The WD repeat protein, Mdv1p, functions as a molecular adaptor by interacting with Dnm1p and Fis1p during mitochondrial fission

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Yeast mitochondrial fission is a multistep process during which the dynamin-related GTPase, Dnm1p, assembles into punctate structures that associate with the outer mitochondrial membrane and mediate mitochondrial division. Steps in the Dnm1p-dependent process of fission are regulated by the actions of the WD repeat protein, Mdv1p, and the mitochondrial outer membrane protein, Fis1p. Our previous studies suggested a model where Mdv1p functions to regulate fission at a post-Dnm1p assembly step and Fis1p functions at two distinct steps, at an early point, to regulate Dnm1p assembly, and later, together with Mdv1p, to facilitate Dnm1p-dependent mitochondrial fission. To test this model, we have examined the physical and functional relationship between Mdv1p and Fis1p and present genetic, biochemical, and two-hybrid data indicating that a Fis1p–Mdv1p complex is required to regulate mitochondrial fission. To further define the role of Mdv1p in fission, we examined the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p. Data from two-hybrid analyses and GFP-tagged domains of Mdv1p indicate that it contains two functionally distinct domains that enable it to function as a molecular adaptor to regulate sequential interactions between Dnm1p and Fis1p and catalyze a rate-limiting step in mitochondrial fission.

Introduction

In the budding yeast Saccharomyces cerevisiae, mitochondria in logarithmically growing cells form a branched and continuous tubular structure distributed at the cell cortex. The formation and maintenance of this branched reticulum requires a balanced frequency of fusion and fission events (Nunnari et al., 1997; Shaw and Nunnari, 2002). Mitochondrial fusion is controlled by the evolutionarily conserved mitochondrial outer membrane GTPase, Fzo1p (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Santel and Fuller, 2001). Mitochondrial fission is a multistep process regulated by the conserved dynamin-related GTPase, Dnm1p, which localizes to the outer mitochondrial membrane and assembles into punctate structures associated with sites of mitochondrial constriction (Otsuga et al., 1998; Smirnova et al., 1998; Bleazard et al., 1999; Labrousse et al., 1999). Members of the dynamin-related GTPase family are required during cellular membrane remodeling events, such as the formation and scission of clathrin-coated vesicles from the plasma membrane during endocytosis. Mutations in DNM1 cause mitochondria to form net-like structures of interconnected mitochondrial tubules that are the result of unopposed mitochondrial fusion (Bleazard et al., 1999; Sesaki and Jensen, 1999).

We and others have identified and characterized two additional proteins, Mdv1p and Fis1p, that act together with Dnm1p to facilitate fission (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Mdv1p is a predicted soluble protein containing at least three regions: an NH2-terminal region of indeterminate structure, a predicted coiled-coil (C-C)* central domain, and a COOH-terminal seven-WD repeat domain. Cytology, genetics, and two-hybrid analyses indicate that Mdv1p interacts with Dnm1p in punctate structures to mediate mitochondrial fission (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Although Mdv1p is not required for the assembly of Dnm1p into punctate structures, Dnm1p-containing structures lacking Mdv1p are not able to complete division.

*Abbreviations used in this paper: AD, activating domain; BD, binding domain; C-C, coiled coil; DSP, dithiobis(succinimidylpropionate); MBP, maltose-binding protein; NTE, NH2-terminal extension; SB, sorbitol buffer.
indicating that Mdv1p function is required at a late step in the fission pathway (Tieu and Nunnari, 2000).

Fis1p is an evolutionarily conserved 18-kD protein that contains a COOH-terminal transmembrane domain that targets and anchors it in the mitochondrial outer membrane (Mozdy et al., 2000). Our previous analysis suggested that Fis1p performs two independent functions during mitochondrial fission (Tieu and Nunnari, 2000). First, Fis1p functions early in the fission pathway to regulate the assembly of Dnm1p into punctate structures and target Dnm1p to mitochondrial membranes (Mozdy et al., 2000; Tieu and Nunnari, 2000). A second function of Fis1p was inferred from the observation that in the absence of Dnm1p, Mdv1p remains associated with mitochondria in a Fis1p-dependent manner (Tieu and Nunnari, 2000). This observation suggests that Fis1p also functions together with Mdv1p later in the fission pathway. Based on these results, we proposed a model of mitochondrial fission where Dnm1p puncta associate and assemble on mitochondria in a Fis1p-dependent manner. Within these structures, Mdv1p interacts with Fis1p or a Fis1p-dependent component, resulting in the catalysis of mitochondrial division (Tieu and Nunnari, 2000).

We have tested this model for mitochondrial fission by examining the functional and physical relationship between Mdv1p and Fis1p and by determining the structural features of Mdv1p required for its activities. Here we present genetic, cytological, and biochemical evidence that Fis1p interacts with Mdv1p to regulate a rate-limiting, Dnm1p-dependent event during mitochondrial fission and report that Mdv1p performs the role of a molecular adaptor by interacting with both Dnm1p and Fis1p.

Results and discussion
Dnm1p puncta assemble in fis1-L80P cells, but fission is impaired

Our previous studies suggested a model where Fis1p functions at two distinct steps in the mitochondrial fission pathway (Tieu and Nunnari, 2000). Specifically, we proposed that Fis1p functions at an early step, to regulate Dnm1p assembly, and at a later step, with Mdv1p, to facilitate Dnm1p-dependent mitochondrial membrane constriction and division. To test this model of Fis1p function, we analyzed strains harboring novel FIS1 alleles for a mutation that would separate these two proposed functions. Interestingly, analysis of one FIS1 mutant indicated that, although mitochondrial fission was blocked, Dnm1p assembled into punctate structures characteristic of wild-type cells. Sequence analysis of the FIS1 locus in these cells revealed a point mutation that results in an amino acid change from leucine 80 to proline present in the conserved cytoplasmic region of Fis1p, in a position to mediate interactions with both Mdv1p and Dnm1p.

Examination of mitochondrial morphology in fis1-L80P cells with mitochondrial-targeted GFP revealed net-like mitochondrial structures, in contrast to the branched tubules seen in wild-type cells (Fig. 1 A; Table I). These structures were indistinguishable from those observed in mdv1Δ, fis1Δ, and dnm1Δ cells and indicate that mitochondrial fission is impaired in fis1-L80P cells (Fig. 1 A). Furthermore, quantification of this mitochondrial morphology defect indicates that the frequency of net-like structures was almost as high as observed in mdv1Δ and fis1Δ cells, indicating that mitochondrial fission is severely defective in fis1-L80P cells (Table I).

To determine whether Dnm1p assembly is affected in fis1-L80P cells, we examined the steady-state localization pattern of Dnm1p–GFP. Interestingly, the characteristics of Dnm1–GFP localization were not significantly altered as compared with wild-type cells (Fig. 1 B; Table I). The ability of Fis1p or a Fis1p-dependent component to support the assembly of Dnm1p into punctate structures is in contrast to what is observed in fis1Δ cells where Dnm1p assembly is aberrant, as indicated by the presence of fewer, brighter Dnm1p–GFP-labeled punctate structures (Fig. 1 B; Table I; Mozdy et al., 2000; Tieu and Nunnari, 2000).

Table 1: Quantification of mitochondrial morphology and Dnm1p-associated puncta

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<td></td>
<td>%</td>
<td>%</td>
<td>No. cells scored</td>
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<td>1</td>
<td>99</td>
<td>30</td>
</tr>
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<td>mdv1Δ</td>
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<td>0</td>
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Figure 1. The fis1-L80P mutation disrupts mitochondrial fission, but Dnm1p-containing puncta have wild-type characteristics. Mito-GFP was used to visualize mitochondrial morphology. Dnm1p was visualized using a Dnm1p–GFP fusion protein. (A) Mitochondrial morphology of representative wild-type, gene deletion, and fis1-l80P cells. (B) Dnm1–GFPp localization pattern in fis1-L80P cells. Bars, 2 μm.
These observations suggest that the fission defect observed in fis1-L80P cells is not the result of a defect in the assembly of Dnm1p-containing puncta, but rather occurs as a result of a defect at a later step in the division pathway. Significantly, the phenotypic characteristics of fis1-L80P cells are similar to those observed in mdv1-Δ cells (Fig. 1, A and B; Tieu and Nunnari, 2000). Together with our previous observation that in dnm1-Δ cells, Mdv1p remains associated with mitochondria in a Fis1p-dependent manner, these results support our hypothesis that Fis1p functions with Mdv1p to regulate fission at a post-Dnm1p assembly step.

**Overexpression of Mdv1p suppresses the fission defect in fis1-L80P cells**

We asked whether overexpression of Mdv1p could suppress the observed fission defect in fis1-L80P cells to further test our hypothesis that a Mdv1p-dependent, post-Dnm1p assembly step is specifically blocked in fis1-L80P cells. To overexpress Mdv1p in cells, we used the GAL1 promoter. When cells containing the GAL1-MDV1 plasmid were grown under inducing conditions, using the carbon source galactose, Mdv1p was overexpressed ~20-fold as assessed by SDS-PAGE and Western blotting with anti-Mdv1p antibodies (Fig. 2 A).

Examination of mitochondrial morphology in wild-type cells with mito-GFP indicated that overexpression of Mdv1p had no effect on mitochondrial structure (Fig. 2, B and C). This observation is in contrast to what has been observed in studies of Dnm1p, where overexpression results in mitochondrial fragmentation and indicates that, unlike Dnm1p, the concentration of Mdv1p in wild-type cells is not rate limiting for fission (Sesaki and Jensen, 1999; Fukushima et al., 2001). As expected, net-like mitochondrial structures observed in mdv1-Δ cells were transformed into tubular branched structures upon Mdv1p overexpression, indicating that fission is restored and that Mdv1p expressed from the GAL1 promoter is functional (Tieu and Nunnari, 2000; Fig. 2, B and C). Significantly, mainly branched reticular mitochondrial structures, characteristic of wild-type cells, were observed when Mdv1p was overexpressed in fis1-L80P, indicating that overexpression of Mdv1p suppresses the fission defect in fis1-L80P cells (Fig. 2, B and C). Mitochondrial nets, however, persisted in fis1-Δ cells when Mdv1p was overexpressed, demonstrating that suppression of the fission defect in fis1-L80P cells is allele specific (Fig. 2, B and C). Taken together, these observations are consistent with our hypothesis that a Mdv1p-dependent post-Dnm1p assembly step is specifically blocked in fis1-L80P and suggest that Mdv1p interacts with Fis1p during fission.

**Mdv1p and Fis1p are in a complex**

We previously used the two-hybrid assay to demonstrate that Dnm1p and Mdv1p interact (Tieu and Nunnari, 2000). In this study, we also used the two-hybrid assay to determine whether Mdv1p and Fis1p interact. Interactions between activating domain (AD) fusion proteins and binding domain (BD) fusion proteins were assessed by monitoring the expression of the stringent GAL2-ADE2 reporter gene. We tested both the full-length AD–FIS1 protein fusion and a construct lacking the transmembrane domain, AD–FIS1-Δ128–155, with BD–MDV1 to assess protein–protein interactions. Cells expressing a combination of AD–FIS1 and BD–MDV1 constructs displayed growth on media lacking adenine, indicating that Fis1p and Mdv1p specifically interact (Fig. 3 A; unpublished data). Cells harboring both the AD–FIS1-Δ128–155 and BD–MDV1 plasmids displayed more robust growth on media lacking adenine. Thus, not surprisingly, the Fis1p transmembrane domain interfered with the Fis1p–Mdv1p interaction in the assay.

To test whether a Mdv1p–Fis1p interaction occurs within the context of the mitochondrial membrane, we performed

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**Figure 2. Overexpression of MDV1 suppresses the mitochondrial fission defect in fis1-L80P cells.** All strains harboring either the empty pGAL1 vector or pGAL1-MDV1 vectors were grown in SKaf media to early log, subcultured into SGal media, and grown for an additional 12 h and analyzed as described. Mitochondrial morphology was visualized with mito-GFP. (A) Western blot analysis of galactose-induced expression of Mdv1p. (B) Mitochondrial morphology in fis1-L80P cells after overexpression of Mdv1p. C. Quantification of mitochondrial morphology phenotypes in cells overexpressing Mdv1p. Bars, 2 µm.
The Mdv1p–Fis1p interaction is abolished in fis1-L80P cells

Taken together, our genetic, biochemical, and two-hybrid analyses suggest that Fis1p interacts with Mdv1p to regulate a rate-limiting, post-Dnm1p assembly step in the fission pathway. In this context, these observations further suggest that an alteration in the Fis1p–Mdv1p interaction in fis1-L80P cells is specifically responsible for the observed defect in mitochondrial fission. To test this idea, we biochemically analyzed the Fis1p–Mdv1p interaction in fis1-L80P cells.

We first examined the stability and intracellular localization of Fis1p and Mdv1p in fis1-L80P cells. Wild-type and fis1-L80P cell extracts were fractionated by differential centrifugation, and analyzed by SDS-PAGE and Western blotting. Consistent with a mitochondrial localization, in extracts from both wild-type and fis1-L80P cells, the majority of Mdv1p and Fis1p cofractionated with porin, the mitochondrial marker, in the mitochondrial-enriched pellet fraction (Fig. 4 A, compare lanes 1–3 with 4–6). In addition, levels of Mdv1p and Fis1p were similar in fis1-L80P and wild-type cell extracts (Fig. 4 A, compare lanes 1–3 with 4–6). These results indicate that both Fis1p and Mdv1p are expressed stably and localized correctly to mitochondria in fis1-L80P cells. Thus, the fission defect ob-
served in fis1-L80P cells is not simply the result of Fis1p and/or Mdv1p instability.

To test whether the Mdv1p–Fis1p interaction is altered in fis1-L80P cells, we determined whether Fis1p coimmunoprecipitated with Mdv1p under conditions where a complex was detected in wild-type cells (Fig. 3). Interestingly, when Mdv1p was immunoprecipitated from DSP–cross-linked extract from fis1-L80P cells with anti-Mdv1p antibodies, we failed to detect Fis1p in the precipitates, in contrast to wild-type cells (Fig. 4 B, pGAL1). These results suggest that a complex containing Mdv1p and Fis1p-L80P fails to form, or that interactions within the complex are weakened and thus harder to detect. Consistent with the latter possibility is our observation that overexpression of Mdv1p in fis1-L80P cells suppresses the mitochondrial fission defect. Thus, we tested whether increasing the amount of Mdv1p in the fis1-L80P cells could, by mass action, restore the Mdv1p–Fis1p interaction observed by coimmunoprecipitation with anti-Mdv1p antibodies.

Wild-type, mdv1-Δ, and fis1-L80P cells harboring pGAL1-MDV1 were grown in galactose to induce overexpression of Mdv1p, DSP cross-linked, and immunoprecipitated with anti-Mdv1p antibodies. Western blot analysis of fractions from wild-type cells indicated that Fis1p coimmunoprecipitated with Mdv1p either with or without overexpression of Mdv1p (Fig. 4 B, pGAL1 and pGAL-MDV1, lanes 1 and 4). Interestingly, overexpression of Mdv1p did not increase the fraction of Fis1p that coimmunoprecipitated with Mdv1p, suggesting that Mdv1p is not limiting for this interaction. As expected, when Mdv1p is overexpressed in mdv1-Δ cells, Fis1p can be observed in the immunoprecipitates, in contrast to immunoprecipitates from mdv1-Δ cells harboring the pGAL1 vector without the MDVI gene (Fig. 4 B). Significantly, overexpression of Mdv1p in fis1-L80P cells restored the Mdv1p–Fis1p interaction as detected by coimmunoprecipitation (Fig. 4 B, pGAL1 and pGAL-MDV1, lanes 1 and 4). These observations indicate that the Mdv1p–Fis1p interaction is defective in fis1-L80P cells and that overexpression of Mdv1p can restore this interaction, as detected by coimmunoprecipitation. These data suggest that the mitochondrial fission defect observed in fis1-L80P cells is the result of a defective Fis1p–Mdv1p interaction, suggesting a role for this interaction at a late post-Dnm1p assembly step in mitochondrial fission.

**Mdv1p functions as a molecular adaptor in fission**

Data presented in this manuscript demonstrate that Mdv1p interacts with Fis1p during fission to catalyze a rate-limiting step. We had previously shown that Mdv1p also interacts with Dnm1p in punctate structures within cells in a Fis1p-independent manner (Tieu and Nunnari, 2000). Thus, to gain further insight into the molecular mechanism of mitochondrial fission, we examined the regions of Mdv1p responsible for its interactions with Fis1p and Dnm1p.

Mdv1p contains at least three distinct regions: a novel NH2-terminal extension region (NTE), a middle region predicted to form a C-C structure, and a COOH-terminal region that contains seven WD repeats predicted to form a seven-bladed propeller structure (WD) (Fig. 5 A; Tieu and Nunnari, 2000). To analyze the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p, we constructed GAL1-regulated GFP fusions to each of these putative domains and examined their localization patterns after expression in wild-type, mdv1-Δ, fis1-Δ, dnm1-Δ, and fis1-Δ dnm1-Δ cells. We also examined mitochondrial morphology, particularly in wild-type and mdv1-Δ cells, to determine whether expression of Mdv1p domains produced dominant negative phenotypes or could complement the loss of MDVI function, respectively.

The localization of GFP-tagged Mdv1p domains and mitochondrial morphology in representative cells are presented and summarized in schematic form in Fig. 5 B. As expected, GFP-tagged Mdv1p was localized to punctate structures primarily associated with mitochondria in wild-type, mdv1-Δ, and fis1-Δ cells, but not in dnm1-Δ, consistent with our previous observations showing that GFP–Mdv1p interacts and colocalizes with Dnm1p in these structures in a Fis1p-independent manner (Fig. 5 B, panels 1–4). In addition, in mdv1-Δ cells, mitochondrial tubular structures characteristic of wild-type cells were observed, indicating that, as previously published, GFP–Mdv1p is functional (Fig. 5 B, panel 2; Tieu and Nunnari, 2000). Also as previously shown, GFP–Mdv1p was observed uniformly localized to mitochondria in dnm1-Δ cells (Fig. 5 B, panel 4; Tieu and Nunnari, 2000). The functional and biochemical data presented in this study indicate that Mdv1p’s mitochondrial localization pattern in dnm1-Δ cells reflects a Dnm1p-independent interaction between Mdv1p and Fis1p. Consistent with this interpretation, in cells lacking both Dnm1p and Fis1p, Mdv1p was observed in a diffuse pattern, associated with the cytosolic fraction (unpublished data; Tieu and Nunnari, 2000).

Mitochondrial net-like structures were observed in mdv1-Δ strains expressing each Mdv1p region alone, indicating that no single region was sufficient for wild-type levels of mitochondrial fission (Fig. 5 B, panels 2, 6, 10, and 14; 100%, n = 50 in all strains). However, in wild-type, mdv1-Δ, and fis1-Δ cells expressing a GFP-tagged version of WD region of Mdv1p, GFP fluorescence was observed in punctate structures, primarily associated with mitochondria (Fig. 5 B, panels 9–12). The localization of GFP–WD to punctate structures was not observed in dnm1-Δ cells, indicating that Dnm1p is required for their formation (Fig. 5 B, panel 12). In addition, punctate structures labeled by GFP–WD also contained Dnm1p, as assessed by colocalization of Dnm1p–dsRed in wild-type cells (Fig. 5 C). Taken together, these observations suggest that the WD region is sufficient to interact with Dnm1p.

GFP–NTE labeled both mitochondria and punctate structures in wild-type cells (Fig. 5 B, panels 5–8). Unlike GFP–WD, however, the punctate structures labeled by GFP–NTE probably do not reflect an interaction of NTE with Dnm1p and may be the result GFP–NTE self-aggregation because they also were observed in dnm1-Δ and dnm1-Δ fis1-Δ cells (Fig. 5 B, panel 12; unpublished data). In addition, GFP–NTE-labeled punctate structures were observed localized at the cell cortex, not associated with mitochondria. The dispersive mitochondrial labeling pattern observed for GFP–NTE in wild-type cells was not observed in fis1-Δ, suggesting that the NTE interacts specifically with Fis1p in cells (Fig. 5 B,
Consistent with this interpretation, GFP–NTE also was observed to be associated with mitochondria in dnm1\-
H9004 cells (Fig. 5 B, panel 8). In contrast, GFP–WD was observed in a diffuse, cytosolic pattern in the majority of dnm1-
H9004 cells (Fig. 5 B, panel 12). Thus, the cytological analysis of Mdv1p domains suggests that the WD and NTE regions are each suf-
ficient to interact with Dnm1p and Fis1p, respectively.

Interestingly, in all cell types examined, the GFP-tagged C-C region of Mdv1p was observed in a diffuse pattern,
consistent with a cytosolic localization, suggesting that this region is not involved in mediating interactions with either Dnm1p or Fis1p (Fig. 5 B, panels 13–16). Interestingly, in contrast to the NTE region, expression of GFP-tagged C-C and WD in wild-type cells caused mitochondrial net-like structures to form (Fig. 5 B, panels 9 and 13; 80% net-like structures, \( n = 58 \), and 19% net-like structures, \( n = 59 \), re-
spectively), indicating that these regions interfere with mito-
chondrial fission in a dominant negative manner.

To independently test our cytological observations, we also examined the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p using the two-hybrid assay. We tested both the full-length Mdv1p and Dnm1p protein fusions and Fis1\,128–155p with regions of Mdv1p to determine protein–protein interactions. An interaction was indicated by cells growing on media lacking adenine (indicated by + in Table II).

As previously shown, full-length Mdv1p was observed to interact with both Fis1\,128–155p and Dnm1p in this as-
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A molecular model for mitochondrial fission

Data presented in this paper support a model, similar to the one recently proposed where Fis1p plays two distinct and separable roles during mitochondrial fission (Shaw and Nunnari, 2002). Early in the fission pathway, Fis1p targets Dnm1p to mitochondrial membranes and regulates its assembly probably via a direct interaction. Dimeric Mdv1p coassembles with Dnm1p in Dnm1p-containing punctate structures where it functions, specifically at a rate-limiting step where fission is triggered. Here we have provided genetic and biochemical evidence that Fis1p also functions at this rate-limiting step, by interacting with Mdv1p. Our observations also indicate that Mdv1p plays the role of a molecular adaptor, whose two functional domains are separated by a C-C region. Our analysis supports a model where the WD domain of Mdv1p mediates an interaction with assembled Dnm1p. Given that expression of the WD region interferes with mitochondrial fission in a dominant negative manner, we infer that the localization of Mdv1p to assembled Dnm1p structures is important for its ability to stimulate fission. Our data also support a role for the NTE region of Mdv1p as a molecular switch that interacts in a regulated manner with Fis1p, triggering conformational changes within an assembled Dnm1p structure that bring about the division of mitochondrial membranes. Elucidation of the stoichiometry of Dnm1p, Fis1p, Mdv1p, and any other factors within the complex(es) we have identified will help answer the question of how these components function in generating the force required to coordinately divide the outer and inner mitochondrial membranes.

Materials and methods

Media and yeast genetic techniques

Yeast strains used in this study are listed in Table III. Standard genetic techniques and yeast media, including VPG (3% glycerol), YPGal (2% galactose), SD, SRaf (2% raffinose), and SGal (2% galactose), were prepared as previously described (Guthrie and Fink, 1991). Yeast transformations were performed as previously described (Gietz and Schiestl, 1991).

Strains, plasmid construction, and yeast two-hybrid analysis

The fis1-L80P allele was isolated as an extragenic suppressor of the fzo1-1 cells as described and identified by analyzing linkage from a cross to an fzo1-1 fis1-1 strain (Tieu and Nunnari, 2000). The mutation was identified by amplifying the FIS1 locus in fis1-L80P cells by PCR using Vent polymerase (New England Biolabs, Inc.) and sequencing the products directly (Davis Sequencing, University of California, Davis). A plasmid containing GAL1-regulated MDV1 was constructed by introducing EcoRI and BamHI sites by PCR amplification of the MDV1 ORF, followed by subcloning into p416GAL1 (American Type Culture Collection [ATCC]), generating the pGAL1-MDV1 plasmid. The GFP-tagged Mdv1p domains were constructed by PCR amplifying S65T GFP lacking the 3′ terminator codon using oligonucleotides engineered with 5′ Spel and 3′ XbaI sites. The amplified products were ligated in frame with the S65T GFP into p416GAL1 (ATCC), yielding plasmids pGAL1-GFP–MDV1, pGAL1-GFP–MDV1(NTE), pGAL1-GFP–MDV1(WD), and pGAL1-GFP–MDV1(C-C), respectively.

Two-hybrid constructs were made by amplifying regions of the MDV1 ORF and introducing EcoRI and BamHI restriction sites by PCR. Full-length MDV1 was amplified from the 5′ ATG initiator to nucleotide 2142. The WD region was amplified from nucleotide 903 to 2142 including a 5′ ATG initiator, the NTE/C-C region was amplified from the 5′ ATG initiator to nucleotide 903, and the C-C region was amplified from nucleotide 723 to

Table III. Yeast strains

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<td>JSY1371</td>
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Table II. Two-hybrid interactions between Mdv1p and Mdv1p domains, Fis1Δ128–155p and Dnm1p

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Five colonies for each construct pair were tested. All constructs paired with empty BD and AD plasmids did not grow on adenine– selective medium. –, no growth on selective medium; +, growth on selective medium.

*Growth observed only for one of the reciprocal AD and BD plasmid pairs.
Cytological analysis

Mitochondrial morphology was analyzed and quantified using MiToTracker CMXR (Molecular Probes) or mito-GFP expressed from the plasmid pYX232 (provided by B. Westermann, Ludwig Maximilians Universitaet, Muenchen, Germany) as previously described (Tieu and Nunnari, 2000). Dnm1p was visualized and quantified by transforming cells with pH520 (DNM1–GFP) (gift from R. Jensen, John Hopkins University, Baltimore, Maryland) or pEClN233 (DNM1–dsRED) as previously described (Tieu and Nunnari, 2000). pGA1 plasmids containing GFP-tagged MDV1 regions were transformed into JY1826, JN854, JN855, and J1371. Transformants were grown in SD, subcultured into SRA media, further subcultured into and scaled up to SGA, supplemented with 1% dextrose to induce expression of GFP-tagged Mdv1p domains, and imaged after 12 h of logarithmic growth. All samples were imaged using either a Leica confocal or DeltaVision microscope with either a 100× 1.4NA or 60× 1.4NA objective.

Biochemical analyses

Soluble MBP–Fis1Δ128–155 was expressed in Escherichia coli (BL21 [DE3]) at 37°C and purified using amylase affinity chromatography (New England Biolabs). Anti-Fis1p polyclonal antibodies were produced in rabbits by injection of the purified MBP–Fis1Δ128–155 fusion protein (Co- Vance Research, Inc.).

Cell extracts were fractionated by differential centrifugation and analyzed by SDS-PAGE and Western blotting as previously described (Tieu and Nunnari, 2000). Immunoprecipitation of Mdv1p or Fis1p was performed from yeast cells grown in rich or SD media to a density of 1 OD600. After washing in water, 10 OD600 equivalents of cells were resuspended in 0.1 ml of sorbitol buffer (12 M sorbitol, 10 mM Heps, 1 mM MgCl2) digested with 10 µl of 10 mg/ml yeast lytic enzyme (80,000 U/g; ICN Biomedicals) for 15 min at 30°C, and washed twice with 10 volumes of cold SB. After treatment with lytic enzyme, cells were resuspended in 0.92 ml of cold SB, and 0.08 ml of 18 mM DSP (Pierce Chemical Co.) in DMSO was added and cells were incubated for 2 h on ice to cross-link proteins. Cross-linking was terminated by quenching with 0.15 ml of 1 M glycine, pH 8.0, and cells were washed twice with two volumes of cold SB. Cross-linked cells were resuspended in 0.6 ml of 20 mM Tris pH 8.0, 50 mM ammonium acetate, 2 mM EDTA, and transferred to Eppendorf tubes containing 1 ml of glass beads (Sigma-Aldrich) and 0.60 ml of 20% TCA. The cells were lysed by bead beating with three cycles of 30-s intervals with cooling on ice between cycles. The cell lysate was removed, collected by centrifugation, resuspended in 120 µl of buffer containing 3% SDS, 100 mM Tris, pH 11, and incubated for 5 min at 65°C and 100°C for 5 min. Insoluble material was pelletted by centrifugation. To 40 µl of the SDS-solubilized sample, 560 µl of solution containing 13.3 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.02% NaN3, 6 µl trysylol, and 6 µl protease inhibitor cocktail (Sigma-Aldrich) was added. The renatured sample was incubated with 50 µl protein A agarose beads equilibrated with buffer containing 0.2% SDS, 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.02% NaN3 (IPS) at 4°C with gentle shaking for 15 min. The cleared supernatant was removed to a new equilibration containing 20 µl of IPS-equilibrated anti-Mdv1p–protein A (anti-Mdv1p) beads were added, and incubated at 4°C with gentle shaking for 45 min. The cross-linked anti-Mdv1p–protein A agarose beads were washed as previously described (Harlow and Lane, 1998). Specifically, a total of 6 mg of crude anti-Mdv1p antibodies were cross-linked to 2.0 ml of protein A agarose beads (Santa Cruz Biotechnology, Inc.) with 20 ml of the water-soluble cross-linker dimethylpimelimidate. 25 µl of anti-Mdv1p–protein A cross-linked beads (antibody–agarose) were washed per immunoprecipitation with 10 OD600 equivalents of cells prepared as described above. The protein-bound resin was washed five times with 1 ml of cold PBS, resuspended with 30 µl SDS-PAGE loading buffer, boiled for 5 min, and subjected to SDS-PAGE and Western blotting.

References


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