolated from cells expressing myc-Ugo1p and treated with trypsin. myc-Ugo1p and OM45p, but not Tim23p and $F_1\beta$ protein, were digested in intact mitochondria (Fig. 8 D). No protected fragments of myc-Ugo1p were seen. Therefore, we conclude that Ugo1p is an outer membrane protein, with its NH_2 terminus facing the cytosol and its COOH terminus in the IMS.

Discussion

We have identified a new outer membrane protein, Ugo1p, required for mitochondrial fusion. Ugo1p was identified using a genetic screen for mutants that maintain mtDNA in the absence of mitochondrial division, but lose mtDNA when division is active. Our screen was based on previous studies with *fzo1* mutants, which are defective in an outer membrane protein that mediates mitochondrial fusion. *fzo1* cells lose mtDNA, but the loss of mtDNA can be suppressed by disruption of *DNM1*, a gene required for mitochondrial division (Bleazard et al., 1999; Sesaki and Jensen, 1999; Jensen et al., 2000). Like *fzo1* mutants, the loss of mtDNA in *ugo1* mutants is suppressed by inactivation of Dnm1p function. *ugo1* cells that carry the dominant negative *DNM1-111* mutant or *ugo1*Δ *dnm1*Δ double mutants maintain mtDNA. Similar to *fzo1* mutants, the mitochondrial morphology defect in *ugo1*Δ cells is suppressed by *DNM1* disruption. Both *fzo1*Δ and *ugo1*Δ mutants contain many small mitochondrial fragments, instead of the few long tubular-shaped mitochondria found in wild-type cells. *ugo1*Δ *dnm1*Δ cells contain mitochondrial tubules similar to those seen in wild-type cells and in *fzo1 dnm1* double mutants (Sesaki and Jensen, 1999). Our results suggest that a balance of fusion and division regulates mitochondrial shape and number. In the absence of mitochondrial fusion, mediated by Fzo1p and Ugo1p, ongoing division produces numerous small organelles. When division is defective, continuous fusion leads to the single interconnected mitochondrial network seen in *dnm1* cells (Bleazard et al., 1999; Sesaki and Jensen, 1999).

We have found that *ugo1*Δ cells, like *fzo1* mutants (Hermann et al., 1998), are defective in mitochondrial fusion. Although *ugo1*Δ *dnm1*Δ double mutants contain tubular-shaped mitochondria, they do not fuse their mitochondria. Similarly, *fzo1*Δ *dnm1*Δ cells remain blocked for fusion (Bleazard et al., 1999; Sesaki and Jensen, 1999). Therefore, the normal-looking mitochondria found in *ugo1 dnm1* and *fzo1 dnm1* mutants (Sesaki and Jensen, 1999) suggest that some aspects of mitochondrial shape result from mechanisms independent of fusion and division. For example, tubular-shaped mitochondria may arise by directed growth of preexisting organelles along cytoskeletal filaments. Alternatively, internal mitochondrial proteins may provide a scaffold for tubulation of mitochondria. Regardless of how tubules are formed, we suggest that a bal-

ance between mitochondrial fusion and division regulates the length, number, and connection of mitochondrial tubules. For example, in *ugo1* and *fzo1* mutants where fusion is blocked, mitochondria form numerous tubular structures, but the length of each tubule is very short. In *dnm1* mutants (Bleazard et al., 1999; Sesaki and Jensen, 1999), *mdv1/gag3/net2* mutants (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001), or *fis1* mutants (Mozdy et al., 2000) where division is defective, mitochondria form a single organelle consisting of interconnected tubules.

Our genetic screen identified five *mgm1* mutants. *mgm1* mutants lose mtDNA (Jones and Fangman, 1992; Guan et al., 1993) and mtDNA loss can be suppressed by inactivation of Dnm1p (Fekkes et al., 2000). Mgm1p is a mitochondrially associated, dynamin-related GTPase, although its exact location in mitochondria is unclear (Shepard and Yaffe, 1999; Wong et al., 2000). In *mgm1* mutants, mitochondria are fragmented like those in *fzo1* and *ugo1* cells, suggesting a role in mitochondrial fusion. Recently, *mgm1* mutants have been shown to be defective in mitochondrial fusion (Wong et al., 2000). However, in contrast to *fzo1 dnm1* (Bleazard et al., 1999; Sesaki and Jensen, 1999) and *ugo1 dnm1* double mutants, *mgm1 dnm1* double mutants are able to fuse their mitochondria, suggesting that Mgm1p plays an indirect role in mitochondrial fusion (Wong et al., 2000).

Ugo1p is embedded in the mitochondrial outer membrane with its COOH terminus of nearly 200 amino acids facing the IMS. Most other proteins involved in membrane fusion, such as SNAREs (Rothman and Warren, 1994; Pelham, 1999), the influenza HA protein (White et al., 1996), and Fzo1p (Hermann et al., 1998; Rapaport et al., 1998), contain few if any residues on the opposite side of the membrane to where fusion takes place. Mitochondria, in contrast to most other organelles, have two membranes. The mitochondrial inner membrane appears to fuse immediately after outer membrane fusion (Okamoto et al., 1998), suggesting a coupling between both fusion events. We speculate that the COOH terminus of Ugo1p may interact with inner membrane fusion machinery. We note that the COOH terminus of Ugo1p contains a mitochondrial energy transfer protein motif (Nelson et al., 1998) which is found in many inner membrane proteins. Studies to determine the role Ugo1p plays in mitochondrial inner and outer membrane fusion are in progress.

Although mitochondrial fusion occurs predominately at the tips of mitochondrial tubules (Nunnari et al., 1997), our studies show that Ugo1p is present throughout the mitochondrial outer membrane. Similarly, Fzo1p shows a uniform distribution along the mitochondrial tubule (Hermann et al., 1998). It is possible that mitochondrial fusion is activated only at sites of fusion. For example, the Fzo1p GTPase may act as a molecular switch that regulates mitochondrial fusion by activating the fusion machinery at the appropriate time. Alternatively, the fusion machinery may be transiently concentrated at fusion sites. It is also possible that mitochondria are competent to fuse anywhere along the tubule, but fusion is directed by controlling contact between organelles. For example, the cytoskeleton may play a crucial role in positioning mitochondria during their fusion.

Is Ugo1p part of a fusion machine? Gel filtration studies of detergent-solubilized mitochondria show that Fzo1p is found in an ~800-kD complex (Rapaport et al., 1998). Since both Fzo1p and Ugo1p are located in the mitochondrial outer membrane and play essential roles in mitochondrial fusion, it is possible that both proteins are part of the same complex. Both proteins appear to be defective in a late step in the fusion pathway. In matings between *ugo1*Δ mutants and mitochondria the two parent cells are closely paired in the neck of the zygote, but do not fuse. Similar connections between unfused mitochondria were seen in matings between *fzo1*Δ cells (Hermann et al., 1998). However, preliminary studies have shown that Fzo1p and Ugo1p do not coimmunoprecipitate (Sesaki, H., unpublished observations). Therefore, it is possible that Fzo1p and Ugo1p mediate distinct steps in mitochondrial fusion and do not physically interact. We note that whereas *fzo1* and *ugo1* mutants both contain fragmented mitochondria, the organelles tend to aggregate in *ugo1* mutants, but mitochondria remain dispersed in *fzo1* cells. Further studies are clearly needed to determine the role of Ugo1p and Fzo1p in mitochondrial fusion.

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