A novel motif in the yeast mitochondrial dynamin Dnm1 is essential for adaptor binding and membrane recruitment

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To initiate mitochondrial fission, dynamin-related proteins (DRPs) must bind specific adaptors on the outer mitochondrial membrane. The structural features underlying this interaction are poorly understood. Using yeast as a model, we show that the Insert B domain of the Dnm1 guanosine triphosphatase (a DRP) contains a novel motif required for association with the mitochondrial adaptor Mdv1. Mutation of this conserved motif specifically disrupted Dnm1–Mdv1 interactions, blocking Dnm1 recruitment and mitochondrial fission.

Introduction

Eukaryotic cells possess a family of dynamin-related proteins (DRPs), each of which is responsible for a specific cellular membrane-remodeling event. For example, the dynamin-related GTPase Drp1 (Dnm1 in yeast) mediates mitochondrial (Bleazard et al., 1999; Labrousse et al., 1999; Sesaki and Jensen, 1999) and peroxisomal fission (Koch et al., 2003; Li and Gould, 2003; Kuravi et al., 2006), Atlastin (Hu et al., 2009; Orso et al., 2009; Moss et al., 2011) and Mitofusins (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998; Chen et al., 2003; Eura et al., 2003) play roles in ER and mitochondrial membrane fusion, respectively, and ARC5 (Gao et al., 2003) facilitates chloroplast membrane division. Like classical dynamin, DRPs self-assemble into highly ordered oligomers that use the energy of GTP hydrolysis to remodel lipid bilayers (Praefcke and McMahon, 2004).

Dynamin and DRPs have conserved GTPase, middle, and GTPase effector domains (see Fig. 1A; van der Bliek, 1999). These domains mediate self-assembly and modulate GTPase activity. Dynamin and DRPs also contain nonconserved domains that, in some cases, have been shown to determine their cellular distribution and heterotypic interactions. For example, a proline-rich domain at the C terminus of dynamin facilitates its binding to a variety of actin-binding proteins. Dynamin also harbors a pleckstrin homology (PH) domain between its GTPase and GTPase effector domain, which is essential for interactions with the plasma membrane. In place of the PH domain, DRPs contain a region called Insert B (InsB) whose length and sequence varies. Whether or not there is a conserved function for InsB is not clear.

Although dynamin interacts directly with the lipid bilayer via its PH domain, DRPs do not initially interact with the lipid bilayers they remodel. Instead, they are recruited to specific cellular sites via interactions with membrane-associated adaptor proteins. In most cases, the DRP domains necessary for adaptor interactions have not been identified. However, structural studies of dynamin and several DRPs (Mears et al., 2007, 2011; Faelber et al., 2011; Ford et al., 2011) suggest that InsB is a likely candidate because it is predicted to reside at the base of the DRP oligomer, closest to the membrane.

Suppressor mutations in Mdv1 that restored Dnm1–Mdv1 interactions and fission identified potential protein-binding interfaces on the Mdv1 β-propeller domain. These results define the first known function for Insert B in DRP–adaptor interactions. Based on the variability of Insert B sequences and adaptor proteins, we propose that Insert B domains and mitochondrial adaptors have coevolved to meet the unique requirements for mitochondrial fission of different organisms.

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Abbreviations used in this paper: colP, coimmunoprecipitation; DRP, dynamin-related protein; DSP, dithiobis(succinimidyl propionate); InsB, Insert B; mt-ffRFP, mitochondrial-targeted fast-folding RFP; PH, pleckstrin homology; WT, wild-type.

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We used the yeast mitochondrial fission machinery as a model to directly test the function of InsB in DRP–adaptor interactions and DRP1 membrane recruitment. In vivo, Dnm1 (the yeast DRP) is recruited to the mitochondrial outer membrane by Mdv1 (the adaptor; Tieu and Nunnari, 2000; Cerveny et al., 2001), which in turn associates with membrane-anchored Fis1 (Mozdy et al., 2000). Once on the membrane, Dnm1 co-assembles with Mdv1 into spirals that encircle and divide the mitochondrial tubule (Bhar et al., 2006; Naylor et al., 2006). We identified a motif in the Dnm1 InsB domain that is required to recruit Dnm1 to mitochondria. The amino acid sequence of this motif is strictly conserved among fungi and is predicted to form a solvent-inaccessible helix (PSIPRED v2.6). Amino acid substitutions in this InsB helix inhibit the recruitment of Dnm1 to mitochondria and block fission. Importantly, these mutations do not impair Dnm1 self-assembly. Instead, they specifically disrupt the Dnm1–Mdv1 interaction. The disrupted interaction is rescued by suppressor mutations in the Mdv1 β-propeller domain to which Dnm1 binds. These findings identify a new functional motif in the Dnm1 InsB domain required for Mdv1 binding and recruitment of Dnm1 from the cytoplasm to its site of action on mitochondria.

**Results**

**Dnm1 InsB contains a conserved sequence motif predicted to be solvent inaccessible**

In a previous genetic screen (Bhar et al., 2006), we identified an InsB mutation (F610L) that interfered with Dnm1 function in mitochondrial fission. Alignment of *Saccharomyces cerevisiae* Dnm1 InsB with its fungal homologues revealed a motif of eight strictly conserved residues encompassing F610 (Fig. 1, A and B). These residues are predicted to form a short helix (PSIPRED v2.6) that is less accessible to solvent than surrounding residues (Fig. 1 C), suggesting that they are part of an interaction interface.

**InsB motif residues are important for Dnm1 function in mitochondrial fission**

Yeast mitochondria form multiple tubular and branched structures that are continually remodeled by fission and fusion events. When fission is disrupted or blocked, mitochondria fuse to form netlike structures or a single interconnected tubule that often collapses to one side of the cell (Bleazard et al., 1999). To evaluate Dnm1 InsB motif function, we individually replaced the eight conserved residues with alanine and quantified the ability of each mutant protein to rescue fission defects in cells lacking the wild-type (WT) Dnm1 protein (dnm1Δ). As shown in Fig. 2 A, expression of WT Dnm1 rescued fission in up to 90% of dnm1Δ cells. In contrast, four Dnm1 mutant proteins (F606A, L607A, F610A, and F611A) failed to rescue mitochondrial morphology in this strain. Hereafter, these four loss-of-function proteins will be collectively referred to as Dnm1<sup>InsBmut</sup>. Western blotting of whole cell extracts indicated that the Dnm1<sup>InsBmut</sup> proteins were expressed at levels similar to WT Dnm1 (Fig. S1 A). Thus, mutations in conserved residues of InsB block Dnm1 function in mitochondrial fission but do not affect protein stability.

In the absence of WT Dnm1, the inability of Dnm1<sup>InsBmut</sup> proteins to support fission could be caused by their failure to self-assemble. To test this possibility, we coexpressed HA- and Myc-tagged versions of the same InsB mutant proteins in dnm1Δ cells and performed coimmunoprecipitation (coIP) assays. As shown in Fig. 2 B, each form of the HA-tagged Dnm1<sup>InsBmut</sup> protein was efficiently coprecipitated with the Myc-tagged version of itself. Thus, the F606, L607, F610, and F611 residues in InsB are not essential for Dnm1 self-assembly.

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The InsB motif is essential for Dnm1 binding to the Mdv1 adaptor

After mitochondrial membrane recruitment and self-assembly, the majority of GFP-tagged Dnm1 can be visualized as puncta on mitochondrial tubules (Fig. 3 A; Otsuga et al., 1998). In contrast, all of the GFP-Dnm1<sup>InsBmut</sup> proteins failed to associate with mitochondria (Fig. 3 A, a representative cell is shown). Instead, GFP-Dnm1<sup>InsBmut</sup> proteins assembled into punctuate structures that moved rapidly through the cytoplasm and could not be captured by digital imaging. In a few cells, GFP-Dnm1<sup>InsBmut</sup> also formed larger, immobile aggregates in the cytoplasm (Fig. 3 A). Importantly, this localization of GFP-Dnm1<sup>InsBmut</sup> proteins is indistinguishable from that observed for GFP-Dnm1 in cells lacking Fis1 or fission adaptor proteins (Mozdy et al., 2000; Griffin et al., 2005). This observation raised the possibility that mutations in Dnm1<sup>InsBmut</sup> proteins were disrupting interactions with the Mdv1 adaptor.

Suppressors of a dnm1<sup>InsBmut</sup> mutation cluster in the Mdv1 β-propeller

Using an integrated form of the dnm1<sup>F610A</sup> mutation, we performed a suppressor screen to identify residues in Mdv1 important for Dnm1–Mdv1 interaction (see Materials and methods and Table S1). Although the screen covered 84% of the MDV1 coding sequence, all but one suppressor mutation fell in the Mdv1<sup>β-propeller</sup> domain (Fig. 4 A). Most of the affected residues localized to the top of the Mdv1 β-propeller model (Fig. 4 B, right),
suggested that they are part of an interaction interface. Suppressors affecting residues on the bottom of the structure may define an additional binding interface. Two additional mutations, S557C and T558I, lay in a short sequence that was eliminated during homology modeling. These results are consistent with our finding that the Mdv1 \(\beta\)-propeller is sufficient for direct interaction with Dnm1 (Fig. S1 B). The final suppressor mutation altered residue Q288 in the Mdv1 coiled-coil domain. This suppressor was not analyzed further, as the structure and function of the coiled-coil domain has been extensively studied (Koirala et al., 2010; Zhang et al., 2012).

In addition to Mdv1, yeast encodes a second fission adaptor protein called Caf4 (Griffin et al., 2005). Although these two adaptors are paralogues and have similar domain structures, Caf4 is not essential for mitochondrial fission. Sequence alignment revealed that most of the suppressor mutations affected conserved or similar amino acids in the Mdv1 and Caf4 \(\beta\)-propeller domains (Fig. 4 C). This conservation is consistent with an important functional role for these amino acids in vivo.

All but one of the residues (S541) identified in our suppressor screen differ from those reported by others (Cerveny and Jensen, 2003; Naylor et al., 2006). The mutations in these previous studies were selected based on homology modeling with known \(\beta\)-propeller structures, whereas the mutations we identified here were selected by the organism in a suppressor screen and are specifically relevant to defects caused by InsB mutations. The exception, S541, was reported not to have a phenotype when replaced by glutamine (Q) in the full-length Mdv1 protein (Naylor et al., 2006). In contrast, we found that an Mdv1 S541G mutant protein was unable to rescue mitochondrial fission defects in an \(mdv1\Delta\) strain (Table 1). This difference may be because of the different yeast strain backgrounds used in these two studies. It is also possible that mutation to glycine has a more significant effect on local protein structure and flexibility than mutation to glutamine.

**Suppressor mutations in the Mdv1 \(\beta\)-propeller rescue mitochondrial fission defects caused by Dnm1\(^{F610A}\)**

In control studies, the Mdv1 suppressor proteins were all stably expressed in vivo (Fig. S1 C). To verify that the Mdv1 suppressor proteins were capable of rescuing fission, we expressed them from a plasmid in cells lacking WT Mdv1 and expressing Dnm1\(^{F610A}\) protein from the genome. All of the Mdv1 suppressors rescued mitochondrial fission defects in this strain (up to 70% rescue). Representative results are shown in Fig. 5 A for the five best rescuing Mdv1 suppressors (Y418C, D539G, D539Y, L540P, and D576G).

The same Mdv1 suppressors also rescued mitochondrial morphology defects caused by other Dnm1\(^{\text{InsBmut}}\) proteins (F606A, L607A, and F611A; Fig. 5 B). To test whether Mdv1 suppressors could rescue the mitochondrial recruitment of Dnm1\(^{F610A}\), Mdv1 suppressors and GFP-Dnm1\(^{F610A}\) were coexpressed in an \(mdv1\Delta dnm1\Delta\) strain. GFP mitochondrial puncta were visible in up to 70% of these cells (Fig. 5 C). The mitochondrial recruitment and puncta formation by GFP-Dnm1\(^{F610A}\) suggested that Dnm1 interaction with the Mdv1 suppressor proteins had been restored. However, this interaction could not be detected in coIP assays, even after chemical cross-linking (unpublished data). Thus, the restored interaction between Dnm1\(^{F610A}\) and Mdv1 suppressors is less robust than WT.

As an alternative, we evaluated Dnm1\(^{F610A}\)–Mdv1 suppressor interactions using the yeast two-hybrid growth assay. Previous studies established that WT Dnm1 and WT Mdv1 interact in

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**Figure 3.** InsB conserved residues are critical for Dnm1–Mdv1 interaction. (A) Representative images of GFP-Dnm1 and GFP-Dnm1\(^{\text{InsBmut}}\) (F606A, L607A, F610A, or F611A) localization in \(dnm1\Delta\) cells. Differential interference contrast microscopy (DIC), mitochondrial matrix-targeted dsRed (mt-ffRFP), GFP, and merged images are shown. Bar, 5 \(\mu\)m. (B) Lysates from cells expressing the indicated C-terminal Myc-tagged Dnm1 were used for immunoprecipitation with anti-Myc agarose beads. Immunoprecipitated fractions (top) and lysates (bottom) were analyzed by SDS-PAGE and Western blotting with anti-Myc and anti-Mdv1 antibodies.
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Figure 4. Suppressors of a dnm1F610A mutation cluster in the Mdv1 β-propeller. (A) Domain structure of Mdv1 with indicated Dnm1F610A suppressor mutations (the number of mutations obtained for each allele is in parentheses). NTE, N-terminal Extension; CC, coiled coil. (B) Top and side views of the Mdv1 β-propeller model (blue). Red indicates residues changed by suppressor mutations. The N terminus is in green. (C) Alignment of the portions of the Mdv1 (aa 408-643) and Caf4 (aa 329-566) β-propeller sequences. Residues affected by suppressor mutations are shown in red. Symbols below the sequence alignment indicate identity (*), strong similarity (:), and weak similarity (.) of amino acids.

Table 1. Characterization of Mdv1 suppressors of the dnm1F610A allele

| Mutations | WT mitochondrial morphologya | GFP mitochondrial puncta b | Interaction with Dnm1c |
|-----------|-------------------------------|-----------------------------|------------------------|
|           | DNM1 dnm1Δ                  | dnm1F610A dnm1Δ            | Dnm1                 | Dnm1F610A |
|           | %                             | %                           | Dnm1                  | Dnm1F610A |
| Mdv1      | 67 ± 7                       | 0 ± 0                       | +                     | +                 |
| Y418C     | 6 ± 3                        | 41 ± 16                     | +                     | +                 | +                  |
| K430R     | 9 ± 8                        | 28 ± 4                      | +                     | +                 | +                  |
| E504G     | 19 ± 8                       | 32 ± 16                     | +                     | +                 | ++                 |
| D539G     | 9 ± 2                        | 68 ± 11                     | +                     | +                 | ++                 |
| D539Y     | 11 ± 1                       | 61 ± 14                     | +                     | +                 | ++                 |
| L540P     | 12 ± 5                       | 58 ± 14                     | +                     | +                 | ++                 |
| S541G     | 8 ± 4                        | 38 ± 12                     | +                     | +                 | ++                 |
| S557C     | 10 ± 8                       | 43 ± 8                      | +                     | +                 | ++                 |
| T558I     | 15 ± 6                       | 31 ± 12                     | +                     | +                 | ++                 |

Numbers are the mean and standard deviation of at least three independent experiments, n = 300.

aGFP puncta localized to mitochondrial tubules.

bYeast two-hybrid assays. – No growth on His or Ade minus medium; +, growth on His but not Ade minus medium; ++, growth on both His and Ade minus media. Growth on Ade minus medium is the more stringent indicator of protein–protein interaction.

dtnsNsite (Fig. 5 D; Tieu and Nunnari, 2000; Cerveny and Jensen, 2003; Karren et al., 2005). Although the interaction was severely disrupted when Dnm1F610A was paired with WT Mdv1, the interaction was partially (D539Y and L540P) or completely (Y418C and D539G) restored by substituting an Mdv1 suppressor protein for WT Mdv1 (Fig. 5 D). Surprisingly, the Mdv1Δ576G
suppressor protein reproducibly rescued both mitochondrial morphology and GFP-Dnm1<sup>F610A</sup> localization (Fig. 5, A–C), but was unable to restore growth in the two-hybrid assay (Fig. 5 D). In the morphology rescue and localization studies, binding of Dnm1<sup>F610A</sup> to this Mdv1 suppressor may be sufficient to recruit Dnm1<sup>F610A</sup> to the membrane, after which oligomerization of both proteins into mitochondrial fission complexes could further stabilize the interaction. Such stabilizing forces may be compromised by the spheroplasting/lysis required for coIP studies or by the fusion of both proteins to nuclear targeting sequences used in the two-hybrid assay. This interpretation is supported by our finding that GFP-Mdv1<sup>D576G</sup> assembled into punctate fission complexes in the presence of Dnm1<sup>F610A</sup> (Fig. S1 D).

**Discussion**

Although Dnm1 binding to Mdv1 and recruitment to the mitochondrial membrane is essential for fission, the Dnm1 domains required for this interaction were not known. Here we identify a novel motif in Dnm1 InsB that is specifically required for interaction with the Mdv1 β-propeller. Second-site suppression studies and cell-based assays confirm that the InsB–β-propeller interaction is critical for Dnm1–Mdv1 binding and Dnm1 membrane recruitment. The Dnm1 InsB motif and Mdv1 adaptor sequences required for this interaction are conserved in fungi but not in mammals or plants (Fig. 6). Thus, different InsB domains and adaptors may have coevolved in different organisms to mediate membrane targeting of mitochondrial dynamin-related GTPases.

Figure 5. Suppressor mutations in the Mdv1 β-propeller rescue mitochondrial fission defects caused by Dnm1<sup>F610A</sup>. (A) Quantification of mitochondrial morphology in mdv1Δ DNM1 and mdv1Δ dnm1::dnm1<sup>F610A</sup> strains expressing the indicated Mdv1 suppressor proteins. (B) Quantification of mitochondrial morphology in mdv1Δ dnm1::dnm1<sup>F610A</sup> strains expressing indicated Mdv1 and Dnm1 proteins from plasmids. The mitochondria were visualized with MitoFluor Red S89 (Molecular Probes). [*] Plasmid-expressed WT Mdv1 rescued Dnm1<sup>F610A</sup> better than genome-expressed WT Mdv1 (Fig. 2 A). This is likely because of the higher steady-state abundance of the plasmid-expressed protein. (C) Quantification of GFP-Dnm1 localization in mdv1Δ dnm1::dnm1<sup>F610A</sup> cells expressing the indicated Mdv1 and GFP-Dnm1 variants. [A and C] Black columns and error bars represent the mean and standard deviation of at least three independent experiments (A, n = 300; C, n = 150). (D) pGBD and pGAD plasmids expressing the indicated fusion proteins were cotransformed into the Y187 yeast two-hybrid reporter strain and grown on S-Dextrose minus histidine plates at 30°C for 3 d.
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residues affected by the suppressor mutations likely delineate regions of the Mdv1 β-propeller in close proximity to Dnm1.

As new mitochondrial fission components were identified, it became clear that different organisms express unrelated adaptor proteins (Fig. 6 A; Tieu and Nunnari, 2000; Cerveny et al., 2001; Griffin et al., 2005; Nishida et al., 2007; Arimura et al., 2008; Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). We propose that the InsB domain provides some of the variability needed to mediate these diverse DRP–adaptor interactions. As shown in Fig. 6 (B and C), sequence alignments reveal little identity between InsB sequences of fungi, algae, plants, and mammals. Conversely, high identity is observed among InsB sequences of representative mammals (Fig. 6 C, 94%). The functional interaction we observe between Dnm1 InsB and the Mdv1 β-propeller in yeast may be recapitulated for DRP–adaptor interactions in other organisms. However, there are almost certainly additional DRP–adaptor interfaces that remain to be identified, especially in mammals, where a single DRP is able to interact with several structurally distinct adaptors (Fig. 6 A; Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010; Palmer et al., 2011; Zhao et al., 2011). A recent study showed that mutation of a conserved residue in the Drp1 middle domain disrupts interaction with a

Our GFP localization and two-hybrid studies established that suppressor mutations in the Mdv1 β-propeller partially restore the interaction of the adaptor with Dnm1F610A. However, this suppression is not allele specific because the suppressor mutations also rescue defects caused by dnm1F606A, dnm1L607A, and dnm1F611A (Fig. 5 B). These results suggest that suppression is not occurring by a classical “lock and key” model. It is possible that the suppressor mutations in the Mdv1 β-propeller increase protein flexibility, allowing them to bind the mutant Dnm1 proteins more efficiently. An alternative explanation is that the suppressor mutations establish new contacts that enhance the interaction between the Mdv1 β-propeller and Dnm1F610A. Although all twelve of the Mdv1 suppressor mutations in the β-propeller were able to recruit and assemble GFP-tagged WT Dnm1 into mitochondrial puncta, ten failed to rescue mitochondrial fission (Table 1, <20% rescue in a DNM1 mdv1Δ strain). Interestingly, these ten Mdv1 suppressor proteins exhibit more robust interaction with WT Dnm1 in a two-hybrid assay (Table 1), likely because of the new contacts. When WT Dnm1 is substituted for Dnm1F610A, these new interactions may interfere with post-recruitment and/or assembly steps of mitochondrial fission. The substituted amino acids in the Mdv1 suppressors must be physically close enough to Dnm1 to establish contacts. Thus, the residues affected by the suppressor mutations likely delineate regions of the Mdv1 β-propeller in close proximity to Dnm1.

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mitochondrial adaptor called Mff (Strack and Cribbs, 2012). The model that InsB domains mediate DRP interactions in other organisms can be directly tested in genetic and cellular studies, as well as structural studies of DRPs bound to their cognate adaptor proteins.

### Materials and methods

#### Yeast strains and plasmids

Yeast strains used in this study include JSY7540 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 mad1Δ::HIS3), JSY1361 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3), JSY9744 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3), JSY9933 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3), JSY9934 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3), JSY1362 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3), JSY9743 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3), and JSY1361 (MATa leu2-1 his3-Δ200 trp1-Δ63 ura3-Δ52 lys2-Δ202 Mdv1Δ::HIS3) strains were identified by their ability to grow on S-dextrose minus histidine or minus uracil. Cells expressing endogenous Dnm1Δ::dnm1 allele (dnm1Δ::dnm1) prevents mitochondrial fragmentation and genome loss, allowing mdv1Δ dnm1Δ::dnm1Δexpressing plasmids were identified by their ability to grow on glycerol at 37°C. Candidate clones with verified phenotypes were sequenced to identify MDV1 mutations. Alleles with multiple amino acid changes, mutations were separated by site-directed mutagenesis. Mutations contributing to growth phenotypes were analyzed for mitochondrial morphology and GFP-Dnm1 localization.

#### Fluorescence microscopy

Mitochondrial morphologies were quantified in WT, dnm1Δ, and mdv1Δ strains expressing the indicated proteins. The WT morphology category includes unbudded or budded cells with more than two free tubules in the mother cell. The formation of GFP-Dnm1 mitochondrial puncta was quantified by analysis of deconvolved epifluorescence images of random fields of cells. Phenotypic quantification is reported as the mean and standard deviation of three independent experiments (total n > 300 cells unless noted). Unless specified in the figure legend, the mitochondria were visualized by expressing mitochondrial-targeted fast-folding RFP (mRFP). Dnm1 InsB variants were expressed in the Dnm1Δ promoter in the pRS415 vector. GFP-tagged Dnm1 variants were expressed from the pRS415 vector. Yeast cells were grown at 30°C in synthetic selective dextrose medium containing 1.0 mg/ml methionine. Overnight cultures were diluted to 0.2 OD600 and grown for 3–5 h (OD600, 0.5–1.0). The mCherry-negative repressible pMD1 promoter is leaky under these conditions and expresses approximately four-fold more protein at steady state than that expressed from the endogenous DNM1 or MDV1 promoters (Koira et al., 2005).

A microscope (Axioplan 2; Carl Zeiss) equipped with a 100× oil immersion objective was used to observe and image cells. For mitochondria and resuspended in 500 µl IP buffer (0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.4, and 1.500 protease inhibitor cocktail set III (EMD)). Cells were lysed with glass bead and cleared by centrifugation at 18,000 g for 10 min. 400 µl of supernatant was incubated with 40 µl of anti-c-Myc-conjugated agarose beads (Sigma-Aldrich) for 1 h at 4°C. Agarose beads were washed and collected using sample buffer lacking β-mercaptoethanol. For mdv1Δ suppression of the dnm1Δ allele

The growth phenotypes of strains used for this screen are summarized in Table S1. The PCR amplification with Taq DNA polymerase was used to introduce random mutations into the MDV1 coding region. The CRISPR products were introduced into linearized pRS415-MET25 using gap repair (Csonka et al., 1983) in mdv1Δ dnm1Δ::dnm1Δ cells. In temperature-sensitive fzo1Δ cells, ongoing mitochondrial fission causes fragmentation, mitochondrial genome loss, and inability to grow on glycerol medium at 37°C (Herrmann et al., 1998). Disrupting fzo1Δ in this strain by introducing an mdv1Δ mutation and expressing dnm1Δ::HIS3 from the endogenous DNM1 locus (dnm1Δ::dnm1Δ) prevents mitochondrial fragmentation and genome loss, allowing mdv1Δ dnm1Δ::dnm1Δ in the PBD fold recognition server (Kelley and Sternberg, 2009). Residues 349–713 of Mdv1 are variably modeled as a seven- or an eight-bladed β-propeller, depending on the structure most recently deposited in the PDB. The eight-bladed β-propeller model shown in Fig. 4 includes the majority of residues identified in the second-site suppressor analysis described here.

#### Yeast two-hybrid analysis

Yeast two-hybrid studies to analyze Dnm1–Mdv1 and Dnm1 self-interactions were performed in the Y187 S. cerevisiae strain background (Takara Bio Inc.) via a growth assay as described previously (Guthrie and Fink, 2002). Yeast cells were co-transformed into the Y187 reporter strain. Interaction between two fusion proteins leads to expression of one of several reporter genes in this strain, allowing the yeast cells to grow on S-dextrose minus histidine or minus adenine. Interaction between two fusion proteins leads to expression of one of several reporter genes in this strain, allowing the yeast cells to grow on S-dextrose minus histidine or minus adenine. WT Dnm1–Dnm1 interactions were performed in cells expressing GAD-Dnm1 WT and GBD-Dnm1 WT. The Dnm1–Mdv1 interaction was tested in both directions. However, the interaction was only detected when Dnm1 and Mdv1 were fused with the GAD and GBD domains, respectively.

#### Online supplemental material

Table S1 shows a screen for mdv1Δ suppressors of dnm1Δ::HIS3. Table S2 shows the plasmids used in this study. Fig. S1 shows expression, interaction, and assembly properties of Dnm1 and Mdv1 variants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201207079/DC1.
References

Arimura, S., M. Fujimoto, Y. Doniwa, N. Kadoya, M. Nakazono, W. Sakamoto, and N. Tsutsui. 2008. Arabidopsis ELONGATED MITOCHONDRIA1 is required for localization of DYNAMIN-RELATED PROTEIN3A to mitochondrial fission sites. Plant Cell. 20:1555–1566. http://dx.doi.org/10.1105/tpc.108.058578

Bhar, D., M.A. Karren, M. Babst, and J.M. Shaw. 2006. Dimeric Dnm1-G385D interacts with Mdv1 on mitochondria and can be stimulated to assemble into fission complexes containing Mdv1 and Fis1p. J. Biol. Chem. 281:17312–17320. http://dx.doi.org/10.1074/jbc.M513530200

Bleazard, W., J.M. McCaflery, E.J. King, S. Bale, A. Mordy, Q. Tieu, J. Nunnari, and J.M. Shaw. 1999. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. Nat. Cell Biol. 1:298–304. http://dx.doi.org/10.1038/13014

Cerveny, K.L., and R.E. Jensen. 2003. The WD-repeats of Net2p interact with Dnm1p and Fis1p to regulate division of mitochondria. Mol. Biol. Cell. 14:4126–4139. http://dx.doi.org/10.1091/mbc.E03-02-0092

Cerveny, K.L., J.M. McCaffery, and R.E. Jensen. 2001. Division of mitochondria requires a novel DNM1-interacting protein, Net2p. Mol. Biol. Cell. 12:309–321.

Chen, H., S.A. Detmer, A.J. Ewald, E.E. Griffin, S.E. Fraser, and D.C. Chan. 2003. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fission and are essential for embryonic development. J. Cell Biol. 160:189–200. http://dx.doi.org/10.1083/jcb.200211046

Eura, Y., N. Ishihara, S. Yokota, and K. Mihara. 2003. Two mitofusin proteins, Mfn1 and Mfn2, direct mitochondrial fission and are essential for embryonic development. Nat. Cell Biol. 15:1190–1202. http://dx.doi.org/10.1038/mbx.1949

Moss, T.J., C. Andreazza, A. Verma, A. Daga, and J.A. McNew. 2001. Membrane fusion by the GTPase DLP1 atlastin requires a conserved C-terminal cytoplasmic tail and dimerization through the middle domain. Proc. Natl. Acad. Sci. USA. 108:11133–11138. http://dx.doi.org/10.1073/pnas.105056108

Moody, A.D., J.M. McCaffery, and J.M. Shaw. 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J. Cell Biol. 151:367–380. http://dx.doi.org/10.1083/jcb.151.2.367

Nishida, K., F. Yagisawa, H. Kuroiwa, Y. Yoshida, and T. Kureiwa. 2007. WD40 protein Mda1 is purified with Dnm1 and forms a dividing ring for mitochondrial fission before Dnm1 in Cyanobioschyzon merolae. Proc. Natl. Acad. Sci. USA. 104:4736–4741. http://dx.doi.org/10.1073/pnas.0609364104

Orso, G., D. Pendin, S. Liu, J. Ingrosso, T.J. Moss, J.E. Faust, M. Micaroni, A. Egorova, A. Martinuzzi, J.A. McNew, and A. Daga. 2009. Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. Nature. 460:978–983. http://dx.doi.org/10.1038/nature08280

Otera, H., C. Wang, M.M. Cleland, K. Setoguchi, S. Yokota, R.J. Youle, and K. Mihara. 2010. MIF is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J. Cell Biol. 191:1141–1158. http://dx.doi.org/10.1083/jcb.201007152

Otsuga, D., B.R. Keegan, B. Brisch, J.W. Thatcher, G.J. Hermann, W. Bleazard, and J.M. Shaw. 1998. The dynamin-related GTPase, Dnm1p, controls mitochondrial morphology in yeast. J. Cell Biol. 143:333–349. http://dx.doi.org/10.1083/jcb.143.2.333

Palmer, C.S., L.D. Oselame, D. Laine, O.S. Koutsopoulos, A.E. Frazier, and M.T. Ryan. 2011. MIF and MID51, new components of the mitochondrial fission machinery. EMBO Rep. 12:565–573. http://dx.doi.org/10.1038/embr.2011.54

Praefcke, G.J., and H.T. McMahon. 2004. The dynamin superfamily: universal membrane tubulation and fission molecules? Nat. Rev. Mol. Cell Biol. 5:133–147. http://dx.doi.org/10.1038/nrm1313

Rapaport, D., M. Brunner, W. Neupert, and B. Westermann. 1998. Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in Saccharomyces cerevisiae. J. Biol. Chem. 273:20150–20155. http://dx.doi.org/10.1074/jbc.273.32.20150

Sesaki, H., and R.E. Jensen. 1999. Division versus fusion: Dnm1p and Fzo1p are anti-orthologues of the mitochondrial division machinery and play an antagonistic role in the generation of the tubular ER network. J. Cell Biol. 140:1141–1158. http://dx.doi.org/10.1083/jcb.140.2.1141

Strack, S., and J.T. Cribbs. 2012. Allosteric modulation of Drp1 mechanoenzyme assembly and mitochondrial fission by the variable domain. J. Biol. Chem. 287:10990–11001. http://dx.doi.org/10.1074/jbc.M112.342105
