Ugo1p Links the Fzo1p and Mgm1p GTPases for Mitochondrial Fusion*

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In yeast, mitochondrial fusion requires Ugo1p and two GTPases, Fzo1p and Mgm1p. Ugo1p is anchored in the mitochondrial outer membrane with its N terminus facing the cytosol and C terminus in the intermembrane space. Fzo1p is also an outer membrane protein, whereas Mgm1p is located in the intermembrane space. Recent studies suggest that these three proteins form protein complexes that mediate mitochondrial fusion. Here, we show that the cytoplasmic domain of Ugo1p directly interacts with Fzo1p, whereas its intermembrane space domain binds to Mgm1p. We identified the Ugo1p-binding site in Fzo1p and demonstrated that Ugo1p-Fzo1p interaction is essential for the formation of mitochondrial shape, maintenance of mitochondrial DNA, and fusion of mitochondria. Although the GTPase domains of Fzo1p and Mgm1p regulate mitochondrial fusion, they were not required for association with Ugo1p. Furthermore, we found that Ugo1p bridges the interaction between Fzo1p and Mgm1p in mitochondria. Our data indicate that distinct regions of Ugo1p bind directly to Fzo1p and Mgm1p and thereby link these two GTPases during mitochondrial fusion.

Mitochondrial fusion is critical for controlling the shape, number, and distribution of this organelle in many organisms (1–7). In the yeast Saccharomyces cerevisiae, mitochondrial fusion requires at least three proteins, Fzo1p (8, 9), Ugo1p (10), and Mgm1p (11, 12). Cells lacking any of these proteins contain many small mitochondrial fragments rather than the few branched mitochondrial tubules seen in wild-type cells. These mutants were directly shown to be defective in mitochondrial fusion using a yeast cell mating assay (8, 10–12). In addition to fusion, Fzo1p, Ugo1p, and Mgm1p are also important to maintain mitochondrial DNA (mtDNA).1 fzo1, ugo1, and mgm1 mutants lose mtDNA and are therefore defective in respiration.

The fragmentation of mitochondria in fzo1, ugo1, and mgm1 mutants is a consequence of ongoing mitochondrial division in the absence of fusion. Mitochondrial division is mediated by Dnm1p (13), Gapg3p/Mdv1p/Net2p (14–16), and Fis1p (16).

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1 The abbreviations used are: mtDNA, mitochondrial DNA; IMS, intermembrane space; MBP, maltose-binding protein; OM, outer membrane; IM, inner membrane; HA, hemagglutinin; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.

dnm1, gag3/mdv1/net2, and fis1 mutants contain a single mitochrondion consisting of interconnected tubules. fzo1 dnm1, ugo1 dnm1, and mgm1 dnm1 double mutants contain nearly wild-type tubular-shaped mitochondria, suggesting that mitochondrial shape and number are normally regulated by a balance between fusion and division (13, 17). Similar to mitochondrial fragmentation, the loss of mtDNA in fzo1, ugo1, and mgm 1 mutants depends on mitochondrial division.

Fzo1p is a GTPase identified as a homolog of Drosophila fuzzy onios, which is required for mitochondrial fusion during fly spermatogenesis (8, 9, 18). Fzo1p is located in the mitochondrial outer membrane (OM) and contains two cytoplasmic domains (Fritz et al. (28); see Fig. 1A). The N-terminal cytoplasmic domain carries the GTPase domain, which is essential for mitochondrial fusion (8), whereas the role of the C-terminal cytoplasmic domain is unknown.

Mgm1p is a dynamin-related GTPase located in the intermembrane space (IMS) (11, 12, 19–21). There are two species of Mgm1p, a 90- and a 100-kDa form; the smaller form is produced by cleavage of the larger form by the rhomboid-like serine protease, Pcp1p/Rbd1p/Ugo2p (22–24). Although the exact functions of these two forms in mitochondrial dynamics are not clear, the 100-kDa form appears to be more important for mitochondrial fusion than the 90-kDa form. Cells that lack the protease express only the 100-kDa form and contain partially fragmented mitochondria but remain competent for mitochondrial fusion (22–24).

UGO1 was isolated in a genetic screen for mutants that lose mtDNA in a Dnm1p-dependent manner (10). Ugo1p is a 58-kDa protein anchored in the OM by a single transmembrane domain (see Fig. 1A). An ~33-kDa N-terminal domain faces the cytosol, whereas an ~23-kDa C-terminal domain is exposed to the IMS. Recent studies have shown that Ugo1p, Fzo1p, and Mgm1p communoprecipitate from detergent-solubilized mitochondria (12) and can be chemically cross-linked in intact mitochondria (11), suggesting that these proteins either coassemble or interact with each other in pairs. In this paper, we examine the role of Ugo1p-mediated protein-protein interactions in mitochondrial fusion. Our data suggest that distinct domains of Ugo1p directly and functionally interact with both Fzo1p and Mgm1p to link these two GTPases during mitochondrial fusion.

EXPERIMENTAL PROCEDURES

Media and Genetic Methods—Yeast cells were grown in media including YEPD (yeast extract peptone medium containing 2% dextrose), YEPGE (yeast extract peptone medium containing 2% glycerol and 2% ethanol), SRaf (synthetic medium containing 2% raffinose), and SGalSuc (synthetic medium containing 2% galactose and 2% sucrose). Standard genetic techniques were used (25).

Strains—Yeast strains used in this study are listed in Table I. fzo1Δ ugo1Δ dnm1Δ cells (YRJ1665) were obtained by crossing dnm1Δ fzo1Δ cells (YRJ1347) to ugo1Δ cells (YRJ1277). mgm1Δ ugo1Δ dnm1Δ cells (YRJ1609) were obtained by crossing dnm1Δ ugo1Δ cells (YRJ1292) to
mgm1Δ cells (YRJ1383). To create FZO1 (YRJ1811 and -1812) and fzo1-825 (YRJ1813 and -1814), pRS304-FZO1 and pRS304-fzo1-825 were digested with NsiI and transformed into MATA and MATA fzo1Δ cells (YRJ1810 and -1811) carrying pRS416-

RESULTS AND DISCUSSION

Fzo1p and Mgm1p Directly Interact with Distinct Domains of Ugo1p—Ugo1p communoprecipitates from detergent-solubilized mitochondria with Fzo1p (the OM-localized GTPase) and Mgm1p (the IMS-localized GTPase) (12). To test whether Ugo1p directly interacts with these two GTPases, we performed an in vitro binding assay using recombinant proteins. MBP and MBP fused to the cytoplasmic domain of Ugo1p (residues 1–294; MBP-cyto Ugo1p) or the IMS domain (residues 312–503; MBP-IMS Ugo1p) were expressed and purified from E. coli (Fig. 1B). Full-length 35S-labeled Fzo1p was synthesized in reticulocyte lysate and incubated with MBP fusion proteins. The MBP fusions were collected on amylose-coupled beads, and bound proteins were separated by SDS-PAGE and detected by autoradiography. As shown in Fig. 1C, ~10% of Fzo1p was found in the pellet fraction along with MBP-cyto Ugo1p but not with MBP-IMS Ugo1p or MBP alone. As a further control, we used 35S-labeled Mmm1p, an OM protein that is not involved in mitochondrial fusion (12), and found that Mmm1p did not interact with any MBP fusion. When 35S-labeled Mgm1p was incubated with MBP-IMS Ugo1p and MBP, Mgm1p specifically precipitated with MBP-IMS Ugo1p but not with MBP (Fig. 1D). These results indicate that the cytoplasmic domain of Ugo1p binds directly to Fzo1p, whereas the IMS domain of Ugo1p directly interacts with Mgm1p.

Identification of the Ugo1p-binding Site in Fzo1p—To map the Ugo1p-binding domain in Fzo1p, different regions of Fzo1p were synthesized and incubated with MBP-cyto Ugo1p. Because Fzo1p has two cytoplasmic domains (28), we first asked if individual cytoplasmic domains (Fzo1p 1–855 and Fzo1p 856–1255) bind to MBP-cyto Ugo1p and found that neither of them precipitated with MBP-cyto Ugo1p (Fig. 1E). Then we made a series of Fzo1p truncations from either its N or C terminus. MBP-cyto Ugo1p bound to Fzo1p fragments lacking the first 449 residues (Fzo1p 1–449) and 629 residues (Fzo1p 1–629) but did not bind to a shorter fragment (Fzo1p 1–629). Therefore, a small part of the N-terminal cytoplasmic domain of Fzo1p is required for its interaction with MBP-cyto Ugo1p. Similarly, the C-terminal cytoplasmic domain of Fzo1p was also essential for the interaction. Fzo1p fragments lacking 12 residues from the C terminus (Fzo1p 1–617) bound to MBP-cyto Ugo1p, but further truncations (Fzo1p 1–605) completely inhibited the interaction. These results indicate that both cytoplasmic domains of Fzo1p are necessary for interaction with Ugo1p and that the Ugo1p-binding domain in Fzo1p is located between amino acid residues 630–843. Because the transmem-

### Table I: Yeast strains

| Strain | Genotype (plasmid) | Ref. |
|--------|-------------------|------|
| YRJ1277 | MATα his3 leu2 lys2 trp1 ura3 ugo1: HIS3 | 10 |
| YRJ1292 | MATα his3 leu2 trp1 ura3 ugo1: HIS3 dnm1::kanMX4 | 10 |
| YRJ1347 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 dnm1::kanMX4 | 12 |
| YRJ1348 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 dnm1::kanMX4 | 12 |
| YRJ1383 | MATα his3 leu2 lys2 trp1 ura3 m3gm1::kanMX4 | 12 |
| YRJ1609 | MATα his3 leu2 lys2 trp1 ura3 m3gm1::kanMX4 ugo1::HIS3 dnm1::kanMX4 | This study |
| YRJ1685 | MATα his3 leu2 lys2 trp1 ura3 ugo1::HIS3 fzo1::kanMX4 dnm1::kanMX4 | This study |
| YRJ1791 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 (pRS416-FZO1) | This study |
| YRJ1811 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1812 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1813 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1814 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1815 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1816 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1817 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1818 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1819 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4-FZO1-TRP1 | This study |
| YRJ1820 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 (pRS314-HA-FZO1) | This study |
| YRJ1821 | MATα his3 leu2 lys2 trp1 ura3 fzo1::kanMX4 (pRS314-HA-fzo1-825) | This study |
brane domains of Fzo1p span residues 704–726 and 737–755 (28), it is likely that the minimal binding domain consists of 74 residues (630–703) in the N-terminal cytoplasmic domain and 90 residues (756–843) in the C-terminal domain.

Our data also show that the GTPase domain of Fzo1p is not required for interaction with Ugo1p in vitro, because Fzo1p fragments lacking the GTPase domain (Fzo1450–630) are competent for binding. Furthermore, when we coimmunoprecipitated Ugo1p and Fzo1p from detergent-solubilized mitochondria, a mutation in the GTPase domain (K200A), which blocks the function of Fzo1p (8), did not affect the Ugo1p-Fzo1p interaction (data not shown). Therefore, Fzo1p interacts with Ugo1p independently of its GTPase domain both in vitro and in vivo.

However, the GTPase domain of Fzo1p might modulate the interaction between Fzo1p and Ugo1p. We found that a Fzo1p fragment lacking the GTPase domain (Fzo1450–630) binds to MBP-cyto Ugo1p more efficiently than full-length Fzo1p (Fig. 1F). Specifically, more than 50% of Fzo1450–630 coprecipitated with MBP-cyto Ugo1p. In contrast, all other fragments examined here bound to Ugo1p similar to full-length Fzo1p, with ~10% of each coprecipitating with Ugo1p. These data suggest that the GTPase domain of Fzo1p partially inhibits the Fzo1p-Ugo1p interaction. Because further truncations of Fzo1450–630 reduced its strong binding activity (compare Fzo1450–630 with Fzo1450–630), it is likely that residues 450–630 are not essential for the Fzo1p-Ugo1p interaction but make the interaction stronger. We speculate that the GTPase domain of Fzo1p regulates the Fzo1p-Ugo1p interaction by masking and exposing these residues (450–630) during GTP binding and hydrolysis.

**Fzo1–825p Is Compromised for Ugo1p Binding**—To determine whether amino acid residues 630–843 in Fzo1p serve as a Ugo1p-binding domain in vivo, we expressed a mutant version of Fzo1p that lacks part of this sequence in yeast cells. We used Fzo1-825p, in which the last 30 residues are deleted, because these residues are dispensable for both the import of Fzo1p into the mitochondria and insertion into the OM with the correct topology (28). For detection, Fzo1p was tagged with the HA epitope at the N terminus. The mitochondria were isolated from the fzo1Δ cells expressing HA-Fzo1p or HA-Fzo1-825p and analyzed by immunoblotting. As expected, HA-Fzo1-825p migrated slightly faster on the gels than wild-type HA-Fzo1p. We found similar amounts of HA-Fzo1p and HA-Fzo1-825p in the mitochondria (Fig. 2A). Thus, HA-Fzo1-825p is normally expressed and localized in mitochondria.

To perform coimmunoprecipitation between Ugo1p and Fzo1p, we expressed Myc-Ugo1p plus either HA-Fzo1p or HA-Fzo1-825p in ugo1Δ fzo1Δ dnm1Δ cells. We disrupted DNM1 because cells expressing Fzo1-825p as the sole copy of Fzo1p lose mtDNA (see Fig. 3). dnm1Δ cells are defective in mitochondrial division and can therefore maintain mtDNA even in the absence of a functional copy of Fzo1p (13). Deletion of DNM1 did not affect interactions between Ugo1p and Fzo1p. When we carried out coimmunoprecipitation using mitochondria isolated from wild-type cells lacking mtDNA, we found equivalent interaction between HA-Fzo1p and Myc-Ugo1p (data not shown). The mitochondria were isolated and solubilized by Triton X-100. We then immunoprecipitated Myc-Ugo1p using anti-Myc antibodies and analyzed the pellet fraction by immunoblotting. As shown in Fig. 2B, almost all of the Myc-Ugo1p was found in the pellet fractions. When we examined the pellet with anti-HA antibodies, a fraction of HA-Fzo1p (~6%) reproducibly coprecipitated with Myc-Ugo1p, confirming our previous observations (12). In contrast, HA-Fzo1-825p showed dramatically reduced interaction with Myc-Ugo1p, and ~7-fold less HA-Fzo1-825p was found in the pellet compared with HA-Fzo1p. Importantly, we noted that similar amounts of Mgm1p coprecipitated with Myc-Ugo1p from mitochondrial extracts contain-
ing either HA-Fzo1p or HA-Fzo1-825p, suggesting that interactions between Mgm1p and Ugo1p are independent of the Ugo1p-Fzo1p interaction. The 100-kDa form of Mgm1p (Fig. 2B, arrow) more efficiently precipitated with Myc-Ugo1p than the 90-kDa form, as reported previously (12). An abundant OM protein, OM45, did not coprecipitate with Myc-Ugo1p. As a further control, we used beads without antibodies, and found no Myc-Ugo1p, HA-Fzo1p, HA-Fzo1-825p, Mgm1p, or Om45p in the pellets. These results clearly show that Fzo1-825p is defective in interaction with Ugo1p in vivo.

Although it is highly reduced, we observed small amounts of HA-Fzo1-825p that coprecipitated with Myc-Ugo1p. It is possi-

![Image 1](https://via.placeholder.com/150)

**Fig. 2.** Immunoprecipitation analysis of Ugo1p and Fzo1p interaction. A, expression of HA-Fzo1p and HA-Fzo1-825p in mitochondria. Mitochondria were isolated from YRJ1820, fzo1Δ cells containing pRS314-HA-FZO1 (lane 1) and YRJ1821, fzo1Δ cells containing pRS314-HA-fzo1-825 (lane 2) and analyzed by immunoblotting with antibodies to the HA epitope and OM45p. B, Fzo1-825p is compromised in its interaction with Ugo1p in mitochondria. Mitochondria were isolated from ugo1Δ fzo1Δ dnm1Δ cells (YRJ1665) containing pRS316-Myc-UGO1 (Sesaki et al. (12)) and pRS314-HA-FZO1 and ugo1Δ fzo1Δ dnm1Δ cells containing pRS316-Myc-UGO1 and pRS314-fzo1-825 and were solubilized by Triton X-100. The extracts were immunoprecipitated with anti-Myc antibodies. Immunoprecipitates (ppt) were analyzed by immunoblotting with antibodies to the Myc epitope (PRB-150; Covance), the HA epitope (12CA5), Mgm1p (24), and OM45p and compared with 10% of the starting mitochondrial extract (ext). An arrow and arrowhead indicate the 100- and 90-kDa forms of Mgm1p, respectively. There is a background band above the 100-kDa form of Mgm1.

**Fig. 3.** The Ugo1p-Fzo1p interaction is required for mitochondrial shape and mtDNA maintenance. A, fzo1Δ cells containing pRS416-FZO1 (YRJ1810) were transformed with pRS314-FZO1 (WT, wild type), pRS314 (fzo1Δ), and pRS314-fzo1-825 (fzo1-825). Transformants were selected for loss of pRS416-FZO1 on medium containing 5-fluoroorotic acid (Boeke et al. (26)). The cells were then transformed with a plasmid KC2, which expresses OM45-GFP/fusion protein (GFP) (29). The cells were grown to log phase in SRAf medium, stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (10), and viewed by differential interference contrast (DIC) and fluorescence microscopy. Bar, 3 μm. B, quantitation of mitochondrial shape (n = 100). Branched tubules (black bar), fragments (white bar), and others (gray bar) were observed. C, quantitation of cells containing mtDNA (n = 100). D, wild-type, fzo1Δ, and fzo1-825 cells were grown to log phase in YEPD medium. Cells corresponding to 2 A600 units were collected and resuspended in 1 ml of YEPD medium. The 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 3 and 6 days, respectively.
Ugo1p-Fzo1p interaction is important for mitochondrial shape. "mented mitochondria (Fig. 3), whereas only 5% of wild-type cells contained fragments. Quantitation of mitochondrial shape showed that more than 95% of wild-type cells were tubular mitochondria with occasional branches. In contrast, fzo1-825 cells contained many mtDNA nucleoids (Fig. 3, A and C). Mitochondria were visualized using an OM-targeted OM45-GFP fusion protein (29). As shown in Fig. 3A, wild-type cells contain 5–10 tubular mitochondria with occasional branches. In contrast, fzo1-825 cells displayed highly fragmented mitochondria. Quantitation of mitochondrial shape showed that more than 95% of fzo1-825 cells contained fragmented mitochondria, whereas only 5% of wild-type cells contained fragmented mitochondria (Fig. 3B). These results suggest that the Ugo1p-Fzo1p interaction is important for mitochondrial shape.

The mitochondrial fragmentation and mtDNA loss in fzo1-825 cells suggested that fzo1-825 cells are defective in mitochondrial fusion. To directly test this possibility, we observed mitochondrial fusion during yeast cell mating (12). The mitochondria in MATa cells were marked using an IM-targeted
Yta10p–CFP fusion protein. In MATα cells, mitochondria were labeled with an IM-targeted Yta10p–YFP fusion. Both fusion proteins were expressed under the control of the inducible GAL1 promoter. MATα and MATα cells were pregrown in galactose-containing medium to induce the expression of the fusion proteins and were transferred to glucose-containing medium to inhibit their further synthesis. MATα and MATα cells were then mated on glucose-containing medium. If mitochondria fuse in the resulting zygotes, the CFP and YFP signals will colocalize. If mitochondria do not fuse, CFP and YFP will be seen in separate organelles.

We found that fzo1-825 and fzo1-825 dm1Δ cells fail to fuse their mitochondria. Fig. 4 shows representative examples of zygotes with a medial diploid bud from each mating mixture. We examined 50 zygotes for each type of cells (wild-type, fzo1-825, and fzo1-825 dm1Δ). When two wild-type haploids mated, the mitochondria in the zygote fused, their IM contents were mixed, and the CFP and YFP fluorescence overlapped.

Mitochondrial fusion also occurred in zygotes formed between dm1Δ mutants. In contrast, when two fzo1-825 cells were mated, mitochondrial fragments containing only CFP or YFP were observed. Although the disruption of DNM1 suppressed the fragmentation of mitochondria and the loss of mtDNA (data not shown), fzo1-825 dm1Δ double mutants were unable to fuse their mitochondria. In fzo1-825 dm1Δ zygotes, we frequently found that two mitochondrial tubules, one derived from each parent, entered the diploid bud and were closely positioned, but nonetheless these mitochondria did not fuse and contained only CFP or YFP. These results clearly demonstrate that the Ugo1p–Fzo1p interaction is required for mitochondrial fusion.

Ugo1p Interacts in Mitochondrial Fusion

As with Fzo1p, we found that the 100-kDa form of Mgm1p is enriched at contact sites using submitochondrial fractionation. Because Fzo1p binds to Mgm1p in an Ugo1p-dependent manner, it is likely that Fzo1p is located at contact sites by interacting with Mgm1p and Ugo1p. We suggest that the Ugo1p regulates the Mgm1p–Fzo1p interaction at a key step that couples OM fusion to IM fusion.

Fzo1p and Mgm1p GTases are conserved from yeast to human, but a mammalian homolog of Ugo1p has not been identified based on its amino acid sequence. Because mitofusin (30–32) and OPA1 (33–36), mammalian homologs of Fzo1p and Mgm1p, respectively, have sub mitochondrial localizations and topologies similar to those of their yeast counterparts, identification of a protein that functionally links these GTases will further our understanding of mitochondrial fusion.

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