Molecular architecture of a dynamin adaptor: implications for assembly of mitochondrial fission complexes

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Recruitment and assembly of some dynamin-related guanosine triphosphatases depends on adaptor proteins restricted to distinct cellular membranes. The yeast Mdv1 adaptor localizes to mitochondria by binding to the membrane protein Fis1. Subsequent Mdv1 binding to the mitochondrial dynamin Dnm1 stimulates Dnm1 assembly into spirals, which encircle and divide the mitochondrial compartment. In this study, we report that dimeric Mdv1 is joined at its center by a 92-Å anti-parallel coiled coil (CC). Modeling of the Fis1–Mdv1 complex using available crystal structures suggests that the Mdv1 CC lies parallel to the bilayer with N termini at opposite ends bound to Fis1 and C-terminal β-propeller domains (Dnm1-binding sites) extending into the cytoplasm. A CC length of appropriate length and sequence is necessary for optimal Mdv1 interaction with Fis1 and Dnm1 and is important for proper Dnm1 assembly before membrane scission. Our results provide a framework for understanding how adaptors act as scaffolds to orient and stabilize the assembly of dynamins on membranes.

Introduction

In eukaryotes, mitochondrial fission regulates organelle copy number and mitochondrial function in metabolism, development, and programmed cell death (Chen and Chan, 2005; Okamoto and Shaw, 2005). Fission begins when a dynamin-related GTPase is recruited from the cytoplasm to the outer membrane, where it assembles into large polymers that hydrolyze GTP and sever the mitochondrial compartment. Protein–protein interactions between the GTPase and a membrane-anchored receptor are essential for the recruitment step and are also thought to provide a structural scaffold that promotes GTPase assembly.

The membrane receptor for yeast mitochondrial fission is a complex composed of two proteins, membrane-anchored Fis1 (Mozdy et al., 2000) and its binding partner, Mdv1 (Tieu and Nunnari, 2000; Cerveny et al., 2001; Tieu et al., 2002; Cerveny and Jensen, 2003). Mdv1 functions as an adaptor to bridge the interaction between Fis1 and the cytoplasmic Dnm1 GTPase. Binding of Dnm1 to Mdv1 nucleates the polymerization of Dnm1 dimers into spirals that encircle and constrict the membrane (Bleazard et al., 1999; Ingerman et al., 2005; Bhar et al., 2006; Lackner et al., 2009). Mdv1 coassembles with Dnm1 in these spirals to generate functional fission complexes (Shaw and Nunnari, 2002). Although fission complexes could, in principle, assemble uniformly on the mitochondrial surface, assembly usually occurs at discrete sites on tubular mitochondria in living cells.

The architectural features of the Mdv1–Fis1 receptor required for Dnm1 recruitment and assembly remain unclear. Structural analysis of the Fis1 cytoplasmic domain reveals a single tetratricopeptide repeat (TPR; Suzuki et al., 2005). The N terminus of Mdv1 contains a short, helix-loop-helix motif that surrounds and clamps the surface of the Fis1 TPR domain (Zhang and Chan, 2007). The C terminus of Mdv1, which is required for Dnm1 binding, is predicted to form a multibladed β-propeller. The N- and C-terminal domains are linked by a predicted heptad repeat (HR). Because Mdv1 acts both to recruit Dnm1 and nucleate Dnm1 assembly, defining the structure,
results

structure of the Mdv1 CC

The Mdv1 sequence was analyzed using the MultiCoil CC prediction program (Wolf et al., 1997). The ends (231–299) for the expression construct were chosen based on the total probability score (P > 0.2). Mdv1 CC
231–299
was expressed as a maltose-binding protein (MBP) fusion protein in Escherichia coli, released by proteolytic cleavage, and purified to homogeneity (see Materials and methods; Fig. 1, A and B). Equilibrium sedimentation (ES) centrifugation (Fig. 1 C) and circular dichroism (CD) analyses (Fig. 1 D) indicated that Mdv1 CC
231–299
is a dimer exhibiting double minima typical of a strong α-helix.

Figure 1. Mdv1 self-assembles via a dimeric, antiparallel CC. (A, top) Domain structure of Mdv1, including the NTE, predicted CC, and WD repeats predicted to form a β-propeller. (bottom) The construct used for purification of the Mdv1 CC domain includes the MBP fused to 10xHis, the PreScission protease cleavage site (PPCS), and Mdv1 residues 231–299 (CC, MBP-10xHis-PPCS-CC). (B) SDS-PAGE analysis of purified MBP-10xHis-PPCS-CC fusion protein stained with Coomassie brilliant blue (lane 1), PreScission protease-cleaved MBP-10xHis-PPCS + CC (lane 2), and purified CC (lane 3). (C) Sedimentation equilibrium profile of the Mdv1 CC fragment at the indicated initial loading concentrations (open symbols) with the corresponding fit of 16,263 D (MW/WMWsolvent = 1.95). Residuals for the nonlinear least-squared fits are shown below. (D) CD wavelength scan of CC
231–299
dimer. (E) Crystal structure of CC
231–292
dimer at a 2.6-Å resolution. Hydrophobic side chains of residues L233, L237, I251, I254, L268, I272, and I275 are shown in magenta.

subsection exposed at the surface of the CC, we show that the CC sequence can function to stabilize the Mdv1–Fis1 complex. The combined data reveal new insights into the formation of functional mitochondrial fission complexes.

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The crystal structure of a selenomethionine-substituted Mdv1 CC\textsuperscript{231–299} construct (L248M and L281M; 2.6 Å; Fig. 1 E and Table S2) revealed that residues 231–292 of this polypeptide fold into a single, 92-Å helix that forms an antiparallel homodimer. This structure is unusual, as intermolecular homodimeric CCs are predominantly parallel. To our knowledge, the structure of only one other homodimeric antiparallel CC of this length (>90 Å) has been reported, also in a protein required for mitochondrial membrane dynamics (Mfn1 HR2, 95 Å; Koshiba et al., 2004). The electrostatic surface of the structure is not highly positive (unpublished data), suggesting that Mdv1 CC\textsuperscript{231–292} does not associate directly with negatively charged lipids exposed at the cytoplasmic face of the outer mitochondrial membrane.

The Mdv1 CC is essential for mitochondrial fission
To determine whether the antiparallel CC interaction in Mdv1 was required for mitochondrial fission, we changed isoleucine and leucine residues contributing to formation of the hydrophobic interface in this structure to glutamate in the full-length protein (Figs. 1 E and 2 A, magenta side chains). Consistent with
for the majority of mitochondrial fission that occurs in vivo, deletion of both Mdv1 and Caf4 is necessary to completely abolish fission. The adaptor Δ strain exhibits severe fission defects, including interconnected, netlike, or collapsed mitochondrial tubules (Fig. 2, D and E). Although expression of Mdv1 restored WT morphology in ∼75% of the population, expression of mdv1CC7 failed to rescue fission defects (Fig. 2, D and E). Thus, dimerization of Mdv1 via its antiparallel CC plays a critical role in mitochondrial fission. When expressed from the uninduced MET25 promoter, steady-state abundance of Mdv1 is fourfold higher than that of Mdv1 expressed from its native promoter (Karren et al., 2005; unpublished data). Although expressing the extended length of this CC, glutamate substitution of all seven isoleucine and leucine residues depicted in Fig. 2 A was necessary to disrupt Mdv1 function (unpublished data). Western blotting of yeast whole cell extracts (WCEs) indicated that the abundance of the mutant adaptor protein, hereafter referred to as mdv1CC7, was similar to that of wild-type (WT) Mdv1 (Figs. 2 B and S4 A). However, these mutations nearly abolished the ability of mdv1CC7 to self-interact in coimmunoprecipitation (co-IP) experiments (Fig. 2 C).

We tested the ability of mdv1CC7 to support mitochondrial fission in adaptor-null (adaptor Δ) yeast cells lacking Mdv1 and its paralogue Caf4 (Griffin et al., 2005). Although Mdv1 is required for the majority of mitochondrial fission that occurs in vivo, deletion of both Mdv1 and Caf4 is necessary to completely abolish fission. The adaptor Δ strain exhibits severe fission defects, including interconnected, netlike, or collapsed mitochondrial tubules (Fig. 2, D and E). Although expression of Mdv1 restored WT morphology in ∼75% of the population, expression of mdv1CC7 failed to rescue fission defects (Fig. 2, D and E). Thus, dimerization of Mdv1 via its antiparallel CC plays a critical role in mitochondrial fission. When expressed from the uninduced MET25 promoter, steady-state abundance of Mdv1 is fourfold higher than that of Mdv1 expressed from its native promoter (Karren et al., 2005; unpublished data). Although expressing
fourfold higher levels of mdv1<sup>CC7</sup> protein did not cause dominant fission defects in WT cells (Fig. 2 F), dominant-negative fission phenotypes began to appear when expression was further elevated by a 4-h induction of the MET25 promoter (Fig. S4 C, ~16-fold overexpression). This dominant-negative effect suggests that mdv1<sup>CC</sup> continues to interact with one or more of its other two binding partners, effectively interfering with the WT fission machinery.

**CC formation promotes mitochondrial recruitment and assembly of the Mdv1 fission adaptor**

We used the mdv1<sup>CC</sup> mutant protein to test the importance of Mdv1 CC formation for its membrane recruitment. Experiments in intact cells expressing GFP-mdv1<sup>CC7</sup> revealed that the localization of the mutant protein was compromised relative to WT. In control experiments, the majority of WT GFP-Mdv1 colocalized with RFP-labeled mitochondrial tubules (Fig. 3, A and C, top), whereas a small fraction of cells also displayed dual localization to mitochondria and the cytoplasm (Fig. 3 A). In contrast, GFP-mdv1<sup>CC7</sup> did not localize exclusively with mitochondria. Instead, yeast cells expressing GFP-mdv1<sup>CC7</sup> exhibited dual mitochondrial and cytoplasmic localization or exclusively cytoplasmic localization of the adaptor protein (Fig. 3, A and C, bottom). Western blotting indicated that this cytoplasmic localization was not caused by proteolysis and release of GFP from the N terminus of the fusion protein (unpublished data).

After membrane recruitment, localization of a subpopulation of Mdv1 appears punctate because of its coassembly with the Dnm1 GTPase into fission complexes (Tieu and Nunnari, 2000; Cerveny et al., 2001; Griffin et al., 2005; Karren et al., 2005; Naylor et al., 2006). Although mitochondrial localized GFP-Mdv1 was largely punctate (Fig. 3 B), GFP-mdv1<sup>CC7</sup> failed to coassemble into punctate fission complexes and uniformly labeled mitochondrial membranes in the majority of cells (Fig. 3, B and C, middle). This uniform mitochondrial labeling resulted from association of Mdv1 with Fis1 and was abolished in cells lacking Fis1 (mdv1<sup>CC7</sup> had no effect on Fis1 localization; unpublished data). These combined data indicate that CC formation promotes both Mdv1 recruitment to Fis1 on mitochondria and subsequent higher order assembly of Mdv1 into fission complexes.

**Mitochondrial recruitment and self-assembly of the Dnm1 GTPase requires Mdv1 CC formation**

The Dnm1 GTPase interacts with the C terminus of Mdv1 (Tieu et al., 2002; Cerveny and Jensen, 2003), which is predicted to form a multibladed β-propeller. This interaction promotes the self-assembly of Dnm1 into rings and spirals (Lackner et al., 2009) that are visualized as mitochondrial puncta (fission complexes) when labeled with a fluorescent marker (Otsuga et al., 1998). We tested whether Mdv1 dimerization in vivo was required for Mdv1–Dnm1 interaction and Dnm1 assembly into mitochondrial puncta. Although HA-tagged Mdv1 efficiently coprecipitated Dnm1 from cell lysates, complex formation between mdv1<sup>CC7</sup>-HA and Dnm1 was significantly reduced (Fig. 4 A). This reduced interaction was also apparent in vivo. Although GFP-Dnm1 efficiently assembled into puncta on mitochondria when cells expressed WT Mdv1 (Fig. 4, B and C, top), the fraction of cytoplasmic GFP-Dnm1 increased in cells expressing mdv1<sup>CC7</sup> (Fig. 4, B and C, bottom). Residual GFP-Dnm1 puncta on mitochondria in mdv1<sup>CC7</sup>-expressing cells were often smaller and less-evenly distributed on the organelle tubules (Fig. 4 C, middle). This may be caused in part by the fact that mdv1<sup>CC7</sup> uniformly labels mitochondrial membranes (RFP-mdv1<sup>CC7</sup>, Fig. 4 D) but does not efficiently coassemble into puncta with Dnm1 (Fig. 3 C, middle and bottom). When combined with our finding that GFP-mdv1<sup>CC7</sup> fails to form mitochondrial puncta and cannot support fission (Fig. 2 E; and Fig. 3 C, middle and bottom), these results provide a direct demonstration that dimeric Mdv1 must coassemble with the Dnm1 GTPase to form functional fission complexes.

**Mdv1 dimerization via a heterologous antiparallel CC partially restores adaptor function**

To determine whether physical CC formation was sufficient for adaptor function, we generated a chimeric protein, which replaced the Mdv1 CC with residues 674–734 of the mammalian mitofusin protein Mfn1 (mdv1<sup>HR2</sup>; Fig. 5 A). In crystallographic experiments, this Mfn1 domain forms an antiparallel CC similar in length to the Mdv1 CC (95 Å; Koshiba et al., 2004). As observed for WT Mdv1, the mdv1<sup>HR2</sup> chimera was stably expressed (Figs. 5 B and S4 A), localized with mitochondria in differential fractionation and fluorescence microscopy experiments (Figs. 5 C and S1 A), and was able to self-interact in co-IP experiments (Fig. 5 D). Unlike WT Mdv1, mdv1<sup>HR2</sup> only partially rescued mitochondrial fission and morphology defects in the adaptor Δ strain (Figs. 5 E and S1 B). This partial rescue could not be attributed to defects in mdv1<sup>HR2</sup>–Dnm1 interactions because mdv1<sup>HR2</sup>-HA coimmunoprecipitated Dnm1 and the WT adaptor (Fig. 5 F), formed mitochondrial puncta in vivo when labeled with GFP (Fig. S1 C), and also promoted assembly of GFP-Dnm1 into punctate mitochondrial fission complexes (Fig. S1 D). These results were surprising because they suggested that dimerization of Mdv1 via an antiparallel CC is important but not sufficient for the adaptor’s function in mitochondrial fission. Instead, the sequence of the Mdv1 CC may also contribute to adaptor function.

**The sequence of the Mdv1 CC is critical for interaction with Fis1**

We noticed that the mitochondrial localization of mdv1<sup>HR2</sup> decreased relative to Mdv1 when Dnm1 was absent (Fig. S1 E). Under these conditions, stable mitochondrial association of mdv1<sup>HR2</sup> depends entirely upon interactions with Fis1. Thus, the inability of mdv1<sup>HR2</sup> to fully rescue fission defects in vivo could result from a reduced Fis1 interaction. It has been unclear whether the Mdv1 CC is necessary for interaction with Fis1. A crystal structure showed that α-helices in the N-terminal extension (NTE) of fission adaptor proteins bind directly to the cytoplasmic tetraarcipeptide domain of Fis1 (Zhang and Chan, 2007). Mutations predicted to disrupt contact sites in this complex interfered with Mdv1–Fis1 interactions and reduced fission in vivo.
Figure 4. Efficient Dnm1–Mdv1 interaction and Dnm1 assembly into functional mitochondrial fission complexes requires Mdv1 CC formation. (A) Lysates from cells expressing the indicated C-terminal HA-tagged Mdv1 proteins were used for IP with anti-HA agarose beads. Lysate (bottom) and immunoprecipitated fractions (top) were analyzed by SDS-PAGE and Western blotting with anti-HA and anti-Dnm1 antibodies. Asterisks mark protein breakdown products. (B) Localization of genomically expressed GFP-Dnm1 in cells expressing Mdv1 or mdv1CC7. Bars and error bars represent the mean and standard deviation of at least three independent experiments (n = 100). (C) Representative images of GFP-Dnm1 localization quantified in B. DIC, mitochondrial matrix–targeted RFP (mito-RFP), GFP-Dnm1, and merged RFP and GFP images are shown. (D) Colocalization of RFP-mdvCC7 and GFP-Dnm1. DIC and merged RFP with GFP images are shown. Bars, 5 µm.

However, genetic suppressor experiments from our laboratory suggested that the Mdv1 CC also contributes to the Mdv1–Fis1 interaction. We previously screened for suppressors of a Fis1 protein–destabilizing mutation (fis1-3; Karren et al., 2005). fis1-3 contains E78D, I85T, and Y88H substitutions in the concave surface of the Fis1 TPR domain that destabilize the protein at an
and mdv1^{HR2} proteins are reduced, whereas Fis1 interaction with the mdv1^{E250G} suppressor is enhanced relative to WT (Fig. 6 B). The straightforward interpretation of these results is that the sequence of the Mdv1 CC (and not just its dimerization capability) is critical for Mdv1 binding to Fis1 during early steps in fission complex assembly.

The length of the Mdv1 CC is optimized for mitochondrial fission

To test the influence of CC length on Mdv1 function, we generated variants lacking two (mdv1^{2HR}) or four (mdv1^{4HR}) HRs of the native Mdv1 CC (Fig. 7 A). Both ΔHR proteins were stably expressed at similar steady-state abundance, continued to self-interact, localized to mitochondria in a Fis1-dependent manner, elevated temperature (37°C). We identified an E250G substitution in the Mdv1 CC domain (at position g in the HR; Fig. S2) that stabilized Mdv1–Fis1 complex formation and restored mitochondrial fission at 37°C in the fis1-3 strain. These findings strongly suggest that the CC sequence is important for Mdv1–Fis1 interaction. To test this idea, we examined the ability of mdv1^{HR2} to interact with Fis1. As shown in Fig. 6, Myc-tagged Fis1 efficiently coprecipitated HA-tagged Mdv1 from cell lysates, whereas coprecipitation of the CC defective mdv1^{CC7}-HA protein was reduced. Significantly, coprecipitation of mdv1^{HR2}-HA with Myc-Fis1 was also reduced despite the fact that this chimeric adaptor protein can self-interact via the heterologous Mfn1 HR2 domain. Quantification of these Fis1–Mdv1 coIP interactions confirmed that Fis1 complex formation with mdv1^{CC7} and mdv1^{HR2} proteins are reduced, whereas Fis1 interaction with the mdv1^{E250G} suppressor is enhanced relative to WT (Fig. 6 B). The straightforward interpretation of these results is that the sequence of the Mdv1 CC (and not just its dimerization capability) is critical for Mdv1 binding to Fis1 during early steps in fission complex assembly.

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As with mdv1/hr2 and mdv1/hr4, we observed that mitochondrial localization of mdv1/hr2 and mdv1/hr4 decreased relative to WT Mdv1 in cells lacking Dnm1 (unpublished data). Because Mdv1 mitochondrial localization in dnm1 is Fis1 dependent, it is likely that shortening the native Mdv1 CC domain compromises the Fis1–Mdv1 interaction. Based on these combined results, we conclude that the overall length of the native CC is optimized for Mdv1 adaptor function in mitochondrial fission complex assembly and fission.

Discussion

Mdv1 is essential for the membrane recruitment and assembly of the mitochondrial dynamin Dnm1. By combining the new structure of the Mdv1 CC domain with a previously reported Fis1–Mdv1 NTE structure (Zhang and Chan, 2007), we provide a working model for the architecture of the Fis1–Mdv1 complex on the membrane. As depicted in Fig. 8, formation of a 92-Å antiparallel CC between two Mdv1 polypeptides forms an extended dimer. The NTE of each Mdv1 subunit is bound to distinct Fis1 TPR-like domains exposed at the cytoplasmic face of the mitochondrial membrane. Cryo-EM structures suggest that dimeric Dnm1 is the basic subunit for assembly of Dnm1 spirals (Hinshaw, J., personal communication), and two-hybrid and promoted Dnm1 assembly into mitochondrial fission complexes in vivo (Fig. S3, A, B, and D; Fig. S4, A and B; and not depicted). However, the ΔHR Mdv1 variants did not form mitochondrial puncta as efficiently as WT Mdv1 and exhibited increased uniform labeling of mitochondrial tubes (Fig. S3 C). Moreover, neither ΔHR protein rescued fission defects in the adaptor Δ strain and WT (Fig. 7 B). Thus, the ΔHR Mdv1 variants have a reduced ability to assemble and function in fission. Like the mdv1/CC7 mutant, the ΔHR variants continued to interact with binding partners in vivo and caused dominant-negative fission defects when overexpressed in a WT strain (Fig. S4 C and not depicted). The dominant-negative defects induced by the ΔHR variants were more severe than those caused by overexpression of mdv1/CC or WT Mdv1, indicating that they have greater ability to interfere with the WT fission machinery in vivo.

We also compared the ability of the ΔHR and E250G Mdv1 variants to restore mitochondrial fission at 25°C and 37°C in cells harboring the fis1-3 allele. In the fis1-3 mdv1Δ strain grown at an elevated temperature, mitochondrial fission is rescued by expression of Mdv1/E250G but not WT Mdv1 (Fig. 7 C). The mdv1/ΔHR and mdv1/E250G proteins rescued mitochondrial fission defects at 37°C but less efficiently than Mdv1/E250G. In addition, neither mdv1/ΔHR nor mdv1/E250G rescued fission defects in WT or Mdv1/E250G at the permissive temperature (25°C).

As with mdv1/hr2, we observed that mitochondrial localization of mdv1/ΔHR and mdv1/E250G decreased relative to WT Mdv1 in cells lacking Dnm1 (unpublished data). Because Mdv1 mitochondrial localization in dnm1 is Fis1 dependent, it is likely that shortening the native Mdv1 CC domain compromises the Fis1–Mdv1 interaction. Based on these combined results, we conclude that the overall length of the native CC is optimized for Mdv1 adaptor function in mitochondrial fission complex assembly and fission.
how this interaction positions Dnm1 dimers to facilitate assembly into spirals. Although the overall length of the native Mdv1 CC is optimized for fission complex assembly and function, our experiments of ΔHR mdv1 variants indicate that maintaining the length of this CC is not essential for Mdv1 function.

In addition to providing structural stability, Mdv1 dimerization via the CC is critical for the accurate assembly of functional Dnm1 fission complexes. The fraction of monomeric mdv1CC7 protein that localizes to mitochondria is sometimes sufficient to recruit and promote the assembly of Dnm1 puncta that resemble fission complexes (Fig. 4C). However, the morphology of these structures is often qualitatively different from those observed in WT cells, as is their distribution along the mitochondrial tubule. Unlike WT Mdv1, which coassembles with Dnm1 during formation of a functional fission complex.

and co-IP analyses indicate that the predicted Mdv1 β-propeller domain is sufficient for Dnm1 interaction (Tieu et al., 2002; Cerveny and Jensen, 2003). Thus, Mdv1 β-propeller domains at each end of the CC would provide interaction sites for dimeric Dnm1 recruited from the cytoplasm. Dnm1 dimer binding could occur at the periphery or on the lateral face of one or multiple Mdv1 β-propeller domains. Alternatively, each Mdv1 dimer could bind a single Dnm1 dimer, as suggested by the ~1:1 Mdv1–Dnm1 stoichiometry of complexes assembled in vitro (Lackner et al., 2009). Previous studies identified residues in the Mdv1 β-propeller important for Dnm1 interaction (Cerveny and Jensen, 2003; Naylor et al., 2006); however, it is not known whether these residues make direct contact with Dnm1. Identification of such contact sites and/or structures of protein complexes will be necessary to model Mdv1–Dnm1 binding and determine
interaction that stabilizes a protein–protein interface. In additional experiments, we have shown that mdv1
E250G and mdv1 E250A substitutions suppress fission defects caused by a mutant Fis1 protein equally well (unpublished data). The E to G substitution is predicted to increase helix flexibility in addition to disrupting a side chain interaction. The E to A substitution should not increase helix flexibility but could also disrupt a side chain interaction. The simplest explanation for these combined findings is that Mdv1 Glu250 negatively regulates Fis1 binding via an Mdv1 self-interaction or interaction with another protein. According to this scenario, both the E to G and E to A substitutions would disrupt this interaction, generating a form of Mdv1 that is constitutively available for partner binding. Because the exact nature of this interaction remains to be determined, it is

mdv1CC7 fails to assemble and form visible puncta in vivo (Fig. 4 D). This last observation is consistent with the idea that a scaffolding function of dimeric Mdv1 is necessary for its inclusion in the Dam1 polymer as it assembles. The scaffolding model is also supported by our finding that restoring Mdv1 dimerization using a heterologous antiparallel CC rescues mdv1HR2 oligomerization and mitochondrial fission defects.

Surprisingly, the rescue provided by mdv1HR2 was incomplete, owing to a reduction in the interaction of the chimeric protein with Fis1 (Fig. 6). This result complements our previous finding that an E250G CC substitution stabilizes complex formation between Mdv1 and both WT and mutant Fis1 proteins (Karren et al., 2005). Glu250 lies at heptad position g in the native CC (Fig. S2) and likely participates in an electrostatic interaction that stabilizes a protein–protein interface. In additional experiments, we have shown that mdv1E250G and mdv1E250A substitutions suppress fission defects caused by a mutant Fis1 protein equally well (unpublished data). The E to G substitution is predicted to increase helix flexibility in addition to disrupting a side chain interaction. The E to A substitution should not increase helix flexibility but could also disrupt a side chain interaction. The simplest explanation for these combined findings is that Mdv1 Glu250 negatively regulates Fis1 binding via an Mdv1 self-interaction or interaction with another protein. According to this scenario, both the E to G and E to A substitutions would disrupt this interaction, generating a form of Mdv1 that is constitutively available for partner binding. Because the exact nature of this interaction remains to be determined, it is

Figure 8. Molecular architecture of the mitochondrial dynamin-related receptor. A structural model of the Mdv1 dimer (green/purple) bound to two Fis1 cytoplasmic domains (red) anchored in the outer mitochondrial membrane (gray). The model was generated from crystal structures of the Fis1 cytoplasmic domain in complex with a fission adaptor NTE (Protein Data Bank [PDB] accession no. 2PQR) and the Mdv1 antiparallel CC (PDB accession no. 2XU6). The C-terminal WD40 domain of Mdv1 is shown as a homology model based on the known structure of the Cdc4 WD40 repeat (PDB 1NEX). Loops of variable length and unknown structure connect these two regions to the central CC dimer.
Materials and methods

Protein production
MdV1 residues 231–299 (MdV1-CC) were fused to the C terminus of MBP via a linker containing 10×His and the PreScission protease cleavage site (MBP-10×HisPPCS-CC231–299). This construct was expressed in BL2[D3(Em)] recA1 751–813- LamB (RIPI, Agilent Technologies). E. coli grown in autoinduction medium (Studier, 2005). Overnight cultures, grown in PA0.5G, were diluted (1:1,000) in ZYP-5052 (MBP-CC) or PASM-5052 (MBP-CC SeMet) and grown at 37°C for 8 h (Studier, 2005). Cultures were transferred to 23°C and grown for an additional 12–36 h before harvesting. All purification steps were performed at 4°C. Proteins were purified by affinity chromatography on amylase resin (New England Biolabs, Inc.) followed by cleavage with PreScission protease (GE Healthcare). After cleavage, MdV1-CC contained two heterologous N-terminal residues ( Gly-Pro). Protein was further purified by means of an exchange chromatography (GE Healthcare). Residual MBP and uncleaved protein were removed by additional nickel Sepharose (GE Healthcare) and amylase affinity chromatography. Purified MdV1-CC was dialyzed in CD/ES buffer (50 mM Na-phosphate, pH 7.4, and 150 mM NaCl) or crystallization buffer (20 mM Tris-Cl, pH 7.5, and 100 mM NaCl) and concentrated to 1–10 mg/ml using Centricon centrifugal concentrators (YM-3, 3 kD MW, Millipore).

CD and ES analysis
CD analysis was performed on a CD spectrometer (410; Aviv Biomedical, Inc.). Wavelength scans (200–260 nm) were performed at 23°C on 10 µM protein samples in 50 mM sodium phosphate, pH 7.4, and 150 mM NaCl using a 1-mm path length cuvette with a 5-s averaging time. ES analysis (Studier, 2005) was measured in 1 mM path length cuvette with a 5-s averaging time. ES analysis was performed at 4°C using a centrifuge (Optima XL-A; Beckman Coulter) at initial protein subunit concentrations of 335.0, 168.0, 84.0, 40.0, 20.0, and 10.0 µM. All analyses were performed at 23°C and 37°C.

Crystallization and structure determination
The protein crystallized in several conditions of the Crystal Screen HT vapor diffusion experiment against a well solution containing 16% PEG 3350, 0.1 M Bis-Tris-Propanol, pH 7.5, and 0.4 M NaCl. Flatplate crystals were cryoprotected in 20% PEG 3350, 0.1 M BTP, pH 7.5, and 25% glycerol and flash frozen in liquid nitrogen. Peak and inflection data were collected on beamline (version 7.1; SSRU) and processed with HKL2000 (Otwinowski and Minor, 1997) to a final Rmerge of 5.3% to 2.6 Å. The selenium sites were identified with SOIIV (Terwilliger and Berendzen, 1999), and solvent flattening and preliminary model building with RESOLVE (Terwilliger and Berendzen, 1999) resulted in 60% of the residues correctly positioned. Final model building and validation was performed using COOT (Emley and Cowtan, 2004). Refinement of the structure of MdV1 CC (Murshudov et al., 1997) within the CCP4 suite (Collaborative Computational Project, 1994) resulted in a final Rmerge/Rfree of 27.1%/31.1% and a model with good geometric statistics (Table S2).

Yeast strains and plasmids
Yeast strains used in this study include JSY5740 (MAA leu2Δ1 his3Δ200 trp1Δ63 ura3-52 lys2Δ202), JSY612 (MAA leu2Δ1 his3Δ200 trp1Δ63 ura3-52 lys2Δ202 mvd1Δ HIS3 caf4:: KanMX; also referred to as the adaptor Δ strain), JSY465 (MAA leu2Δ1 his3Δ200 trp1Δ63 ura3-52 lys2Δ202 mvd1Δ HIS3 caf4:: KanMX dnm1:: HIS3), JSY9541 (MAA leu2Δ1 his3Δ200 trp1Δ63 ura3-52 lys2Δ202 mvd1Δ HIS3 caf4:: KanMX fis1:: HIS3), and JSY7674 (MAA leu2Δ1 his3Δ200 trp1Δ63 ura3-52 fis1:: 3-mdv1:: HIS3).

A list of plasmids used in this study is provided in Table S1. To construct pMalC-2x-mdv1-CC, DNA sequences encoding residues 231–299 of MDV1 were PCR amplified with a forward primer that introduced an EcoRI site, 10×His, a PreScission Protease cleavage site, and a reverse primer that introduced three stop codons and a HindIII site. The resulting fragment was cloned into the EcoRI and HindIII sites of the pMalC-2x vector (New England Biolabs, Inc.). The pMalC-2x-mdv1-CC construct was made by site-directed mutagenesis (QiikClone kit, Agilent Technologies) of pMalC-2x-mdv1-CC to change leucine codons to methionine codons at positions encoding residues 248 and 281 in the full-length MDV1 protein. Construction of pRS416MET25-MDV1 was described previously (Karren et al., 2005). For pRS416MET25-mvd1Δ1137, sequence-directed mutagenesis of pRS416MET25-MDV1 template was used to introduce sequences encoding the substitutions L233E, L237E, I251E, I254E, L268E, I272E, and I275E. For pRS415MET25-GFP-MDV1, pRS415MET25-GFP-mvd1Δ1137, and pRS415MET25-GFP-mvd1Δ1137, PCR-amplified MDV1, mvd1Δ1137, and mvd1Δ1137 sequences were cloned into the BamHI and SalI sites of pRS415MET25-GFP. pRS415MET25-mvd1Δ1137 was created using the following five steps: (1) site-directed mutagenesis was used to introduce two silent restriction endonuclease sites into MDV1 at nucleotides 628–633 (XmaI) and nucleotides 901–906 (XhoI) in the pRS415MET25-MDV1 vector; (2) an existing XhoI site in the multiple-cloning site of pRS416MET25-MDV1 was removed by site-directed mutagenesis; (3) A XhoI-MVD1-3′MFN1 sequence (CC) chimeric sequence was synthesized by MR. GENE. The nucleotide sequence of the synthesized MDV1-3′MFN1 CC chimera was XmaI-MDV1(634–687)MFN1(2022–2222)MDV1(183–900)XhoI, (4) the CC chimera provided in a vector was excised by XmaI and XhoI digestion and cloned into the same sites of the pRS416MET25-MDV1 plasmid generated in step 1. To construct, pRS416MET25-MDV1-13MYC, MDV1 was PCR amplified, digested with SpeI and SacII, and cloned into a vector that fused sequence encoding 13MYC in frame at the C terminus of MDV1. pRS416MET25-mvd1Δ1137-13MYC and pRS416MET25-mvd1Δ1137-13MYC were cloned by similar strategies. To create pRS416MET25-MDV1-3HA, MDV1 was PCR amplified, digested, and cloned into the BamHI-XhoI site of pRS416MET25-MDV1-13MYC. In the resulting clone, the C-terminus ORF was replaced by the MDV1 ORF, and sequence encoding 3HA was fused in frame to the C terminus of Mdv1. A similar strategy was used to generate clones encoding C-terminal 3HA-tagged forms of mvd1Δ1137, mvd1Δ1137, and mvd1Δ250. For pRS416MET25-MDV1-9MYC-FI51, 9MYC-FI51 was excised from pRS415MET25-MYMCYC-FI51 (Karren et al., 2005) using SpeI and XhoI and cloned into pRS416MET25. To create pRS416MET25-mvd1Δ1137 and pRS416MET25-mvd1Δ250, DNA encoding mvd1Δ1137 (XmaI–343–708–XhoI) and mvd1Δ250 (XmaI–343–708–ΔXhoI) was synthesized and cloned by Integrated DNA Technologies, Inc. After digestion of the provided plasmids with XmaI and XhoI, the following fragments were ligated into either side of the encoded Mdv1 CC in pRS416MET25-MDV1. pRS415MET25-mvd1Δ250-3HA, pRS415MET25-mvd1Δ250-13MYC, pRS415MET25-GFP-mvd1Δ250, and pRS415MET25-GFP-mvd1Δ250 were generated using strategies similar to those described for WT and mutant Mdv1 variants. For pRS416MET25-MvDI-FRFP-mvd1Δ1137, a PCR-amplified fragment containing mvd1Δ1137 was digested with BamHI and Sali and ligated into pRS416MET25-FRFP.
After centrifugation at 18,000 g, beads in 500 µl IP buffer (0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.5–1.0 µM) were washed in IP buffer, and bound proteins were released as described in the previous paragraph. Steady-state abundance of HA-tagged WT and mutant Mdv1 proteins, cells grown for 4 h after dilution in selective medium, were adjusted using only linear operations applied to the entire image.

Analysis of protein expression and targeting
Protein expression was analyzed in WCEs prepared by the alkaline extraction method (Kushnirov, 2000). To analyze subcellular localization of proteins, cells grown in the appropriate synthetic galactose dropout medium were spheroplasted and homogenized to produce WCE, which was subsequently subjected to differential sedimentation to isolate post-mitochondrial supernatant and mitochondrial pellet enriched fractions (Kondo-Okamoto et al., 2003). Either 0.5 OD600 equivalents of alkaline extract or 50 µg protein of WCE, 50 µg postmitochondrial supernatant, and 25 µg mitochondrial pellet fractions were separated by SDS-PAGE and analyzed by Western blotting using anti-HA (1:2,000; Invitrogen), anti-porin (1:2,000; Invitrogen), and HRP-conjugated secondary goat anti–mouse antibody (1:10,000; Sigma-Aldrich). Proteins were detected by ECL (GE Healthcare) and HRP-conjugated secondary goat anti–mouse antibody (1:10,000; Sigma-Aldrich). Antibodies were visualized on imaging software (version 4.6; Carl Zeiss, Inc.). Three-dimensional projections of mitochondria were generated with the transparency (voxel) setting and converted to a single image. Final images were processed and assembled using Photoshop and Illustrator (CS3; Adobe). Brightness and contrast were adjusted using only linear operations applied to the entire image.

Online supplemental material
Fig. S1 shows additional analysis of mdv1− induced localization and function. Fig. S2 shows that Glu250 is exposed on one face of the dimeric antiparallel βCC. Fig. S3 shows an additional analysis of mdv1− and mdv1−Dnm1 proteins. Fig. S4 shows dominant-negative effects of WT and mutant Mdv1 proteins. Table S1 shows plasmids used in this study. Table S2 shows x-ray data and model statistics. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201005046/DC1.

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