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Domain Interactions within Fzo1 Oligomers Are Essential for Mitochondrial Fusion*

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Mitofusins are conserved GTPases essential for the fusion of mitochondria. These mitochondrial outer membrane proteins contain a GTPase domain and two or three regions with hydrophobic heptad repeats, but little is known about how these domains interact to mediate mitochondrial fusion. To address this issue, we have analyzed the yeast mitofusin Fzo1p and find that mutation of any of the three heptad repeat regions (HRN, HR1, and HR2) leads to a null allele. Specific pairs of null alleles show robust complementation, indicating that functional domains need not exist on the same molecule. Biochemical analysis indicates that this complementation is due to Fzo1p oligomerization mediated by multiple domain interactions. Moreover, we find that two non-overlapping protein fragments, one consisting of HRN/GTPase and the other consisting of HR1/HR2, can form a complex that reconstitutes Fzo1p fusion activity. Each of the null alleles disrupts the interaction of these two fragments, suggesting that we have identified a key interaction involving the GTPase domain and heptad repeats essential for fusion.

The balance between fusion and fission maintains mitochondria as a dynamic tubular reticulum. In the absence of fusion, ongoing fission results in complete fragmentation of the mitochondrial network. Fragmentation leads to a loss of mtDNA and respiratory incompetence in yeast (1, 2) and accumulation of poorly functional mitochondria in mammals (3). Mice deficient for mitochondrial fusion die during mid gestation (4), and mutations in the mitochondrial fusion genes *Mfn2* and *OPA1* cause the neurodegenerative diseases Charcot-Marie-Tooth disease type 2A and dominant optic atrophy, respectively (5–9). Importantly, the mitochondrial fusion machinery is dedicated solely to mitochondrial fusion and does not include SNARE[2] proteins or NSF (*N*-ethylmaleimide-sensitive protein), which mediate most other intracellular membrane fusion events. Thus, mitochondrial fusion likely involves a unique mechanism.

There are three essential components of the fusion pathway in yeast. The yeast mitofusin Fzo1p is a 98-kDa transmembrane GTPase uniformly distributed in the mitochondria outer membrane (1, 2). Both the N- and C-terminal portions of Fzo1p are oriented toward the cytosol, in position to mediate important steps during fusion (1, 2). Mgm1p is a dynamin-related protein in the mitochondrial intermembrane space (10–12). It remains unclear if Mgm1p possesses the membrane severing activity of other dynamin family members, and if so, how this activity relates to its requirement for fusion. The third component of the fusion pathway is Ugo1p, a 58-kDa protein that spans the outer membrane once and interacts with both Fzo1p and Mgm1p (10, 12–14). In this capacity, Ugo1p may anchor a fusion complex that coordinates the mitochondrial outer and inner membranes during fusion.

Fzo1p is the best candidate to directly mediate fusion of the mitochondrial outer membrane. Unlike Mgm1p, it is positioned in the outer membrane and unlike Ugo1p its requisite role is conserved from yeast to mammals. In addition, Fzo1p has three hydrophobic heptad repeat (HR) regions that are predicted to form coiled-coils. Heptad repeat regions are directly involved in SNARE and Class I viral membrane fusion. In both cases, membrane fusion is driven when a *trans* complex that spans both membranes undergoes a structural transition to a highly stable fusogenic conformation. These fusogenic structures use coiled-coil-like helical bundles to position the two membranes in close apposition, leading to fusion (15–18).

Given these similarities, it is likely that the heptad repeat regions form interactions critical for mitochondrial fusion. Indeed, the mammalian mitofusins Mfn1 and Mfn2 form homotypic and heterotypic complexes, and several intermolecular interactions have been identified. Mfn1 HR2 is known to form a *trans* anti-parallel, dimeric coiled-coil that tethers mitochondria together (19). There is also evidence that Mfn1 HR1 and HR2 interact with each other (20). Moreover, Fzo/mitofusins have similarity to dynamins, a family of large membrane-associated GTPases characterized by oligomerization-stimulated GTP hydrolysis and multiple inter- and intramolecular interactions (21). It has not been established if Fzo1p forms similar intermolecular interactions that contribute to mitochondrial fusion.

In this study, we show that Fzo1p functions as an oligomer during mitochondrial fusion. We demonstrate that each of the Fzo1p heptad repeats is essential and that the GTPase domains and heptad repeats from different Fzo1p molecules within an oligomer function together to mediate fusion. Finally, we define a novel interaction between the HRN/GTPase and HR1/HR2 regions of Fzo1p that is essential for mitochondrial fusion.

EXPERIMENTAL PROCEDURES

Media and Yeast Genetic Techniques—Standard genetic techniques and media were used. SD and YP media supplemented with either 2% dextrose (YPD) or 3% glycerol (YPG) were prepared as described previously (22).

DCY538 (*MATa*, *ura3–52*, *his3*Δ*200*, *leu2*Δ*1*, *trp1*Δ*63*, *lys2*Δ*202*, *fzo1*::HIS3, *leu2*:: GPD-mitoGFP-*LEU2*, pRS416-*FZO1*) was derived from JSY2287 (kindly provided by J. Shaw) (1) by integration of BstEII-linearized EG686 (pRS405 plus mito-GFP) into the *leu2*Δ*1* locus. DCY328 (*MAT*α, *ura3–52*, *his3*Δ*200*, *leu2*Δ*1*, *trp1*Δ*63*, *fzo1*::HIS3, rho^0) was derived from JSY2793 (1) by selecting for the loss of pRS414-*fzo1–1*.

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[2] The abbreviations used are: SNARE, soluble NSF attachment protein receptors; HR, heptad repeat; SD, synthetic media with 2% dextrose; YP, yeast extract peptone; mito-GFP, mitochondrially targeted green fluorescent protein; mito-DsRed, mitochondrially targeted red fluorescent protein from *Discosoma*; HA, hemagglutinin.

Because the loss of mtDNA in *fzo1*Δ yeast is a non-revertible phenotype, a plasmid-shuffle strategy was used to generate yeast strains for structure-function and intragenic allelic complementation analyses. Briefly, DCY538, a *rho*$^+$ yeast strain containing a chromosomal deletion of *FZO1* covered by an *FZO1*-expressing plasmid (pRS416-*FZO1*), was transformed with a plasmid encoding an *FZO1* under *TRP1* selection (structure-function experiments) or two *FZO1* plasmids under *TRP1* and *LYS2* selection (intragenic complementation experiments). The transformation plates were replica-plated, and the pRS416-*FZO1* plasmid was removed by counterselection with 5-fluoroorotic acid. The resulting yeast colonies were replica-plated onto YPD and YPG plates. The glycerol growth phenotype was reproducibly evident after 3 days of growth. In the intragenic complementation experiments, for example, all colonies showed growth on YPG plates when a pair of null alleles were complemented. For quantitative serial dilution and morphological analysis, representative colonies were isolated and re-analyzed. The possibility of recombination events was excluded by the presence of many non-complementing pairs of alleles (for example, *fzo1*(*V172P*) and *fzo1*(*L819P*)), the robust complementation between non-overlapping fragments (Fzo1p-HRN/GTPase plus Fzo1p-HR1/HR2), and the reproducibility of the glycerol growth phenotype among all colonies at the replica plating stage. In addition, in the complementation experiment involving Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2, Western blot analysis confirmed the expression of the proper fragments.

Mitochondrial Morphology and Glycerol Growth Analysis—Yeast strains expressing the indicated *FZO1* constructs were generated through a plasmid shuffle strategy and grown at 30 °C in appropriate SD medium overnight. Cultures were diluted 1:20 in fresh YPD medium and counted live during logarithmic growth. For glycerol growth assays, cultures grown overnight in SD medium were pelleted, resuspended at a density of 1 A_{600} unit/ml and diluted 1:5 six times in YP. 3 μl of the dilutions was spotted to plates and grown for 2 days (YPD) or 4 days (YPG) at 30 °C.

Plasmid Construction—pRS414-9XMyc-*FZO1* (EG36) was constructed as follows. The *FZO1* terminator was amplified from genomic DNA using the oligonucleotides Eg5 (CTCTATACGAAGGAACCGTGGCTC) and Eg6 (GCAGAATTCTTGTTGTCTTTTAAATGGAG) and cloned as a ClaI/EcoRI fragment. The *FZO1* coding sequence was cloned as a SalI and ClaI fragment using the *FZO1* forward primer Eg3 (ACCATGGTCGACTCTGAAGGAAAACAACAATTC) to engineer a SalI restriction site following the initiator ATG. The *FZO1* 5′-untranslated region was amplified using the primers Eg1 (GCAGGTACCAAGGAGTTTGTGTCGTTTTTCAC) and Eg2 (CAGAGTCGACCATGGTCGTTAAATGAGCCTACCGTTTTGCC) and cloned as a KpnI/SalI fragment. These fragments were all subcloned into pRS414 (Stratagene) to yield pRS414-*FZO1* (EG32). Next, a 9XMyc cassette was cloned into the SalI site to yield pRS414-9XMyc-*FZO1* (EG36). The entire 9XMyc-*FZO1* cassette was moved as a KpnI (blunted)/EcoRI fragment into SmaI and EcoRI sites of pRS317 to generate pRS317-9XMyc-*FZO1* (EG197). The 9XMyc cassette in EG36 was replaced with a 6XHA cassette (XhoI/SalI fragment) to generate pRS414-6XHA-*FZO1* (EG594). The entire 6XHA-*FZO1* fragment was moved as a KpnI/EcoRI fragment into pRS416, resulting in pRS416-6XHA-*FZO1* (EG600).

pRS416 plus 6XHA-*FZO1-HR1/HR2* (residues 416–855) was generated using the primer Eg253 (GCAGTCGACCATTATCATAATGAAAATG). pRS414-9XMyc-*FZO1-HRN/GTPase*-(1–415) was generated with the primer Eg226 (GCAATCGATTGGCAATTCATCTCCGTT). Mutations were initially generated in the pRS414-9XMyc-*FZO1* vector with the QuikChange site-directed mutagenesis kit (Stratagene) or by subcloning PCR products containing the indicated mutations. Primer sequences are available upon request.

Mating Assay—The indicated yeast strains were grown overnight in the appropriate SD medium, mixed by gently centrifuging together and incubated in YPD at 30 °C for 5 h. The cells were then fixed for 10 min with 3.7% formaldehyde and washed four times with 1 ml of phosphate-buffered saline. Large budded zygotes were scored for the complete overlap of the mito-GFP and mito-DsRed signals. Plasmids were expressed in DCY1440 (*MAT*α, *his3*Δ*200*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *dnm1*::HIS5, *fzo1*::KANMX6, *lue2*::GPD mito-DsRed-*LEU2*) and DCY1553 (*MATa*, *his3*Δ*200*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *dnm1*::*HIS5*, *fzo1*::KANMX6, *leu2*::GPD mito-GFP-*LEU2*). DCY1451 (*MAT*α, *his3*Δ*200*, *met15*Δ*0*, *trp1*Δ*63*, *ura3*Δ*0*, *dnm1*::*HIS5*, *leu2*::GPD mito-DsRed-*LEU2*) and DCY2312 (*MATa*, *his3-*, *met15*Δ*0*, *ura3*Δ*0*, *dnm1*::kanMX6, *leu2*::GPD mito-GFP-*LEU2*) were used for the two *FZO1 dnm1*Δ strains.

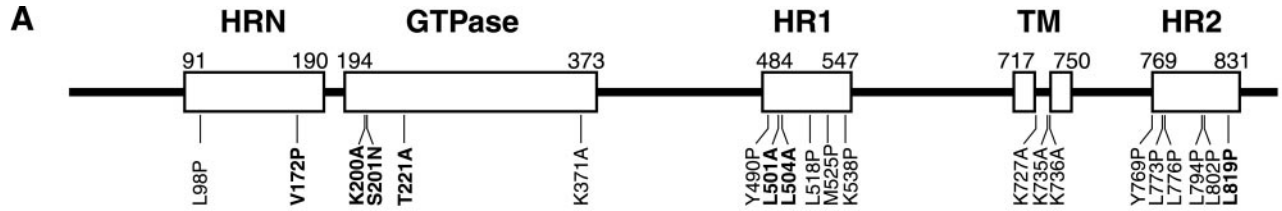
Immunoprecipitations—9XMyc- and 6XHA-tagged Fzo1p constructs were expressed in a *rho*^{0} *fzo1*Δ strain (EG328). The use of a *rho*^{0} strain eliminated differences in mtDNA levels supported by functional and null alleles of *FZO1*. Overnight cultures were grown in the appropriate SD medium, diluted into YPD and harvested at A_{600} ~ 1.5. Then, ~50 A_{600} units of cells was disrupted by glass bead lysis (40 s with a vortex mixer, four times) in 500 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 0.2% Triton X-100) supplemented with fungal protease inhibitors (Sigma-Aldrich). Lysates were cleared by centrifugation at 5,000 rpm for 5 min and 14,000 rpm for 15 min at 4 °C. A total lysate sample was taken, and 400 μl of the remaining lysate was incubated with to 20 μl (bead volume) of 9E10-conjugated protein A-Sepharose beads (Sigma-Aldrich) for 90 min at 4 °C. The beads were then washed four times with 1 ml of lysis buffer and eluted in SDS gel loading buffer for 5 min at 95 °C. Western blot analysis was performed with 9E10 hybridoma supernatant (anti-Myc) or 12CA5 ascites fluid (anti-HA).

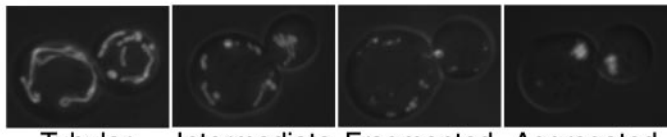
Immunofluorescence—The indicated 9XMyc-Fzo1p constructs were introduced in DCY538 by a plasmid shuffle. YPD cultures at ~1.5 A_{600} were fixed for 10 min with 3.7% formaldehyde. The cells were washed four times with water and spheroplasted with Zymolyase 100T (ICN). Spheroplasts were permeabilized for 10 min in blocking buffer (phosphate-buffered saline containing 1% bovine serum albumin and 1% Tween 20) and incubated for 1 h with 9E10 hybridoma diluted 1:1 in blocking buffer. After four 5-min washes with blocking buffer, the cells were incubated with a Cy3-conjugated anti-mouse secondary antibody for 1 h. After four 5-min washes, Gel Mount (BioMedia) was added to preserve fluorescence. All incubations were performed at room temperature.

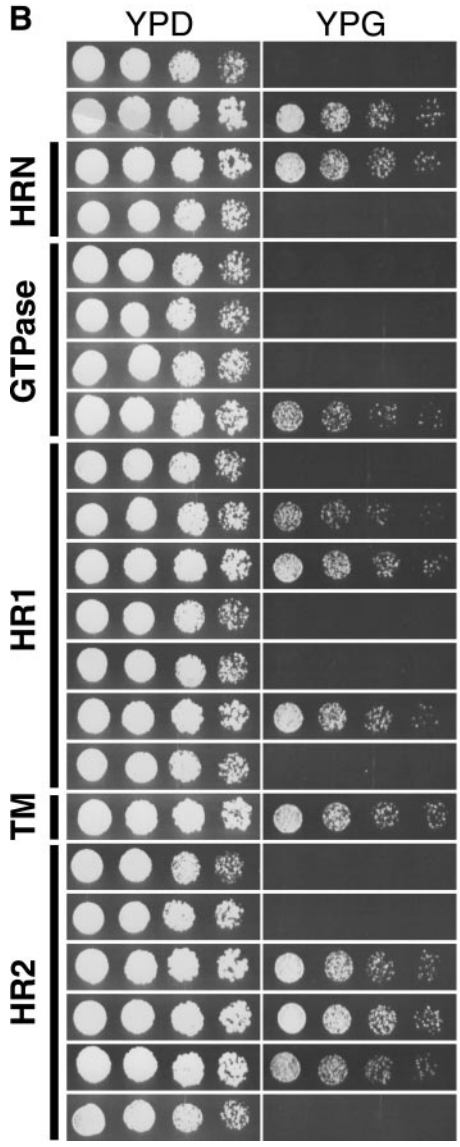
Imaging—Images were acquired on a Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) equipped with a 100× Plan-Apochromat, numerical aperture 1.4, oil-immersion objective and controlled by Axiovision 4.2 software. Z-stacks were collected at 100- to 200-μm intervals with an ORCA-ER camera (Hamamatsu) at room temperature. Iterative deconvolutions and maximum intensity projections were performed using Axiovision 4.2. Fluorescent images were overlaid with differential interference contrast images set at 50% opacity (Fig. 1) using Adobe Photoshop CS.

RESULTS

Identification of Essential Fzo1p Domains—Fzo1p has a bipartite transmembrane domain that resides in the mitochondrial outer membrane and divides the protein into N- and C-terminal regions, both of which face toward the cytosol (Fig. 1*A*) (1, 2, 23). The N-terminal region







		Tubular	Intermediate	Fragmented	Aggregated
	*fzo1*Δ	0	5	88.5	6.5
	WT	74.5	9.5	5.5	10.5
HRN	L98P	65.5	9	15	10.5
HRN	**V172P**	0	12	42	46
GTPase	**K200A**	0	10	80	10
GTPase	**S201N**	0	10.5	81	8.5
GTPase	**T221A**	0	8	52.5	39.5
GTPase	K371A	37.5	28	29	5.5
HR1	Y490P	0	8	37	55
HR1	L501A	33.5	22	25.5	19
HR1	L504A	42	16.5	28.5	13
HR1	**L501A, L504A**	0	5.5	80.5	14
HR1	L518P	0	4.5	48.5	47
HR1	M525P	45	15.5	24	15.5
HR1	K538P	3	13	74	10
TM	K727A, K735A, K736A	44.5	17.5	26	12
HR2	Y769P	0	10	81.5	8.5
HR2	L773P	0	16.5	71	12.5
HR2	L776P	50.5	15	20	14.5
HR2	L794P	44	19.5	25	11.5
HR2	L802P	35.5	19.5	27	18
HR2	**L819P**	0	9	69.5	21.5

FIGURE 1. **Identification of critical regions of Fzo1p.** *A*, schematic of Fzo1p, including HRN (residues 91–190), the GTPase domain (residues 194–373), HR1 (residues 484–547), the bipartite transmembrane (*TM*) domain (residues 717–750), and HR2 (residues 769–831). The point mutations analyzed are indicated below. *B*, a serial dilution growth assay to analyze *fzo1*Δ yeast expressing the indicated alleles of *FZO1*. Growth on rich dextrose (YPD) and glycerol (YPG) plates are shown. These strains were generated through a plasmid-shuffle strategy in which pRS416-*FZO1* is replaced by a pRS414-9XMyc-*FZO1* plasmid containing the indicated mutations. Null alleles analyzed further in this report are indicated in *bold*. *C*, summary of mitochondrial morphologies in yeast expressing the indicated *fzo1* mutant. Examples of the morphology classes are shown in the images above. 200 live cells were analyzed during log phase growth in minimal selective media; percentages are indicated.

contains a GTPase domain that is required for fusion (1). Fzo1p also contains three domains with hydrophobic heptad repeats. Like other mitofusins, including the mammalian Mfn1 and Mfn2, Fzo1p contains the hydrophobic heptad repeat regions HR1 and HR2, which flank the transmembrane domain (Fig. 1*A*). However, Fzo1p also contains an additional heptad repeat region near the N terminus that we term HRN. Because the role of these heptad repeats in Fzo1p-mediated mitochondrial fusion has not previously been assessed, we performed a structure-function analysis of Fzo1p HRN, HR1, and HR2. Our analysis made use of the fact that loss of Fzo1p function causes complete fragmentation of

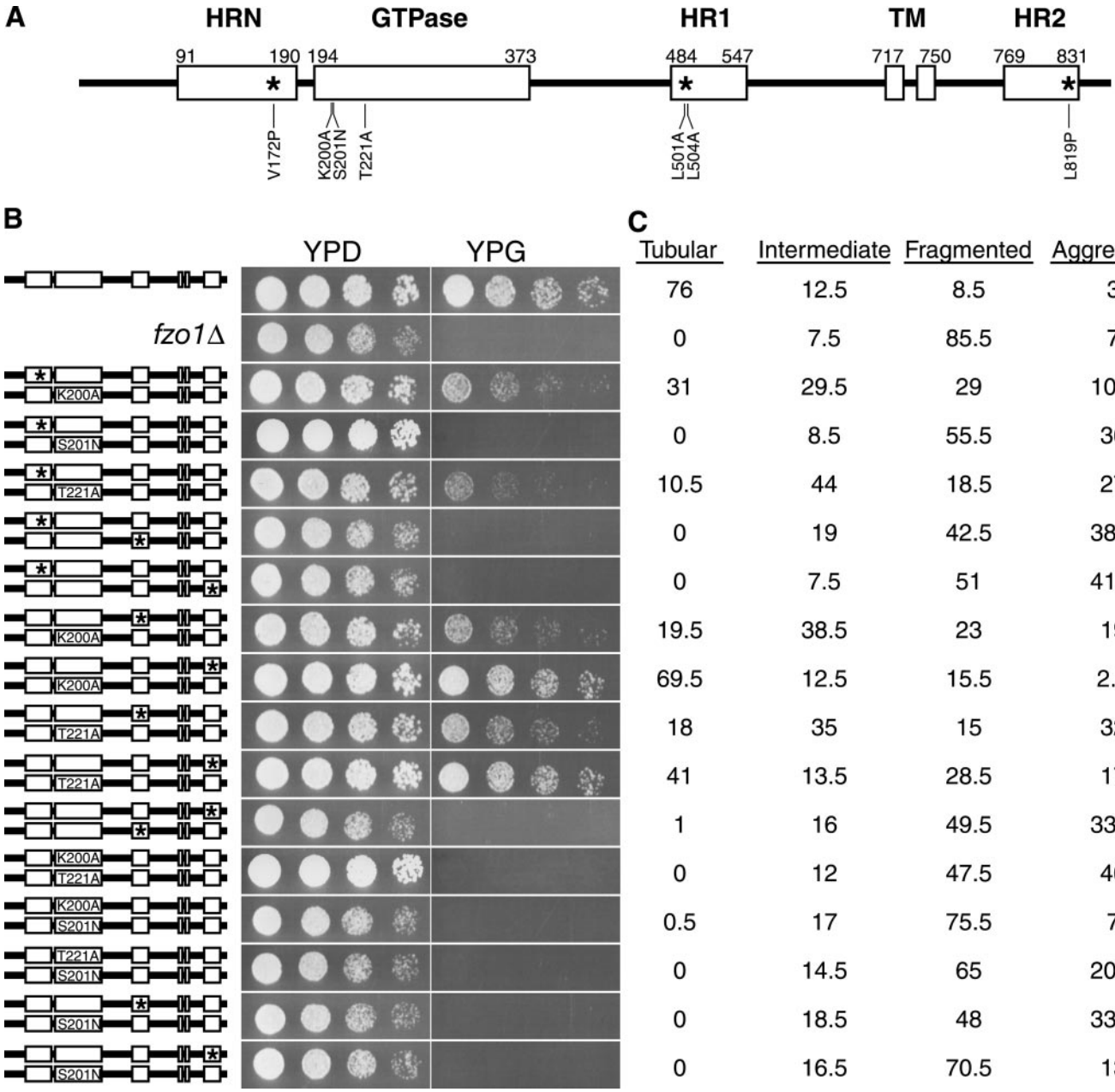


FIGURE 2. **Allelic complementation between null alleles of *FZO1*.** *A*, schematic of the null mutations used for allelic complementation analysis. *Asterisks* are used to indicate the mutations in HRN (V172P), HR1 (L501A,L504A), and HR2 (L819P) in *B*. *B*, serial dilution assay for growth on dextrose and glycerol plates. The assays were performed as in Fig. 1, except that the null *FZO1* alleles were co-expressed from pRS414-9XMyc-*FZO1* and pRS317-9XMyc-*FZO1* plasmids. A schematic of the different co-expressed alleles is indicated on the *left*. *C*, summary of mitochondrial morphologies in yeast expressing the indicated alleles. 200 live cells were counted for each strain; percentages are indicated.

the mitochondrial reticulum due to ongoing mitochondrial fission (1, 24). In addition, because mitochondrial fragmentation leads to a secondary loss of mitochondrial DNA, fusion mutants can grow on the fermentable carbon source dextrose but not the non-fermentable carbon source glycerol (1). Thus, we assessed the function of Fzo1p mutants by monitoring mitochondrial morphology and growth on glycerol plates.

HR1 is 64 residues long (residues 484–547 as predicted by COILS) and lies between the GTPase domain and transmembrane domain (Fig. 1*A*) (25). We substituted proline residues along HR1 to disrupt the predicted coiled-coil structure (Y490P, L518P, M525P, and K538P). Proline residues impose a kink in the protein backbone and are often incompatible with a helical fold, although sometimes such substitutions can be tolerated by local distortions in backbone geometry. The mutant *fzo1*(*M525P*) retained significant function, whereas *fzo1*(*Y490P*), *fzo1*(*L518P*), and *fzo1*(*K538P*) were strong loss-of-function alleles, incapable of supporting growth on glycerol plates or rescuing fragmentation of the mitochondrial network (Fig. 1, *B* and *C*). In addition, we substituted alanine for leucine at two *a* and *d* positions, Leu-501 and Leu-504. Bulky hydrophobic residues at the *a* and *d* positions of the heptad repeat (*abcdefg*) form a stable hydrophobic interface between the interacting helices of a coiled coil; their substitution by the smaller residue alanine is predicted to destabilize this interface. Individually, *fzo1*(*L501A*) and *fzo1*(*L504A*) behaved as mild loss-of-function alleles. However, the double mutant *fzo1*(*L501A,L504A*) behaved as a null allele, showing no growth on glycerol and completely fragmented mitochondrial morphology (Fig. 1, *B* and *C*). Taken together, our mutagenesis of HR1 generated an allelic series and demonstrated that HR1 is essential for Fzo1p function.

TABLE 1

Summary of intragenic complementation data presented in Fig. 2

For simplicity, each data point is presented twice. "+" indicates growth on glycerol plates and tubular mitochondrial morphology. "−" indicates failure to grow on glycerol plates and fragmented mitochondrial morphology.

	K200A	S201N	T221A	V172P	L501A,504A	L819P
K200A		−	−	+	+	+
S201N	−		−	−	−	−
T221A	−	−		+	+	+
V172P	+	−	+		−	−
L501A,504A	+	−	+	−		−
L819P	+	−	+	−	−	

In mammalian Mfn1, HR2 forms an anti-parallel dimer that tethers mitochondria together at an early step in fusion (19). Proline substitutions in Mfn1 HR2 prevent mitochondrial tethering and block mitochondrial fusion (19). Proline substitutions at residues Leu-776, Leu-794, and Leu-802 of Fzo1p HR2 (residues 764–826) were tolerated with only modest effects on *FZO1* function (Fig. 1, *B* and *C*). However, proline substitutions at positions Tyr-769, Leu-773, and Leu-819 in HR2 caused a complete loss of function and resulted in no growth on glycerol plates and mitochondrial fragmentation (Fig. 1, *B* and *C*). Thus, like Mfn1 HR2, Fzo1p HR2 is essential for mitochondrial fusion.

HRN (residues 91–190) is also required for *FZO1* function. Initial experiments showed that N-terminal truncations that spared HRN had little effect on *FZO1* activity, whereas truncations that deleted portions of HRN behaved as null alleles.[3] In addition, *fzo1*(*L98P*) had only slight loss of function, whereas *fzo1*(*V172P*) behaved as a null allele (Fig. 1, *B* and *C*). Taken together, these results indicate that like HR1 and HR2, HRN is essential for Fzo1p activity.

We made several further observations in our structure-function analysis. First, consistent with previous reports, mutations in the GTPase domain of *FZO1* (*fzo1*(*K200A*), *fzo1*(*S201N*), and *fzo1*(*T221A*)) all caused complete fragmentation of the mitochondrial reticulum (1). Second, in several of the null mutants (including *fzo1*(*V172P*), *fzo1*(*T221A*), *fzo1*(*Y490P*), and *fzo1*(*L518P*)) the mitochondrial fragments frequently formed loose aggregates (Fig. 1*C*). The basis of this aggregation is unclear, but may reflect the trapping of tethered intermediates. Finally, the charged residues present in the bipartite transmembrane domain are not essential for fusion. Fzo1p has three lysines (Lys-727, Lys-735, and Lys-736) in the region of the transmembrane domain thought to form a U-turn in the intermembrane space. The triple mutant *fzo1*(*K727A,K735A,K736A*) was highly functional (Fig. 1, *B* and *C*). Thus, although the loop residing in the intermembrane space is important for Fzo1p function (23), its charged nature is not essential.

Intragenic Complementation between FZO1 Null Alleles—Mammalian mitofusins have been shown to form higher order complexes (4, 26), and Fzo1p has been shown to be present in a high molecular weight complex in yeast (2). In unusual cases, proteins that self-assemble can show intragenic complementation, a genetic phenomenon that results from formation of a functional complex from individually nonfunctional subunits (27). To test whether yeast Fzo1p could exhibit intragenic complementation, we used a plasmid-shuffle strategy to construct *fzo1*Δ strains co-expressing two null *fzo1* alleles. The function of the co-expressed null alleles was evaluated by growth on glycerol plates and mitochondrial morphology. Based on our results above, we used *fzo1*(*V172P*), *fzo1*(*L501A, L504A*), and *fzo1*(*L819P*) as representative null alleles with mutations in HRN, HR1, and HR2, respectively (Fig. 2*A*). These mutants were tested along with the three GTPase mutants.

[3] E. Griffin and D. Chan, unpublished results.

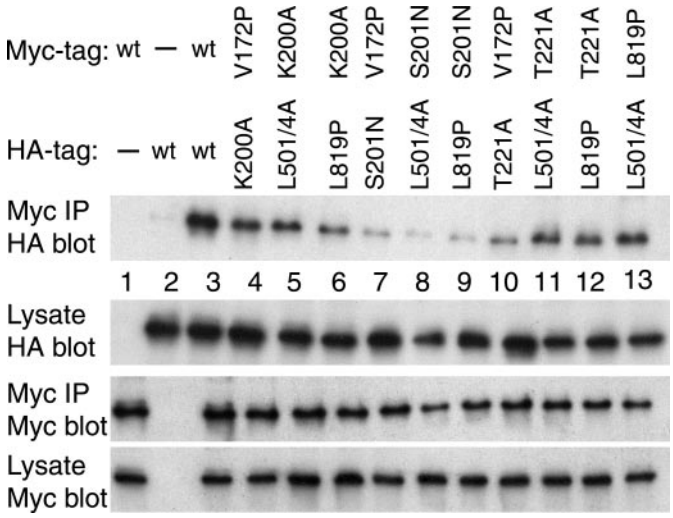


FIGURE 3. **Self-association of Fzo1p.** Lysates (labeled "*Lysate*") from yeast expressing the indicated HA-Fzo1p and Myc-Fzo1p alleles were immunoprecipitated with anti-Myc antibodies (labeled "*Myc IP*") and analyzed by Western blotting with anti-Myc (9E10) and anti-HA (12CA5) antibodies. The immunoprecipitate samples are loaded at 10 equivalents of the lysate samples.

With these 6 mutants, we constructed 15 pairwise combinations, and intragenic complementation was reproducibly observed in 6 cases (Table 1 and Fig. 2). Co-expression of either of two GTPase mutants with a heptad repeat (HR) mutant restored Fzo1p function. In repeated trials, the GTPase mutants *fzo1*(*K200A*) or *fzo1*(*T221A*) showed strong complementation with either *fzo1*(*V172P*), *fzo1*(*L501A, L504A*), or *fzo1*(*L819P*), resulting in tubular mitochondrial morphology that corresponded with a restored ability to grow on glycerol plates (Fig. 2, *B* and *C*). Thus, Fzo1p activity can occur when the function of the GTPase and heptad repeat regions reside on different Fzo1p molecules. These complementation data also indicate that the null mutations do not have global effects on Fzo1p structure.

In contrast, many pairs of alleles failed to complement. The GTPase mutant *fzo1*(*S201N*) did not complement any of the heptad repeat mutants (see next section). No complementation was observed when an HR1 mutant (*fzo1*(*L501A,L504A*)) was co-expressed with an HR2 mutant (*fzo1*(*L819P*)) or an HRN mutant (*fzo1*(*V172P*)) (Fig. 2, *B* and *C*). In addition, when an HRN mutant was co-expressed with an HR2 mutant, *FZO1* activity was not restored (Fig. 2, *B* and *C*). Finally, co-expression of any two GTPase mutants did not restore mitochondrial fusion.

The simplest interpretation of these data is that Fzo1p functions as an oligomer in which GTPase and heptad repeat activity can be donated by different molecules. However, it is also possible that the GTPase and heptad repeat regions act independently (for example, sequentially) at different steps in the fusion pathway. We sought to distinguish these possibilities by testing directly for Fzo1p oligomerization.

Fzo1p Functions as an Oligomer—We sought evidence for Fzo1p oligomerization by using a co-immunoprecipitation assay. In these experiments, HA-Fzo1p and Myc-Fzo1p were co-expressed at endogenous levels in *fzo1*Δ cells. When Myc-Fzo1p was immunoprecipitated, ~10% of HA-Fzo1p co-immunoprecipitated (Fig. 3, *lane 3*). Similarly, the mammalian orthologs Mfn1 and Mfn2 have previously been shown to form homotypic and heterotypic complexes (4). Taken in conjunction with our intragenic complementation results, these interaction data strongly suggest that Fzo1p functions as an oligomer during mitochondrial fusion.

If intragenic complementation is due to oligomerization between mutant Fzo1p molecules, we would expect complementing mutant

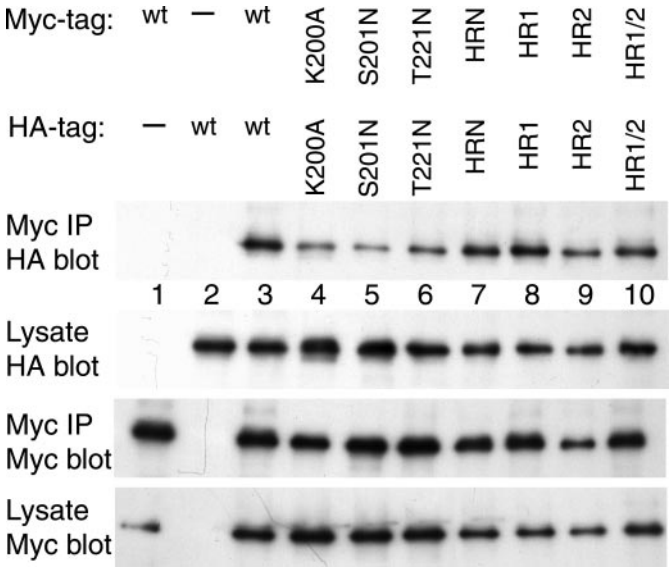


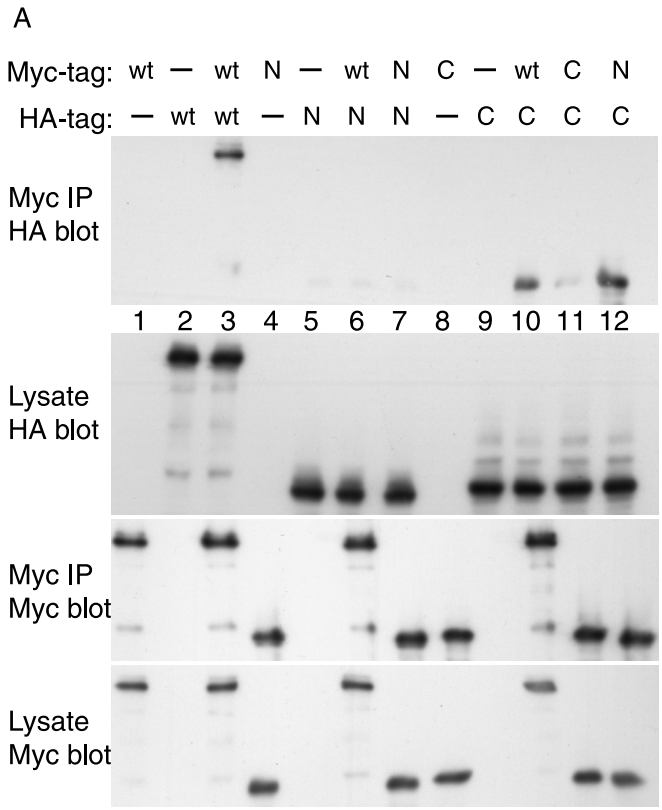
FIGURE 4. **Fzo1p null mutants form homotypic oligomers.** Co-immunoprecipitations were performed as in Fig. 3. Yeast strains contained Myc- and HA-tagged alleles of Fzo1p as indicated. *HRN*, V172P; *HR1*, L501A,L504A; *HR2*, L819P; *HR1/2*, L501A,L504A,L819P.

pairs to co-immunoprecipitate. Indeed, the Fzo1p GTPase mutants *fzo1*(*T221A*) and *fzo1*(*K200A*) interacted with each of the heptad repeat mutants *fzo1*(*V172P*), *fzo1*(*L501A, L504A*), and *fzo1*(*L819P*) (Fig. 3, *lanes 4–6* and *10–12*). In contrast, *fzo1*(*S201N*) interacted weakly with all the heptad repeat mutants (Fig. 3, *lanes 7–9*) and was unable to complement (Table 1 and Fig. 2), suggesting efficient oligomerization is necessary for intragenic complementation.

Surprisingly, all of our null mutants retained the ability to form homotypic interactions. Mutations in HRN, HR1, HR2, or in both HR1 and HR2 had little effect on Fzo1p self-assembly (Fig. 4, *lanes 7–10*). More of an effect was seen with the GTPase mutants, most notably S201N, which displayed the lowest levels of interaction (Fig. 4, *lanes 4–6*). Given that mutations in no single domain completely disrupt Fzo1p interactions, we conclude that oligomerization of Fzo1p likely involves interactions between multiple domains, and that the null phenotypes of the heptad repeat and GTPase mutants are not a result of Fzo1p dissociation.

Interaction between Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2—Because several domains likely work in concert to stabilize full-length Fzo1p oligomerization, we attempted to develop a simplified system for examining domain interactions. We tested whether fragments of Fzo1p could physically interact with each other. We analyzed an N-terminal Fzo1p fragment containing HRN and the GTPase domain (Fzo1p-HRN/GTPase, residues 1–415) and a C-terminal fragment that includes HR1, the TM and HR2 (Fzo1p-HR1/HR2, residues 416–855). Fzo1p-HR1/HR2, like full-length Fzo1p, interacted efficiently with full-length Fzo1p (Fig. 5, *lanes 3* and *10*). A weaker interaction was also seen between two Fzo1p-HR1/HR2 fragments (Fig. 5, *lane 11*). Most interestingly, the non-overlapping Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 fragments interacted with each other as efficiently as the full-length Fzo1p-Fzo1p interaction (Fig. 5, *lane 12*). Thus, Fzo1p-HR1/HR2 has at least two modes of interaction: a homotypic interaction with itself and a heterotypic interaction with Fzo1p-HRN/GTPase.

We confirmed the interaction between Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 by showing that Fzo1p-HR1/HR2 can recruit Fzo1p-HRN/GTPase to mitochondria. Full-length Myc-Fzo1p ($n = 200$, Fig. 5, *B–D*) and Fzo1p-HR1/HR2 (data not shown) displayed a uniform mitochondrial localization pattern in all cells. In contrast, Myc-Fzo1p-HRN/



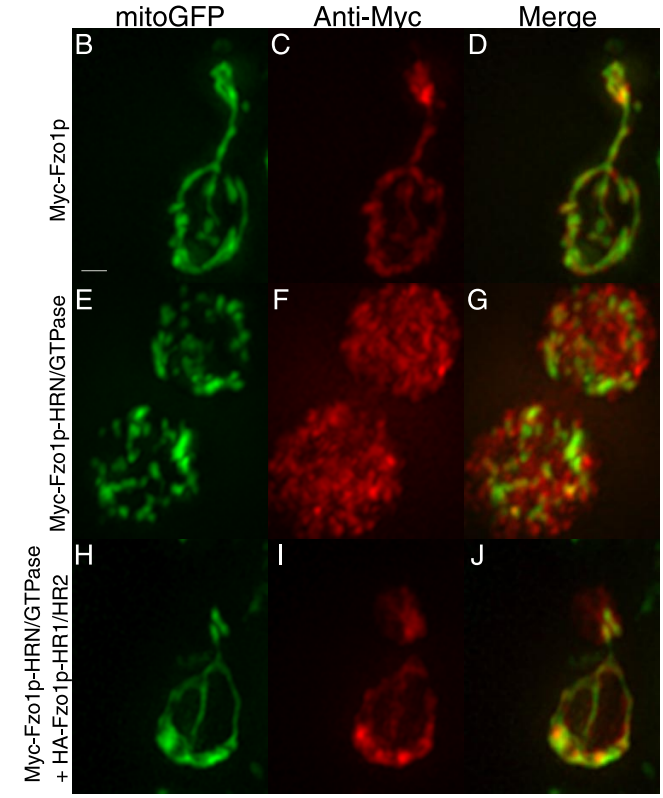


FIGURE 5. **Two interacting fragments of Fzo1p.** *A*, co-immunoprecipitations were performed as in Fig. 3. Fzo1p-HRN/GTPase (residues 1–415) is referred to as '*N*' and Fzo1p-HR1/HR2 (residues 416–855) is referred to as '*C*'. *wt*: full-length Fzo1p. *B–J*, immunofluorescence (*red*, *middle panels*) was used to localize Myc-tagged full-length Fzo1p (Myc-Fzo1p, *B–D*), Myc-Fzo1p-HRN/GTPase (*E–G*), and Myc-Fzo1p-HRN/GTPase when co-expressed with HA-Fzo1p-HR1/HR2 (*H–J*) in *fzo1*Δ cells. Mitochondria were labeled with mito-GFP (*left panels*). Overlays of the two signals are shown in the merged images (*right panels*). *Bar*, 1 μm.

GTPase localization was non-mitochondrial and probably cytosolic in all cells ($n = 200$, Fig. 5, *E–G*). However, co-expression with the mitochondrial HA-Fzo1p-HR1/HR2 caused redistribution of Myc-Fzo1p-HRN/GTPase to mitochondria in 41% (7% completely mitochondrial, 34.5% mitochondrial and cytosolic) of cells ($n = 200$, Fig. 5, *H–J*). These data confirm the physical interaction between Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 and indicate that this interaction occurs on mitochondria.

Having identified an interaction between these two Fzo1p fragments, we tested whether this interaction is functionally important. The null mutations identified previously were tested for their effect on the binding of Fzo1p-HRN/GTPase to Fzo1p-HR1/HR2. When mutations in either HR1 or HR2 were introduced into Fzo1p-HR1/HR2, its interaction with Fzo1p-HRN/GTPase was completely abolished (Fig. 6, *lanes 11–13*). Similarly, when mutations in HRN or the GTPase domain were introduced into Fzo1p-HRN/GTPase, its interaction with Fzo1p-HR1/HR2 was abolished (Fig. 6, *lanes 7–10*). Therefore, our null alleles indicate that the interaction of Fzo1p-HRN/GTPase with Fzo1p-HR1/HR2 is essential for fusion.

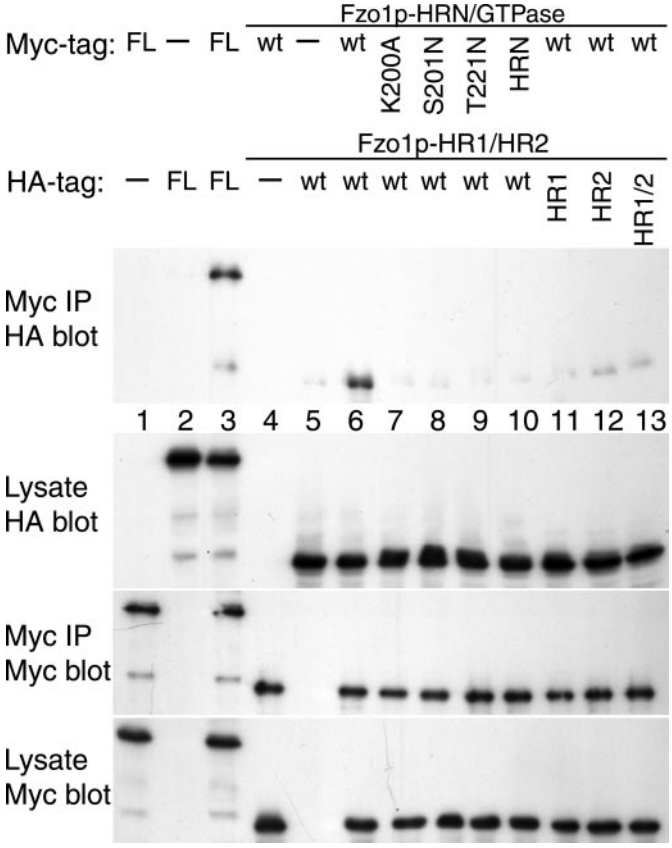


FIGURE 6. **The GTPase and heptad repeat regions are required for the interaction between Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2.** Co-immunoprecipitations were performed and labeled as in Fig. 3. The indicated mutations were present in either Fzo1p-HRN/GTPase (HRN, V172P, K200A, S201N, and T221A) or HA-Fzo1p-HR1/HR2 (HR1: L501A,L504A; HR2: L819P). *FL*: full-length wild-type Fzo1p.

Functional Complementation between Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2—Given their physical interaction on mitochondria, we tested if co-expression of Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 could restore mitochondrial fusion. When expressed individually, neither fragment supported growth on glycerol or rescued mitochondrial morphology (Fig. 7, *A* and *B*). Remarkably, the co-expression of Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 was sufficient to restore fusion. These cells grew robustly on glycerol plates and displayed largely tubular mitochondria (Fig. 7, *A* and *B*). Moreover, cells co-expressing the Fzo1p fragments show significant mitochondrial fusion in the zygotic fusion assay (Table 2) (28). These data indicate that Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 form a complex competent to support mitochondrial fusion.

DISCUSSION

In this study, we show that Fzo1p functions as an oligomer during mitochondrial fusion. In support of this conclusion, Fzo1p forms homotypic oligomers detectable by co-immunoprecipitation. In addition, specific pairs of *fzo1* null alleles are capable of intragenic complementation. Intragenic complementation can occur between pairs of mutants if each mutant affects a different domain of an oligomerized multidomain protein. Within the oligomer, different functional domains are donated from distinct molecules, resulting in the restoration of activity (27). An alternative scenario for intragenic complementation is that the GTPase and heptad repeat domains are not required on the same molecule, because they act at different steps in the fusion reaction. Although we cannot formally exclude this latter explanation, the observation that Fzo1p molecules physically associate strongly favors the former model.

Our intragenic complementation data provide insight into the requirements for the activity of different domains on Fzo1p molecules

TABLE 2
Strains used in this study

Yeast expressing mito-GFP and mito-DsRed were mated in YPD for 5 h. Following fixation, 200 large-budded zygotes were scored for mitochondrial fusion.

Strains mated	Mitochondrial fusion
	%
*FZO1 dnm1*Δ x *FZO1 dnm1*Δ	96
*fzo1*Δ *dnm1*Δ x *fzo1*Δ *dnm1*Δ	0
*fzo1*Δ *dnm1*Δ + pRS414-*9XMYC-FZO1* x *fzo1*Δ *dnm1*Δ + pRS414-*9XMYC-FZO1*	86
*fzo1*Δ *dnm1*Δ + pRS414-*9XMYC-FZO1(HRN/GTPase)* + pRS416-6XHA-*FZO1(HR1/HR2)* x *fzo1*Δ *dnm1*Δ + pRS414-*9XMYC-FZO1(HRN/GTPase)* + pRS416-6XHA-*FZO1(HR1/HR2)*	38.5

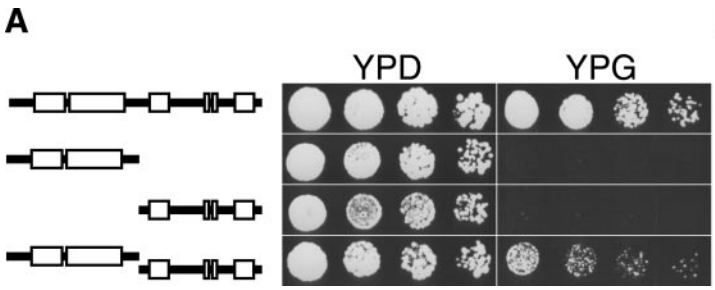


FIGURE 7. **Co-expression of the Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 fragments of Fzo1p complements *fzo1*Δ.** *A*, serial dilution assay for growth on glycerol was performed as described in Fig. 1. *B*, percentage of cells displaying the indicated mitochondrial morphologies during log phase growth in selective minimal dextrose media (images of the morphological classes are provided in Fig. 1). 200 live cells were counted for each strain.

within these oligomers. Within an Fzo1p oligomer, one molecule must possess the activity of all three heptad repeats (for example, a GTPase mutant). A second molecule must possess GTPase activity but not necessarily full heptad repeat activity (for example, an HRN, HR1, or HR2 mutant). Thus co-expression of Fzo1p molecules with heptad repeat activity and Fzo1p molecules with GTPase activity restores fusion, whereas co-expression of two heptad repeat mutants or two GTPase mutants does not restore fusion. Because Fzo1p is required on adjacent mitochondria to support fusion (29), an interesting question that remains to be addressed is whether the intragenic complementation we observe occurs in *cis* (in a complex formed on one mitochondrion) or in *trans* (in a complex that spans two mitochondria prior to fusion). In principle, this issue could be determined using an *in vitro* fusion assay, but unfortunately the available assay does not work for mitochondria isolated from yeast that are incompetent for respiration (29).

We have also identified binding of Fzo1p-HRN/GTPase to Fzo1p-HR1/HR2 as an interaction critical for mitochondrial fusion. First, co-expression of Fzo1p-HRN/GTPase and Fzo1p-HR1/HR2 supports mitochondrial fusion. Second, these Fzo1p fragments physically interact with each other. Finally, mutations in the GTPase domain or heptad repeat regions block this interaction and cause complete loss of mitochondrial fusion activity.

Future work will help to resolve the complex interactions occurring between Fzo1p molecules. Fzo1p is a large membrane-associated GTPase with some similarities to the dynamin family of mechanochemical membrane-severing enzymes. Dynamins are characterized by their oligomerization-dependent stimulation of GTP hydrolysis. Thus, it will be interesting to test the possibility that intermolecular interactions within an Fzo1p oligomer regulate its GTPase activity. Given that co-expression of a functional GTPase domain on one Fzo1p molecule and functional heptad repeat regions on another molecule is sufficient to support fusion, it is possible that the heptad repeats could play a role in regulating GTPase activity. For example, based on its location near the C terminus, it has been suggested HR2 could function analogously to the GTPase effector domain of dynamin (21). *In vitro* characterization of recombinant Fzo1p fragments can be used to address this possibility. Ultimately, structural information will be required to fully understand the nature of the Fzo1p oligomer.

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