Gag3p, an Outer Membrane Protein Required for Fission of Mitochondrial Tubules

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Abstract. Mitochondrial morphology and function depend on *MGM1*, a *Saccharomyces cerevisiae* gene encoding a dynamin-like protein of the mitochondrial outer membrane. Here, we show that mitochondrial fragmentation and mitochondrial genome loss caused by lesions in *MGM1* are suppressed by three novel mutations, *gag1*, *gag2*, and *gag3* (for glycerol-adapted growth). Cells with any of the *gag* mutations displayed aberrant mitochondrial morphology characterized by elongated, unbranched tubes and highly fenestrated structures. Additionally, each of the *gag* mutations prevented mitochondrial fragmentation caused by loss of the mitochondrial fusion factor, Fzo1p, or by treatment of cells with sodium azide. The *gag1* mutation mapped to *DNM1* that encodes a dynamin-related protein required for mitochondrial fission. *GAG3* encodes a novel WD40-repeat protein previously found to interact with Dnm1p in a two-hybrid assay. Gag3p was localized to mitochondria where it was found to associate as a peripheral protein on the cytosolic face of the outer membrane. This association requires neither the *DNM1* nor *GAG2* gene products. However, the localization of Dnm1p to the mitochondrial outer membrane is substantially reduced by the *gag2* mutation, but unaffected by loss of Gag3p. These results indicate that Gag3p plays a distinct role on the mitochondrial surface to mediate the fission of mitochondrial tubules.

Key words: mitochondria • membrane dynamics • *Saccharomyces cerevisiae* • organelle division • mitochondrial division

Introduction

Mitochondria are complex organelles that perform essential metabolic functions in nearly all eukaryotic cells. These functions depend on a highly conserved composition and internal structure, yet the shape and distribution of mitochondria vary enormously depending on cellular type, function, and nutritional status (Yaffe, 1999a). In many proliferating cells, mitochondria comprise dynamic reticular networks whose tubules undergo frequent division, fusion, and redistribution in the cytoplasm (Bereiter-Hahn and Voth, 1994). Recent studies have uncovered a few of the components that facilitate these changes in mitochondrial shape and distribution (Hermann and Shaw, 1998; Yaffe, 1999b), but many mechanisms mediating mitochondrial dynamics have yet to be described.

Among key factors regulating mitochondrial behavior are three GTP-binding proteins that act at the mitochondrial surface. One of these, the fuzzy onions protein in *Drosophila* or its yeast homologue, Fzo1p, mediates mitochondrial fusion (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998). Another component, the dynamin-related yeast protein Dnm1p or its animal cell homologue, Drp1, facilitates the fission of mitochondrial tubules (Otsuga et al., 1998; Smirnova et al., 1998; Bleazard et al., 1999; Labrousse et al., 1999; Sesaki and Jensen, 1999). A third factor, Mgm1p, another dynamin-like protein, is essential for the maintenance of mitochondrial tubules and also plays a role in mitochondrial inheritance in *Saccharomyces cerevisiae* (Guan et al., 1993; Shepard and Yaffe, 1999). This latter protein is also essential for the maintenance of mitochondrial DNA (Jones and Fangman, 1992; Guan et al., 1993), and cells depleted of Mgm1p rapidly become respiration-deficient.

To further investigate the role of Mgm1p, genetic suppressors that prevent mitochondrial DNA loss in *mgm1* mutant cells were isolated. The analysis of these suppressors has led to the identification of a novel protein that mediates the fission of mitochondrial tubules.

Materials and Methods

Strains and Genetic Techniques

Yeast strains used in this study are listed in Table I. Strain JSY1361 was a gift from J. Shaw (University of Utah, Salt Lake City, UT). All other strains are isogenic to MYY290 (Smith and Yaffe, 1991). Strain MYY971 was described previously (Shepard and Yaffe, 1999). Strains MYY993, MYY994, and MYY995 were isolated as described below. Strain MYY1200 was constructed as described below, and strain MYY1201 was derived as a haploid segregant

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Table I. Strains Used in This Study

Strain	Genotype	Source
MYY290	*MAT a, his3, leu2, ura3*	Smith and Yaffe, 1991
MYY291	*MAT α, his3, leu2, ura3*	Smith and Yaffe, 1991
MYY971	*MAT a, mdm17, his3, leu2, ura3*	Shepard and Yaffe, 1999
MYY977	*MAT a, gag1, his3, leu2, ura3*	This study
MYY981	*MAT a, gag2, his3, leu2, ura3*	This study
MYY986	*MAT a, gag3, his3, leu2, ura3*	This study
MYY993	*MAT a, mdm17, gag1, his3, leu2, ura3*	This study
MYY994	*MAT a, mdm17, gag2, his3, leu2, ura3*	This study
MYY995	*MAT a, mdm17, gag3, his3, leu2, ura3*	This study
MYY1200	*MAT a, ura3::c4GFP-LEU2, his3, leu2*	This study
MYY1201	*MAT α, ura3::c4GFP-LEU2, his3, leu2*	This study
MYY1202	*MAT a, ura3::c4GFP-LEU2, dnm1::DNM1myc-HIS3, his3, leu2*	This study
MYY2000	*MAT a, fzo1::URA3, his3, leu2, ura3*	This study
MYY2001	*MAT α, fzo1::URA3, his3, leu2, ura3*	This study
MYY2005	*MAT a, fzo1::URA3, his3, leu2, ura3::c4GFP-LEU2*	This study
MYY2007	*MAT a, fzo1::URA3, dnm1::HIS3, his3, leu2, ura3::c4GFP-LEU2*	This study
MYY2009	*MAT a, fzo1::URA3, gag2, his3, leu2, ura3::c4GFP-LEU2*	This study
MYY2011	*MAT a, fzo1::URA3, gag3, his3, leu2, ura3::c4GFP-LEU2*	This study
MYY2013	*MAT a, dnm1::HIS3, his3, leu2, ura3::c4GFP-LEU2*	This study
MYY2014	*MAT α, dnm1::HIS3, his3, leu2, ura3::c4GFP-LEU2*	This study.
MYY2016	*MAT α, his3, leu2, ura3::c4GFP-LEU2, gag3::GAG3HA-HIS3*	This study
MYY2017	*MAT a, his3, leu2, ura3, gag3::KanMX4*	This study
MYY2019	*MAT a, his3, leu2, ura3::c4GFP-LEU2, gag3::KanMX4*	This study
MYY2029	*MAT a, his3, leu2, ura3::c4GFP-LEU2, gag2*	This study
MYY2030	*MAT α, his3, leu2, ura3::c4GFP-LEU2, gag2*	This study
MYY2031	*MAT a, his3, leu2, ura3::c4GFP-LEU2, gag3*	This study
MYY2033	*MAT a, his3, leu2, ura3::c4GFP-LEU2, gag1*	This study
JSY1361	*MAT a, dnm1::HIS3, his3, leu2, ura3*	Otsuga et al., 1998

from a cross of MYY1200 to MYY291. Strains MYY977, MYY981, and MYY986 were isolated as haploid segregants from crosses of MYY993, MYY994, and MYY995, respectively, to MYY291. Strains MYY2033, MYY2029, MYY2030, and MYY2031 were derived from crosses of MYY977, MYY981, and MYY986 to MYY1201. Strain MYY2001 was created as described below. MYY2000 was a haploid segregant from a cross of strain MYY2001 to MYY290. Strain MYY2005 was derived from a cross of MYY2000 to MYY1201. MYY2007, MYY2009, and MYY2011 were haploid segregants derived from crosses of strain MYY2001 to strains MYY2013, MYY2029, and MYY2031, respectively. Strain MYY2013 and MYY2014 were derived from a cross of strain JSY1391 to MYY1201. Epitope-tagged strains MYY2016 and MYY1202 were created as described below. Strains MYY2017 and MYY2019 were derived from a cross of yeast disruption strain 11311 (Research Genetics, Inc.) to strain MYY1201.

Culture conditions and genetic analysis of yeast followed standard procedures (Rose et al., 1990). Plasmid DNA was prepared from *Escherichia coli* strain DH5α.

Gene Disruption and Tagging

The *fzo1:URA3* deletion (MYY2001) was created by PCR-mediated gene disruption as described by Baudin et al. (1993). In brief, the primers fzo-ko1, 5′-ATGTCTGAAGGAAAACAACAATTCAAAGACAGCAATAAAGA-TTGTACTGAGAGTGCACC-3′; and fzo-ko2, 5′-CTAATCGATGTC-TAAATTTATTTCTTCCACCATCAATTTTGTGCGGTATTTCCAC-CGC-3′ were used to amplify the *URA3* gene from plasmid pRS306 (Sikorski and Hieter, 1989). This cassette was used to transform strain MYY291, Ura⁺ transformants were selected, and the disruption was verified by PCR and phenotypic analysis. Mating of the disruptant strain to MYY290, sporulation, and isolation of meiotic progeny allowed for identification of a *fzo1-URA3* spore of the opposite mating type (MYY 2000). Strains MYY1202 and MYY2016 were created with the PCR-mediated gene tagging technique described by Knop et al. (1999) using strains MYY1200 and MYY1201.

A version of green fluorescent protein (GFP)[1] fused in frame with the mitochondrial targeting sequence of cytochrome oxidase subunit 4

(COX4) was created as follows. Sequences corresponding to GFP were amplified by PCR from plasmid pS65T-C1 (Clontech) using primer C4-GFP, 5′-CGGGATCCGTCGACATGCTTTCACTACGTCAATC-TATAAGATTTTTCAAGCCAGCCACAAGAACTTTGTGTAGCT-CTAGAGTGGGTAAAGGGAGAAGAACT-3′, which encoded the mitochondrial targeting sequence of COX4, and primer GFP-3P, 5′-TGC-CCGGGATCCCTAGTATAGTTCATCCATGCC-3′. The resulting PCR product was digested with BamHI, made blunt-ended with Klenow, and ligated into the blunted EcoRI site downstream of the ADH1 promoter sequence of plasmid pAC1 (Hurt et al., 1985). The resulting plasmid was named pAC1-c4GFP. Plasmid YIp5-LEU2 was created by inserting the *LEU2*-containing XhoI–SalI fragment from plasmid YEp13 (Broach et al., 1979) into the SalI site of plasmid YIp5 (Struhl et al., 1979). Plasmid YIp5-LEU2-c4GFP was constructed by isolating the EcoRV fragment encoding ADH1-c4GFP from plasmid pAC1-c4GFP, and ligating this fragment into the SmaI site of YIp5-LEU2. Then, strain MYY1200 was created by transformation of MYY290 with YIp5-LEU2-c4GFP that had been linearized with StuI. Colonies that were auxotrophic for leucine were verified for the presence of green mitochondria by fluorescence microscopy.

The GFP-tagged DNM1 vector, pHS20, was a gift from H. Sesaki and R. Jensen (Johns Hopkins University Medical School, Baltimore, MD).

Isolation of the gag Mutants

mdm17 cells (MYY971) were plated on YPG-agar medium (1% yeast extract, 2% bactopeptone, 3% glycerol) and cultured at 37°C for 3 d. Colonies that grew were backcrossed to wild-type cells, and meiotic progeny were analyzed for growth on YPG-medium at 37°C and by microscopy for defects in mitochondrial morphology. Cells from these colonies were stained with 2-(4-dimethylaminostryl)-1-methylpyridinium iodide (DASPMI) and examined by fluorescence microscopy as previously described (McConnell et al., 1990). Candidates with abnormal mitochondrial morphologies, including MYY993, MYY994, and MYY995, were backcrossed to wild-type cells to determine whether restored growth was due to reversion or second site suppressors. Candidates harboring mutations that caused recessive mitochondrial morphology defects similar to those described for *dnm1* (Otsuga et al., 1998) were crossed to one another and assigned to complementation groups *gag1*, *gag2*, and *gag3* (for glycerol-adapted growth).
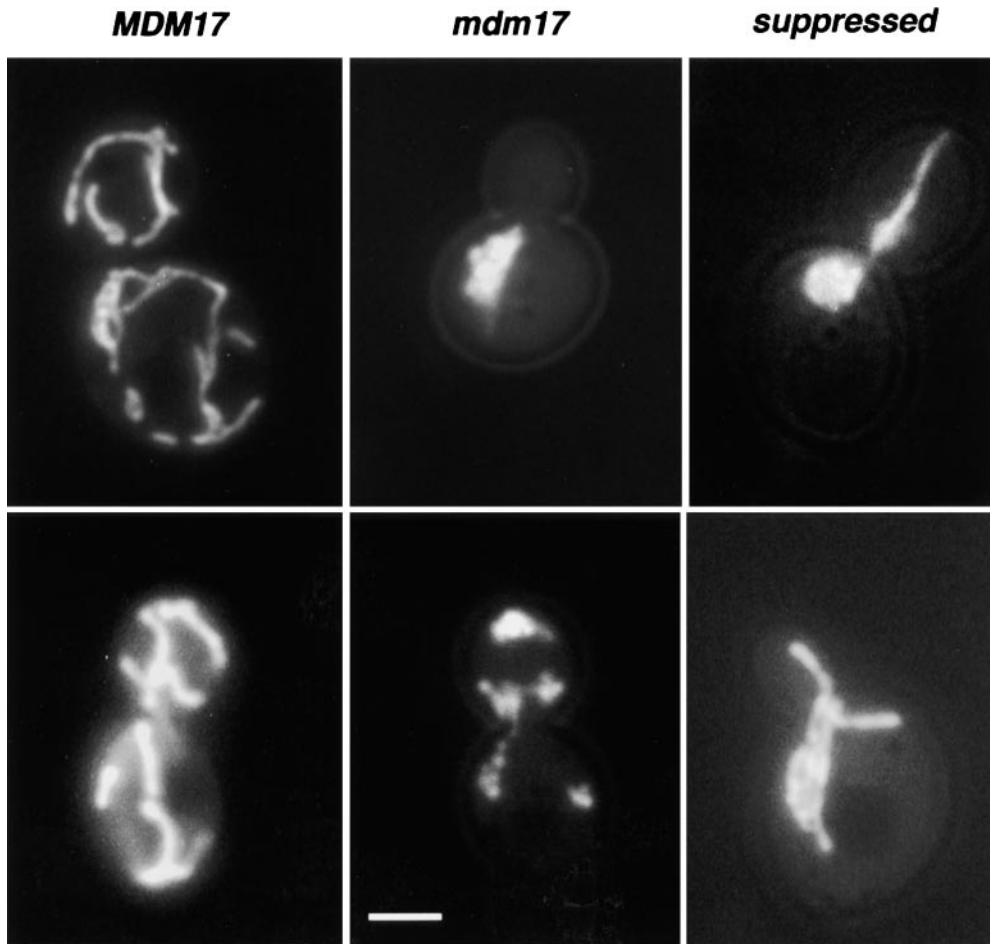
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[1]*Abbreviations used in this paper:* DASPMI, 2-(4-dimethylaminostryl)-1-methylpyridinium iodide; gag, glycerol-adapted growth (mutant); GFP, green fluorescent protein; YPG, yeast extract/peptone/glycerol.

MDM17	mdm17	suppressed

Figure 1. Suppressing mutations prevent mitochondrial fragmentation in the *mdm17* mutant. Wild-type (MYY290; left), *mdm17* (MYY971; center), and suppressed *mdm17* (MYY993) cells were grown at 37°C on YPD-medium, stained with DASPMI, and visualized by fluorescence microscopy. Bar, 2 μm.

Subcellular Fractionation and Analysis

Cells were grown in semisynthetic lactate medium (Daum et al., 1982), converted to spheroplasts, homogenized, and subcellular fractions were isolated by differential centrifugation as previously described (Schauer et al., 1985; Yaffe, 1991). Mitochondria were treated with trypsin, sodium chloride, sodium carbonate, and urea as described previously (Sogo and Yaffe, 1994), except that the extraction time was reduced to 10 min.

Quantification of Dnm1p Binding to Mitochondria

To quantify the presence of GFP-labeled Dnm1p on mitochondria, cells freshly transformed with plasmid pHS20 were stained with 5 μg/ml DASPMI and examined by fluorescence microscopy. Mitochondria appeared yellow and GFP–Dnm1p appeared green when viewed on the fluorescein channel. For each sample, >300 cells were examined by two independent observers, and the fractions of GFP–Dnm1p dots that were associated with the mitochondria were determined. To visualize the mitochondria separately from GFP–Dnm1p, cells were labeled with MitoTracker red CMXRos (Molecular Probes).

Results

gag Mutations Suppress mgm1 Defects in Mitochondrial Morphology and Function

MGM1 was shown previously to encode a mitochondrial outer membrane protein required for mitochondrial genome maintenance, mitochondrial inheritance, and the determination of normal mitochondrial morphology (Shepard and Yaffe, 1999). To identify components that act together with Mgm1p to mediate these functions, second-site suppressors that restored growth of *mgm1* mutant cells on glycerol-containing media were isolated. These suppressors were isolated in a strain harboring *mdm17*, a temperature-sensitive allele of *MGM1*. From $\sim 10^9$ cells, 30 colonies able to grow at 37°C on glycerol-medium were identified. Fluorescence microscopy revealed that cells harboring the suppressing mutations possessed tubular mitochondria at 37°C, in contrast to unsuppressed *mdm17* cells that contained fragmented and spherical mitochondria (Fig. 1). However, suppression of the mitochondrial morphology defects was not complete, as mitochondrial tubules appeared bundled, aggregated, and restricted in their lateral distribution (Fig. 1).

To determine whether the "glycerol-adapted" strains harbored second-site suppressor mutations, each candidate was crossed to a wild-type strain, and the meiotic progeny were analyzed for defects in mitochondrial morphology. The suppressors were found to be genetically unlinked to *MGM1* and were designated *gag* (glycerol-adapted growth) mutations. The *gag* lesions defined three distinct complementation groups, *gag1*, *gag2*, and *gag3*. Interestingly, each of the *gag* mutations was able to rescue the respiration deficiency associated with a null allele of *MGM1*, suggesting that the mechanism of suppression involved a bypass of normal *MGM1* function. Additionally, crossing the suppressed *mdm17* cells to an *mgm1*-null strain resulted in diploids with an *mdm17*-like phenotype, indicating that *gag* suppression is recessive (data not shown).
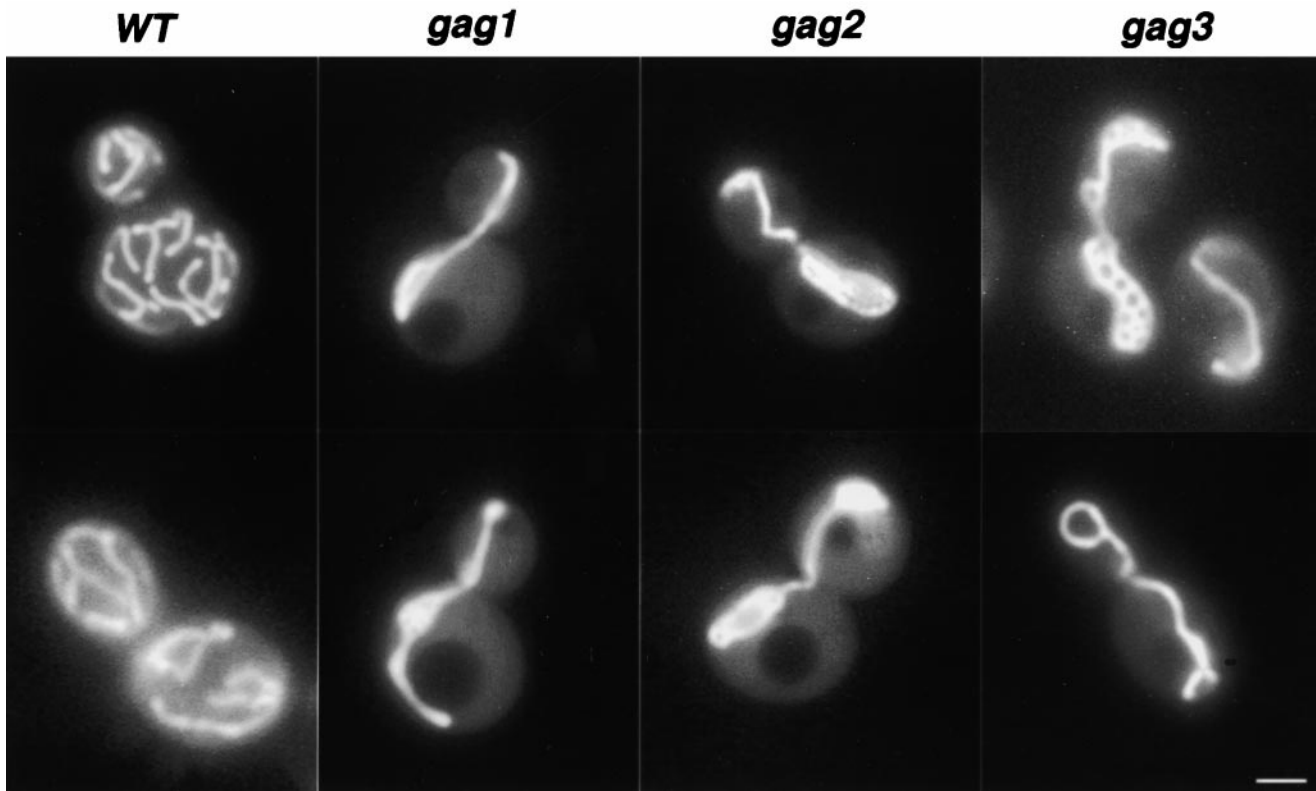
Figure 2. gag Mutants display abnormal mitochondrial morphology. Wild-type (MYY1200), *gag1* (MYY2033), *gag2* (MYY2029), or *gag3* (MYY2031) cells with GFP-labeled mitochondria were cultured on YPD-medium and examined by fluorescence microscopy. Two representative cells are shown for each mutant strain. Bar, 2 μm.

Haploid cells harboring any of the *gag* mutations, together with wild-type *MGM1*, displayed aberrant mitochondrial morphology characterized by large reticular bundles and net-like sheets of highly branched tubules (Fig 2). Despite their unusual appearance, these abnormal mitochondrial structures caused no apparent defect in growth under a variety of conditions (data not shown). These phenotypes were remarkably similar to those described for cells mutant for *DNM1/MDM29*, another gene known to control mitochondrial dynamics (Otsuga et al., 1998). To determine whether any of the *gag* mutants mapped to the *dnm1* locus, *gag1*, *gag2*, and *gag3* cells were crossed to a strain bearing a *dnm1*-null allele, and their meiotic progeny were analyzed. No recombination was detected between *gag1* and *dnm1*, indicating that *gag1* is a mutation in *DNM1*. The double mutant recombinants *dnm1 gag2*, *gag2 gag3*, and *dnm1 gag3*, and the triple mutant, *dnm1 gag2 gag3*, were recovered and displayed the same mitochondrial phenotype as the single mutants (data not shown), suggesting that the mutations are located in genes that participate in the same cellular process.

gag Mutants Are Defective for Mitochondrial Fission

Recently, two groups have demonstrated that Dnm1p is an essential component of the mitochondrial fission machinery (Bleazard et al., 1999; Sesaki and Jensen, 1999). The similarity of the *gag2* and *gag3* mutant phenotypes to that of *dnm1* suggested that they also participate in mitochondrial fission. To test this possibility, the *gag* mutants were crossed to a yeast strain deleted for the mitochondrial fusion factor gene, *FZO1*. Cells lacking Fzo1p display frag-

mented mitochondria that readily lose mitochondrial DNA (Hermann et al., 1998; Rapaport et al., 1998), and the development of these phenotypes depends on Dnm1p (i.e., a *dnm1* mutation prevents mitochondrial fragmentation and genome loss in an *fzo1* mutant; Bleazard et al., 1999; Sesaki and Jensen, 1999). Analysis of *gag2 fzo1* and *gag3 fzo1* double mutants revealed that these new *gag* lesions similarly prevented the fragmentation of mitochondrial tubules (Fig. 3 A) and allowed growth on glycerol-containing medium (Fig. 3 B), indicating that mitochondrial genome loss was prevented.

Further evidence that *GAG2* and *GAG3* gene products participate in mitochondrial fission was revealed by examination of mitochondria in cells treated with sodium azide. Treatment of wild-type cells with this energy poison leads to fragmentation of mitochondrial tubules (Fig. 3 C). However, such fragmentation does not occur in cells harboring *dnm1* (*gag1*), *gag2*, or *gag3* mutations (Fig. 3 C). The absence of fragmentation in *dnm1* cells indicates that the mitochondrial fission apparatus mediates the alterations in mitochondrial morphology induced by sodium azide. Furthermore, the absence of fragmentation in *gag2* or *gag3* cells support the role of these novel *GAG* genes in the fission process.

GAG3 Encodes a WD40 Domain Protein

A recent global screen for protein interactions in *S. cerevisiae* by two-hybrid analysis revealed an interaction between Dnm1p and the product of an uncharacterized open reading frame (ORF), YJL112w (Uetz et al., 2000). The predicted product of this ORF is an 80-kD (714 aa)
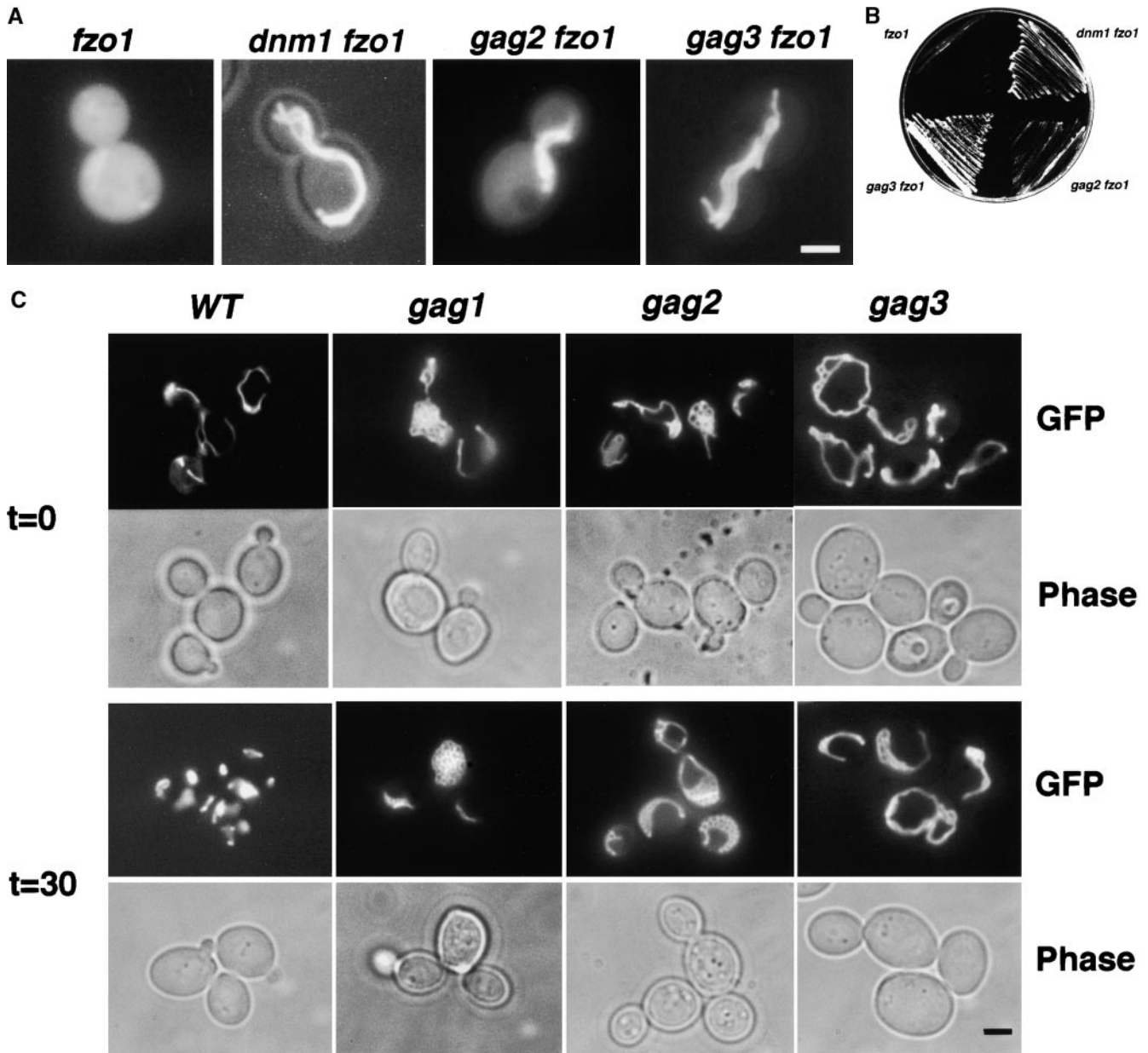
Figure 3. GAG genes participate in mitochondrial fission. A, Cells harboring GFP-labeled mitochondria and mutant for *FZO1* alone (strain MYY2005) or together with either *dnm1/gag1* (strain MYY2007; left), *gag2* (strain MYY2009; center), or *gag3* (strain MYY2011; right) mutations were visualized by fluorescence microscopy. B, Cells with the *fzo1* mutation (MYY2005) or the double mutants *dnm1 fzo1* (MYY2007), *gag2 fzo1* (MYY2009), or *gag3 fzo1* (MYY2011) were plated on glycerol-medium and cultured for 3 d at 30°C. C, Wild-type (MYY1200), *dnm1/gag1* (MYY2013), *gag2* (MYY2029), and *gag3* (MYY2031) cells with GFP-labeled mitochondria were treated with 0.5 mM sodium azide and examined immediately (top) or after 30 min (bottom) by fluorescence microscopy. Bar, 2 μm.

polypeptide possessing six WD40 repeats in the COOH-terminal half of the protein and a region in the NH$_2$-terminal half predicted to form a coiled-coil. Since proteins that interact with Dnm1p might comprise other components of the mitochondrial fission machinery, the function of YJL112w and its relationship to *gag2* and *gag3* were examined. Cells bearing a null-mutation in YJL112w displayed mitochondrial morphology and distribution defects identical to those found in *gag* mutant cells (data not shown). Additionally, genetic analysis involving crosses of strains deleted for YJL112w to *gag2* or *gag3* strains, revealed that *gag3* maps to YJL112w. This identity was further supported by DNA sequence analysis of YJL112w isolated from the *gag3* mutant, which revealed a deletion of nucle-

otides 1453 and 1454. This lesion results in a frameshift mutation at amino acid residue 485 and a truncation of the protein after five additional residues. These results demonstrate that *GAG3* corresponds to YJL112w.

Gag3p Is a Peripheral Protein of the Mitochondrial Outer Membrane

To determine the site of Gag3p activity, a yeast strain was created in which the chromosomal copy of *GAG3* was replaced by a version of the gene encoding a form of Gag3p with an HA-epitope tag at the COOH terminus. This tagged protein was fully functional, as cells expressing only this variant gene displayed mitochondrial morphology in-
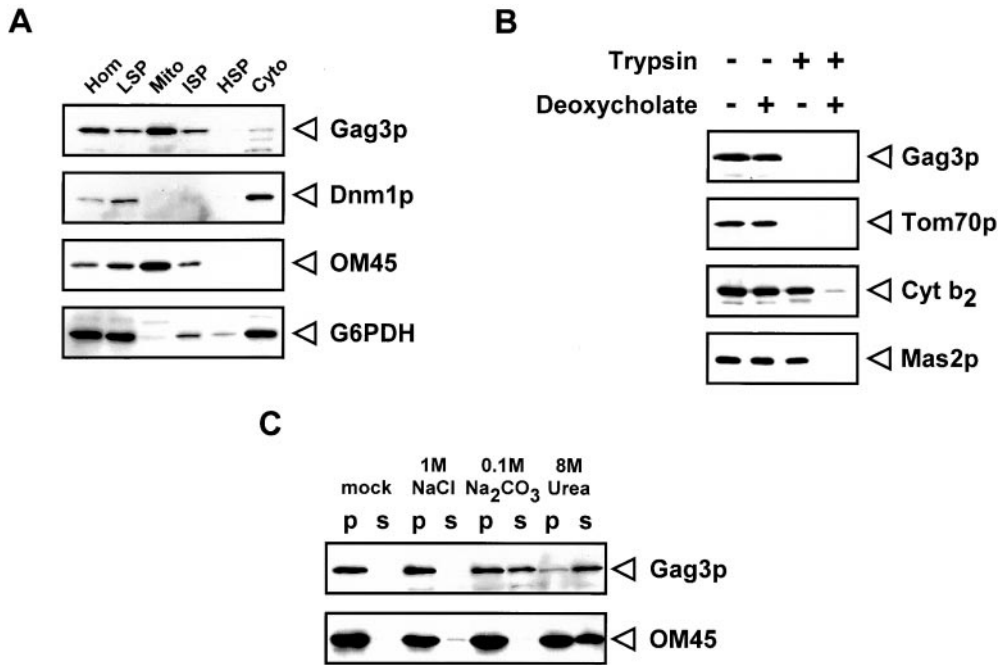
A

Hom LSP Mito ISP HSP Cyto

◁ Gag3p

◁ Dnm1p

◁ OM45

◁ G6PDH

B

Trypsin   − − + +
Deoxycholate − + − +

◁ Gag3p

◁ Tom70p

◁ Cyt b_2

◁ Mas2p

C

mock | 1M NaCl | 0.1M Na_2CO_3 | 8M Urea

p s   p s   p s   p s

◁ Gag3p

◁ OM45

Figure 4. Gag3p is a peripheral protein of the mitochondrial outer membrane. A, Cells expressing HA-tagged Gag3p (MYY2016) or myc-tagged Dnm1p (MYY1202) were grown on semisynthetic lactate medium (Daum et al., 1982). Cells were homogenized and subcellular fractions were isolated by differential centrifugation. The homogenate (hom), low-speed pellet (LSP), mitochondrial (mito), intermediate-speed pellet (ISP), high-speed pellet (HSP), and the cytosolic fractions (cyto) were analyzed by SDS-PAGE and immunoblotting to detect Gag3p, Dnm1p, the mitochondrial outer membrane protein OM45, and the cytosolic protein glucose-6-phosphate dehydrogenase (G6PDH). Each lane contained 10 μg total protein. B, Trypsin treatment of mitochondria. Isolated mitochondria containing HA-tagged Gag3p were treated with trypsin in the presence (+) or absence (−) of deoxycholate detergent and analyzed by SDS-PAGE and immunoblotting. Samples were tested for the presence of Gag3p, the outer membrane protein Tom70p, the intermembrane space protein cytochrome b_2, and the mitochondrial matrix protein Mas2p. C, Gag3p is peripherally associated with mitochondrial membranes. Isolated mitochondria containing HA-tagged Gag3p were treated with 1 M NaCl, 0.1 M Na_2CO_3, or 8 M urea. Mitochondria were centrifuged, and the resulting pellet (p) and supernatant (s) fractions were analyzed by SDS-PAGE and immunoblotting for Gag3p and OM45.

distinguishable from that of wild-type cells (data not shown). Cells expressing the tagged protein were homogenized and subcellular fractions were isolated by differential centrifugation. Immunoblot analysis revealed that Gag3p was enriched in the mitochondrial fraction (Fig. 4 A). This pattern was similar to that observed for the mitochondrial outer membrane protein, OM45, but contrasted with that found for glucose-6-phosphate dehydrogenase, a cytosolic protein. An identical pattern of Gag3p fractionation with mitochondria was observed in Δdnm1, *gag2*, and double mutant Δ*dnm1 gag2* cells, indicating that the mitochondrial association of Gag3p is independent of functional Dnm1p or Gag2p (data not shown). In contrast to Gag3p, Dnm1p was essentially absent from the mitochondrial fraction and largely recovered in the cytosolic fraction (Fig. 4 A). This distribution agrees with that previously reported for Dnm1p in subcellular fractions (Otsuga et al., 1998), although indirect immunofluorescence microscopy revealed a mitochondrial localization for Dnm1p (Otsuga et al., 1998; Sesaki and Jensen, 1999). This discrepancy between microscopic localization and recovery in isolated subcellular fractions may reflect a weak association or transient interaction of Dnm1p with mitochondria. Although we have been unable to detect Gag3p by indirect immunofluorescence microscopy (data not shown), its association with mitochondria is strong enough to persist through purification of the organelle.

To determine the submitochondrial location of Gag3p, the protein's accessibility to the protease trypsin was assessed (Fig. 4 B). Mild trypsin treatment of isolated mitochondria readily digested Gag3p. The same treatment also cleaved Tom70p, a mitochondrial outer membrane protein with a large cytosolic domain. Proteins of the mitochondrial matrix and intermembrane space, Mas2p and cytochrome b_2, respectively, were protected from the protease treatment unless membranes were disrupted with the detergent deoxycholate (Fig. 4 B). These results suggest that Gag3p is located on the mitochondrial outer membrane.

The association of Gag3p with the mitochondrial outer membrane was investigated by chemical treatment of isolated mitochondria (Fig. 4 C). Gag3p remained associated with mitochondria in the presence of 1 M NaCl, was partially extracted by washing with 0.1 M Na_2CO_3, and was mostly removed by treatment with 8 M urea (Fig. 4 C). Although both Na_2CO_3 and urea treatment released Gag3p from the mitochondria, a substantial fraction remained associated under these conditions, suggesting a strong interaction between Gag3p and mitochondria. All together, these data demonstrate that Gag3p is a peripheral protein bound to the cytosolic surface of the mitochondrial outer membrane.

Localization of Dnm1p Depends on GAG2, but Not on GAG3

To examine the role of the *GAG2* and *GAG3* gene products in localization of Dnm1p to the mitochondrial surface, the distribution of GFP-tagged Dnm1p was determined in *gag2* and *gag3* mutant cells by fluorescence microscopy (Fig. 5). Mitochondria in these same cells were labeled with MitoTracker red dye. As described previously (Otsuga et al., 1998; Sesaki and Jensen, 1999), in wild-type cells, Dnm1p was localized to punctate structures largely associated with mitochondrial tubules (Fig 5). This pattern
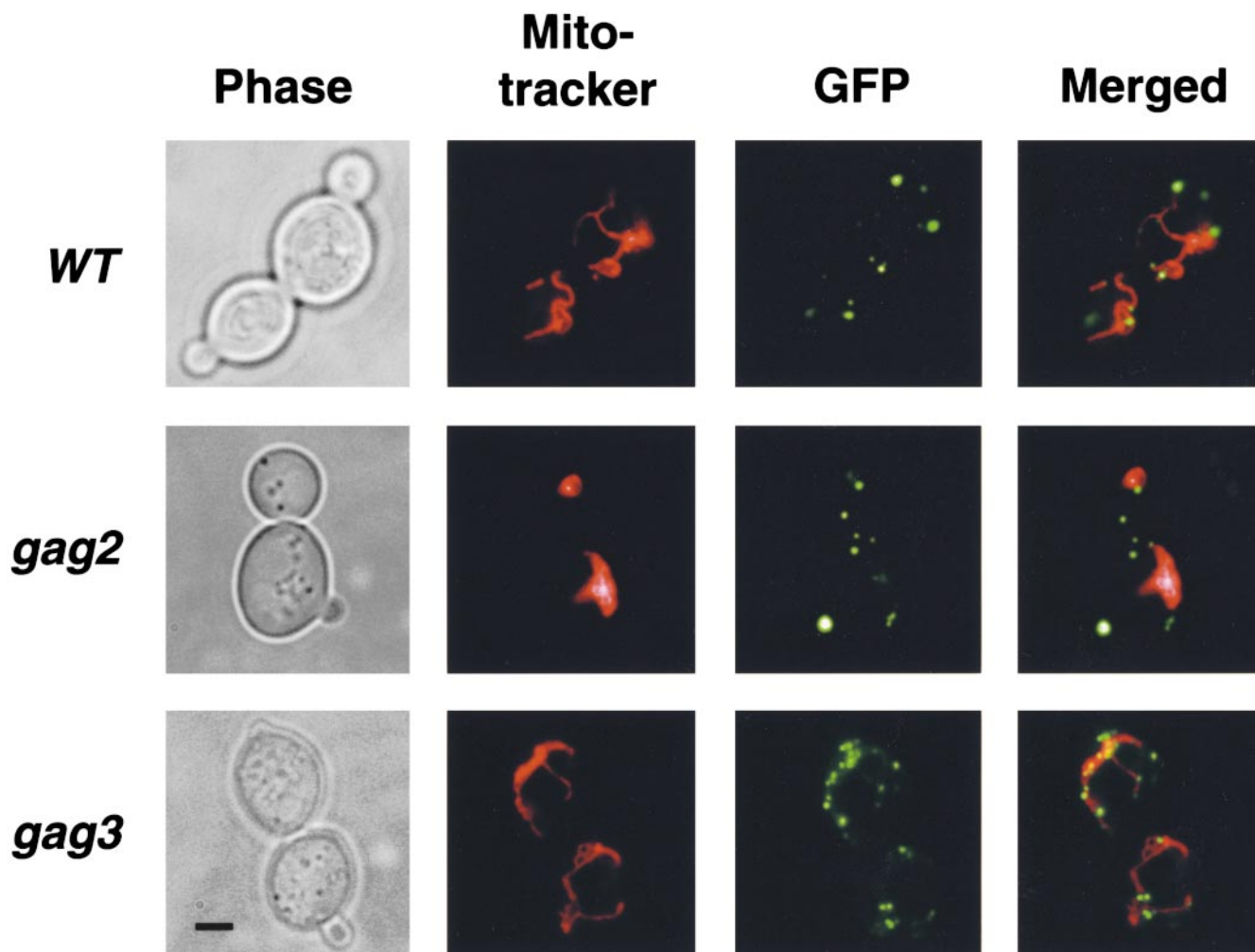
Figure 5. Localization of Dnm1p in *gag* mutant cells. Wild-type (MYY290), *gag2* (MYY981), and *gag3* (MYY2017) cells expressing GFP-tagged Dnm1p were cultured on minimal glucose medium, stained with MitoTracker Red CMXRos, and analyzed by fluorescence microscopy. Pseudocolor was added to the digitized image. GFP-labeling is shown in green, MitoTracker is shown in red, and the merged images are shown on the right. Bar, 2 μm.

was also observed in *gag3* cells, despite their abnormal mitochondrial morphology (Fig. 5). In *gag2* cells, however, a substantial fraction of Dnm1p appeared to be displaced from the mitochondrial network and randomly distributed through the cytoplasm. Interestingly, Dnm1p appeared to be associated in punctate structures, even when not localized to the mitochondria (Fig. 5). Quantitative analysis of this distribution indicated that 94% (± 4%, $n = 319$) of fluorescent Dnm1p spots were associated with mitochondria in wild-type cells, and 89% (± 5%, $n = 384$) in *gag3*-null cells. In contrast, only 46% (± 14%, $n = 642$) of Dnm1p structures were localized to mitochondria in *gag2* cells. These results indicate that the *GAG2* gene product plays a role in promoting the interaction of Dnm1p with the mitochondrial surface, but that Gag3p does not mediate this interaction.

Discussion

Division of mitochondrial tubules contributes to the overall morphology of the mitochondrial reticulum (Yaffe,

1999a). Our findings reveal that the *gag* mutations define components that mediate mitochondrial fission. *gag1* mapped to *DNM1* that previously was shown to encode a dynamin-related protein essential for mitochondrial division (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999). Three observations indicate that *GAG2* and *GAG3* also encode fission factors. First, the *gag2* and *gag3* mutants display defects in mitochondrial morphology that are essentially identical to those observed in *dnm1* mutant cells (Fig. 2). Second, as previously shown for *DNM1*, mutations in both *GAG2* and *GAG3* prevent mitochondrial fragmentation caused by loss of the fusion factor, Fzo1p (Fig. 3 A). Finally, mitochondria in *gag2*, *gag3*, and *dnm1* mutants remain tubular upon addition of sodium azide, an agent that stimulates the fragmentation of mitochondrial tubules in wild-type cells (Fig. 3 C).

Both Gag3p and Dnm1p function on the outer membrane to mediate mitochondrial division, yet they display distinct characteristics in their association with the mitochondrial surface. Gag3p is tightly bound to the mitochondrial outer membrane, whereas the association of Dnm1p

appears weaker, since the protein is recovered with the cytosolic fraction during subcellular fractionation (Fig. 4 A; Otsuga et al., 1998). Additionally, the *gag2* mutation causes displacement of a major fraction of Dnm1p to the cytosol in intact cells (Fig. 5), yet Gag3p remains tightly bound to mitochondria isolated from *gag2* mutant cells (data not shown). Although two-hybrid analysis and localization studies are consistent with an interaction of Dnm1p and Gag3p, such an association is not required for the localization of the two proteins to the outer membrane, since neither protein is displaced in the absence of the other. These differences suggest that Gag3p and Dnm1p play distinct roles in the fission process.

The specific molecular function of Gag3p in mitochondrial division is unknown. Unlike Dnm1p, whose homologue dynamin mediates the scission step at the neck of coated pits in animal cells (Schmid et al., 1998, van der Bliek, 1999), no proteins related to Gag3p have been implicated in other membrane fission events. One clue to the protein's function might lie in its two distinct structural domains, a coiled-coil region in the NH_2-terminal half of the protein and six WD40-repeats in the COOH-terminal half. These features are likely to comprise protein interaction domains (Smith et al., 1999) and suggest a role for Gag3p in bringing together or organizing multiple components on the mitochondrial surface. One interacting partner is likely to be Dnm1p, although the dissimilar behavior of these components during subcellular fractionation and our inability to detect binding with coimmunoprecipitation analysis (data not shown) suggest a transient or unstable interaction of these proteins. Gag3p is likely to bind other outer membrane proteins and, in particular, one or more integral membrane proteins. The identification of these binding partners and an analysis of the dynamic interactions of fission components will lead to the elucidation of molecular mechanisms that mediate mitochondrial division.

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