The WD40 protein Caf4p is a component of the mitochondrial fission machinery and recruits Dnm1p to mitochondria

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The mitochondrial division machinery regulates mitochondrial dynamics and consists of Fis1p, Mdv1p, and Dnm1p. Mitochondrial division relies on the recruitment of the dynamin-related protein Dnm1p to mitochondria. Dnm1p recruitment depends on the mitochondrial outer membrane protein Fis1p. Mdv1p interacts with Fis1p and Dnm1p, but is thought to act at a late step during fission because Mdv1p is dispensable for Dnm1p localization. We identify the WD40 repeat protein Caf4p as a Fis1p-associated protein that localizes to mitochondria in a Fis1p-dependent manner. Caf4p interacts with each component of the fission apparatus: with Fis1p and Mdv1p through its NH$_2$-terminal half and with Dnm1p through its COOH-terminal WD40 domain. We demonstrate that mdv1$^{\Delta}$/H9004 yeast contain residual mitochondrial fission due to the redundant activity of Caf4p. Moreover, recruitment of Dnm1p to mitochondria is disrupted in mdv1$^{\Delta}$/caf4$^{\Delta}$/H9004 yeast, demonstrating that Mdv1p and Caf4p are molecular adaptors that recruit Dnm1p to mitochondrial fission sites. Our studies support a revised model for assembly of the mitochondrial fission apparatus.

Introduction

Mitochondria are dynamic organelles that undergo fusion and fission. These processes intermix the mitochondria within cells and control their morphology. In addition to controlling mitochondrial shape, recent studies have also implicated components of the fission machinery in regulation of programmed cell death (Frank et al., 2001; Fannjiang et al., 2004; Jagasia et al., 2005). Genetic approaches in Saccharomyces cerevisiae have identified DNM1, FIS1, and MDV1 as components of the mitochondrial fission pathway (Shaw and Nunnari, 2002). Dnm1p and its mammalian homologue Drp1 are members of the extensively studied dynamin family of large, oligomeric GTPases. Although the precise mechanism remains controversial, dynamins may couple GTP hydrolysis to a conformational constriction that causes membrane scission (Praefcke and McMahon, 2004). In yeast cells, Dnm1p dynamically localizes to dozens of puncta that are primarily associated with mitochondria (Otsuga et al., 1998; Bleazard et al., 1999; Sesaki and Jensen, 1999; Legesse-Miller et al., 2003). A subset of these puncta are sites of future fission.

The assembly of functional Dnm1p complexes on mitochondria is a critical issue in understanding the mechanism of mitochondrial fission. The mitochondrial outer membrane protein Fis1p is required for the formation of normal Dnm1p puncta on mitochondria. In fis1$^{\Delta}$/H9004 cells, Dnm1p puncta are primarily cytosolic or form abnormally large aggregates on mitochondria (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000). Mdv1p interacts with Fis1p through its NH$_2$-terminal half and with Dnm1p through its COOH-terminal WD40 domain. However, Mdv1p appears dispensable for Dnm1p assembly on mitochondria because mdv1$^{\Delta}$/H9004 cells show little or no change in Dnm1p localization, even though mitochondrial fission is disrupted (Fekkes et al., 2000; Tieu and Nunnari, 2000; Tieu et al., 2002; Cerveny and Jensen, 2003). These observations have led to two important features of a recently proposed model for mitochondrial fission (Shaw and Nunnari, 2002; Tieu et al., 2002; Osteryoung and Nunnari, 2003). First, Fis1p acts to assemble and distribute Dnm1p on mitochondria in an Mdv1p-independent step. Second, Mdv1p acts downstream of Dnm1p localization to stimulate membrane scission. An alternative model proposes that Dnm1p marks the site of mitochondrial fission and recruits Fis1p and Mdv1p into an active fission complex (Cerveny and Jensen, 2003). Again, in this model Mdv1p functions downstream of Dnm1p localization.
Despite extensive efforts, however, there is no evidence that Fis1p can interact directly with Dnm1p. We speculated that there may be an additional component of the mitochondrial fission pathway required for the Fis1p-dependent assembly of Dnm1p puncta on mitochondria. Because a genome-wide screen for mitochondrial morphology mutants (Dimmer et al., 2002) did not yield obvious candidates, we used a biochemical approach to identify additional components of the mitochondrial fission machinery. Using immunopurification and mass spectrometry, we have identified the WD40 repeat protein Caf4p as a Fis1p-interacting protein. Caf4p localizes to mitochondria and associates with Fis1p, Mdv1p, and Dnm1p. Moreover, we show that mdv1Δ cells are only partially deficient in mitochondrial fission due to the redundant activity of Caf4p. Importantly, Caf4p mediates recruitment of Dnm1p puncta to mitochondria in mdv1Δ yeast. Inclusion of CAF4 significantly clarifies the current models for mitochondrial fission.

Results

Caf4p is associated with Fis1p

To identify Fis1p-associated proteins by multidimensional protein identification technology (MudPIT) (Link et al., 1999; Graumann et al., 2004), we constructed a yeast strain containing endogenous Fis1p with an NH2-terminal tandem affinity tag (Fig. 1 A). NH2-terminal tagging is necessary because FIS1 is nonfunctional when COOH-terminally tagged (unpublished data). We first designed a recombination cassette containing 9XMyc/TEV/URA3/TEV/His8 modules (Fig. 1 A). After targeted integration into the FIS1 locus, spontaneous and precise recombination between the flanking ~50-bp tobacco etch virus (TEV) protease sites excises URA3. This strategy was used to generate a yeast strain (DCY1557) that expresses a functional Fis1p with an NH2-terminal 9XMyc/TEV/His8 tag (M9TH-Fis1p) from the endogenous locus.

Tandem affinity-purified M9TH-Fis1p was subjected to MudPIT analysis in two independent experiments (see Materials and methods). Fis1p was identified in both experiments (61.3% coverage, 14 unique peptides; 58.7% coverage, 9 unique peptides). Mdv1p, a previously identified member of the mitochondrial fission pathway and a known Fis1p-interacting protein, was also identified in both experiments (22.1% coverage, 12 unique peptides; 10.2% coverage, 5 unique peptides). These data confirmed that our MudPIT procedure could preserve and identify Fis1p complexes relevant to mitochondrial fission. Dnm1p was not observed in either dataset, in agreement with previous immunoprecipitation experiments (Mozdy et al., 2000). The complete datasets are presented in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200503148/DC1).

Interestingly, peptides derived from the WD40 repeat protein Caf4p were identified in both Fis1p MudPIT experiments (24.4% coverage, 9 unique peptides; 8.5% coverage, 3 unique peptides). CAF4 (YKR036C) was first identified in a yeast two-hybrid screen for CCR4p-interacting proteins (Liu et al., 2001). CCR4p is a central component of the CCR4-NOT transcriptional regulator and cytosolic deadenylase complex (Denis and Chen, 2003). Caf4p is the nearest homologue of Mdv1p in S. cerevisiae (38% identity and 57% similarity), and the two proteins show extensive sequence identity throughout their lengths (Fig. 1 B). Both proteins share a unique NH2-terminal extension (NTE) (25.3% identity), a central coiled-coil (CC) domain (19% identity) and a COOH-terminal WD40 repeat domain (44.4% identity). The Caf4p CC scores significantly more weakly (~0.3 probability) than the Mdv1p coiled coil (~1.0 probability) in the MultiCoil prediction program (Wolf et al., 1997).

Caf4p interacts with components of the mitochondrial fission machinery

We sought independent confirmation of the physical interaction between Fis1p and Caf4p. For immunoprecipitation experiments, Caf4p-HA or Mdv1p-HA were expressed from their endogenous promoters in strains carrying chromosomal M9TH-FIS1 (3XMyc/TEV/His8-FIS1) and deleted for CAF4 or MDV1, respectively. When M9TH-Fis1p was immunoprecipitated, ~5% of both Caf4p-HA and Mdv1p-HA coprecipitated (Fig. 2 A, lanes 7 and 10).
Previous yeast two-hybrid analysis determined that the NTE/CC region of Mdv1p (residues 1–300) is responsible for its interaction with Fis1p (Tieu et al., 2002). We detected the same interaction by coimmunoprecipitation (Fig. 2 A, lane 11). Additionally, we found that the analogous region of Caf4p (residues 1–274) also interacted with Fis1p (Fig. 2 A, lane 8). A shorter Caf4p fragment lacking the majority of the predicted coiled coil (residues 1–250) interacted equally well with Fis1p (unpublished data). In contrast, Fis1p did not bind to the COOH-terminal regions of either Mdv1p or Caf4p (Fig. 2 A, lanes 9 and 12). These data suggest that both Caf4p and Mdv1p likely interact with Fis1p through a common mechanism involving the NTE domain.

We also used a yeast two-hybrid assay to analyze the interaction of Caf4p and Mdv1p with Fis1p and Dnm1p (Table I). Full-length Caf4p and an NTE/CC fragment of Caf4p interacted strongly with the cytosolic portion of Fis1p (residues 1–128), consistent with our immunoprecipitation data. Similar interactions were observed between Fis1p and both full-length Mdv1p and the NTE/CC region of Mdv1p, as has been previ-
Table I. *Caf4p* and *Mdv1p* interact with *Dnm1p* and *Fis1p* in a yeast two-hybrid assay

<table>
<thead>
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<th>Genotype</th>
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<th>Collapsed net</th>
<th>Spread net</th>
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*Caf4p*, *Mdv1p*, *Fis1p*, and *Dnm1p* fragments were scored for growth (+), no growth (−), or poor growth (weak) on adenine-deficient plates. All constructs showed no growth when paired with empty activation domain or DNA-binding domain vector. Binding domain fusions are listed across the top of the table and activation domain fusions are listed down the left. *Caf4p* and *Mdv1p* N and C fragments are defined in Fig. 2.

**Figure 3.** *CAF4* regulates mitochondrial morphology. Strains expressing mitochondrial-targeted GFP were grown in YP dextrose to mid-log phase and fixed. The percentage of cells (n = 400) with mitochondria having wild-type (A), collapsed net (B), or spread net morphology (C and D) is tabulated. The spread net phenotype encompasses a distribution of morphologies ranging from simple structures containing one or two loops (C) to complexly fenestrated mitochondria with dozens of loops (D). For both wild-type and *caf4Δ* strains, the wild-type category includes 1% fragmented cells. Bar, 1 μm.

Mdv1p wt fragments are defined in Fig. 2. Activation domain fusions are listed down the left. *Caf4p* and *Mdv1p* N and C showed no growth when paired with empty activation domain or DNA-binding domain vector. Binding domain fusions are listed across the top of the table and activation domain fusions are listed down the left. *Caf4p* and *Mdv1p* N and C fragments are defined in Fig. 2.

We next tested whether *caf4Δ* cells show synthetic defects in mitochondrial morphology when other components of the fission machinery are deleted. Yeast defective in mitochondrial fission display net-like mitochondrial morphology due to unopposed mitochondrial fusion (Bleazard et al., 1999; Sesaki and Jensen, 1999). These mitochondrial nets can have a spread morphology (Fig. 3, C and D), or they can collapse to one side of the cell (Fig. 3 B). Although *FIS1*, *DNM1*, and *MDV1* are all involved in mitochondrial fission, we found that *mdv1Δ* cells have a distribution of mitochondrial profiles that can be readily distinguished from both *fis1Δ* and *dnm1Δ* cells (Fig. 3). In rich dextrose medium, almost all *fis1Δ or dnm1Δ* cells (93 and 90%, respectively) contain collapsed mitochondrial nets. In contrast, less than half of *mdv1Δ* cells contain collapsed nets, with the majority displaying a spread net morphology. The spread nets range in morphology from interconnected tubules with several loops (Fig. 3 C) to networks with complex fenestrations (Fig. 3 D). *mdv1Δ dnm1Δ* cells behave identically to *dnm1Δ* cells, with >90% collapsed nets in dextrose (Fig. 3). This observation indicates that the *dnm1Δ* collapsed net phenotype is epistatic to the *mdv1Δ* spread net phenotype. In rich galactose medium (unpublished data), a greater portion of all strains contain spread nets, but again *mdv1Δ* cells have a higher percentage of cells with spread nets (80%) compared with *fis1Δ* (45.5%), *dnm1Δ* (53%), or *mdv1Δ dnm1Δ* cells (40.5%). These results agree with a previous report that *mdv1Δ* cells have more spread nets compared with *dnm1Δ* cells in galactose medium (Cerveny et al., 2001). However, this study found that the *mdv1Δ* spread net phenotype is epistatic to the *dnm1Δ* collapsed net phenotype (Cerveny et al., 2001). The reason for this discrepancy is unclear, but we note the *mdv1Δ* morphology is most distinct in dextrose cultures.

Most interestingly, we found that *mdv1Δ caf4Δ* cells have mitochondrial net distributions indistinguishable from...
either *dnm1Δ* cells or *fis1Δ* cells. Deletion of *CAF4* in *mdv1Δ* cells markedly shifts the distribution to one composed almost entirely of collapsed mitochondrial nets (>90% in dextrose, Fig. 3). Our results support a model in which partial reduction of mitochondrial fission results in predominantly spread mitochondrial nets, and complete loss of fission eventually results in collapse of the nets. That is, *mdv1Δ* cells retain residual mitochondrial fission, whereas *mdv1Δ caf4Δ* cells are devoid of fission, similar to *dnm1Δ, fis1Δ, or mdv1Δ dnm1Δ* cells. An analogous situation appears to exist in mammalian cells, in which weak Drp1 dominant-negative alleles cause the formation of spread nets, whereas strong dominant-negative alleles cause nets to collapse (Smirnova et al., 2001).

We tested this model by reanalyzing mitochondrial morphologies in the presence of latrunculin A, which disrupts the actin cytoskeleton. Disruption of the actin cytoskeleton leads to rapid fragmentation of the mitochondrial network due to ongoing mitochondrial fission (Boldogh et al., 1998; Jensen et al., 2000). Latrunculin A treatment rapidly resolves a fraction of collapsed nets into spread nets (Jensen et al., 2000; Cerveny et al., 2001), and allows a closer examination of the degree of connectivity in mitochondrial nets. Similarly, in mammalian cells, collapsed mitochondrial nets induced by overexpression of dominant-negative Drp1 can be spread by the microtubule-depolymerizing agent nocodazole (Smirnova et al., 2001). Both wild-type and *caf4Δ* yeast treated with latrunculin A show

<table>
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Figure 4. *CAF4 mediates residual fission in mdv1Δ cells.* Top: mid-log cultures grown in YP dextrose were treated for 60 min with 200 μM latrunculin A (+) or vehicle (−). For each strain, 200 cells were scored into the following phenotypic categories: wild-type (A), fragments and short tubules (B), collapsed net (C), spread net (D), or partial net (E). Numbers shown are percentages. The fragments and short tubules category encompasses a range of morphologies from completely fragmented (as shown in B) to a mixture of fragments and short tubules. (F–H) Still images from time-lapse movies showing fission events in *mdv1Δ* yeast treated with 200 μM latrunculin A. The boxed area in the first frame is magnified in the subsequent sequence of five images. Arrows indicate fission events. Mitochondria were visualized with the outer membrane marker OM45-GFP. Bars, 1 μm.
mitochondrial fragmentation (Fig. 4). 80% of mdv1Δ cells treated with latrunculin A contain partial mitochondrial nets (Fig. 4 E, partial net) that are less interconnected and have fewer fenestrations than the collapsed or spread nets that predominate in latrunculin A–treated dnm1Δ or fis1Δ cells. 95% of latrunculin A–treated mdv1Δ caf4Δ cells show either collapsed nets or highly fenestrated spread nets, a profile indistinguishable from that in dnm1Δ or fis1Δ cells (Fig. 4). Thus, after disruption of the actin cytoskeleton, mdv1Δ yeast display a distribution of mitochondrial morphologies that suggest an incomplete defect in mitochondrial fission. In contrast, mdv1Δ caf4Δ yeast have mitochondrial morphologies similar to that in fis1Δ and dnm1Δ yeast. We conclude that Caf4p mediates low levels of mitochondrial fission in mdv1Δ cells.

We next monitored the mitochondrial network in mdv1Δ cells by time-lapse microscopy to assess the levels of mitochondrial fission. In pilot experiments, we found that free mitochondrial ends produced by fission events in mdv1Δ cells were rapidly involved in fusion events, making unambiguous documentation of fission difficult. Because latrunculin A reduces the levels of fusion and thereby should prolong the presence of free mitochondrial ends, we monitored mitochondrial dynamics in latrunculin A–treated mdv1Δ cells carrying the outer membrane marker OM45-GFP. In 8 out of 10 mdv1Δ cells, we observed at least one fission event in a 30-min recording period (Fig. 4, F–H; Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200503148/DC1). Due to the complexity and rapid rearrangements of the mitochondrial networks in these cells (see Videos 1 and 2), these numbers likely underestimate the actual levels of fission. In contrast, no fission events were observed in 8 mdv1Δ caf4Δ cells. We conclude that the ability of Caf4p to mediate mitochondrial fission events contributes significantly to the spread net morphology of mdv1Δ cells.

**Mitochondrial fission is blocked by overexpression of Caf4p or Caf4p fragments**

Because overexpression of Mdv1p or Mdv1p fragments inhibits mitochondrial fission (Cerveny and Jensen, 2003), we next tested the effects of Caf4p overproduction. Caf4p-HA under control of the GalL promoter was expressed ~20 times above endogenous levels in rich galactose medium (unpublished data). Spread mitochondrial nets formed in 23.5% of cells (Fig. 5 C). An additional 38% of cells had an intermediate phenotype that we termed “connected tubules,” consisting of a completely interconnected mitochondrial network in which no tubular ends were detected (Fig. 5 B). Overexpression of an NH2-terminal fragment that interacts with Fis1p (residues 1–250; unpublished data) had a similar effect (9% spread nets, 33% connected tubules; Fig. 5), suggesting that the formation of mitochondrial net-like structures may result from a dominant-negative effect on Fis1p function. A similar distribution of mitochondrial phenotypes resulted from 20-fold overproduction of Mdv1p-HA (7.5% spread nets and 24.5% interconnected tubules) and an Mdv1p-HA NH2-terminal fragment (5% spread nets and 39% interconnected tubules; unpublished data). These data confirm that Caf4p interacts with the mitochondrial fission apparatus.

**Full bypass suppression of fzo1Δ requires loss of both MDV1 and CAF4**

Yeast fission mutants are able to suppress the glycerol growth defect of cells deficient in mitochondrial fusion (Blezard et al., 1999). Indeed, MDV1 was originally identified because of
its ability to suppress the glycerol growth defect of strains carrying temperature-sensitive fzo1 or mgm1 alleles (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Deletion of MDV1 has previously been reported to suppress the glycerol growth defect of fzo1Δ cells less efficiently than deletion of DNM1 (Cerveny et al., 2001). To further test our hypothesis that mdv1Δ cells have only a partial loss of mitochondrial fission, we compared the efficiencies with which the mdv1Δ and dnm1Δ mutations suppress the glycerol growth defect of fzo1Δ cells. Diploids were sporulated, genotyped, and scored by serial dilution for their ability to grow on glycerol plates relative to dextrose plates (Fig. 6). As expected, all wild-type and no fzo1Δ spores grew on glycerol plates. Of 17 mdv1Δ fzo1Δ spores tested, 7 showed no detectable growth on glycerol and an additional 4 spores grew very poorly, with <1% cell survival on glycerol. Only 3 of the 6 remaining spores showed >20% survival on glycerol. More than half of dnm1Δ fzo1Δ spores grew robustly on glycerol plates, with between 20 and 50% cell survival. Most importantly, the triple mutant mdv1Δ caf4Δ fzo1Δ spores grew much more robustly than the mdv1Δ fzo1Δ spores, with all spores growing on glycerol and the majority between 20 and 50% cell survival. The markedly enhanced bypass suppression of fzo1Δ by mdv1Δ caf4Δ double mutations compared with the mdv1Δ mutation provides genetic evidence that mdv1Δ cells retain residual mitochondrial fission due to the activity of Caf4p.

**Caf4p localizes to mitochondria in a Fis1p-dependent manner**

We next sought to determine the subcellular localization of Caf4p. Caf4p was detected in highly purified mitochondrial preparations (Sickmann et al., 2003), and a Caf4p-GFP fusion generated in a genome-wide analysis localizes to mitochondria (Huh et al., 2003). We confirmed the mitochondrial localization of Caf4p-GFP, but did not study it further because the GFP fusion protein was not functional when expressed from the CAF4 locus (unpublished data). We instead used immunofluorescence to localize Myc-tagged versions of Caf4p and Mdv1p (termed Caf4p-HTM and Mdv1p-HTM) that are expressed from the endogenous loci and are functional. Mitochondria were labeled with mitochondrially targeted GFP [A–R, left, green]. The majority of Dnm1p-GFP puncta colocalize with Caf4p-HTM (S–U). Overlays of the two signals are shown in the merged images (right). Note that both Caf4p and Mdv1p localize to mitochondria in wild-type cells, but are diffusely cytosolic in fis1Δ cells. Cells were grown in YM dextrose (A–L) or YM galactose (M–U). Representative maximum intensity projections of deconvolved z-stacks are shown. Bar, 1 μm. [V] Caf4p-HTM and Mdv1p-HTM were analyzed by subcellular fractionation. The total cell lysate (Total), high-speed supernatant (Cyto), and mitochondrial pellet (Mito) were analyzed by Western blot with an anti-Myc antibody. PGK (3-phosphoglycerate kinase) is a cytosolic marker, and porin is a mitochondrial outer membrane marker.

**Figure 7.** Mitochondrial localization of Caf4p and Mdv1p requires Fis1p. Immunofluorescence [red, middle panels] was used to localize Myc-tagged Caf4p (Caf4p-HTM; A–F and M–U) and Mdv1p (Mdv1p-HTM; G–L and P–R) in wild-type [A–C, G–I, and M–R] and fis1Δ cells (D–F and J–L). Caf4p-HTM and Mdv1p-HTM are expressed from the endogenous loci and are functional. Mitochondria were labeled with mitochondrially targeted GFP [A–R, left, green]. The majority of Dnm1p-GFP puncta colocalize with Caf4p-HTM (S–U). Overlays of the two signals are shown in the merged images (right). Note that both Caf4p and Mdv1p localize to mitochondria in wild-type cells, but are diffusely cytosolic in fis1Δ cells. Cells were grown in YM dextrose (A–L) or YM galactose (M–U). Representative maximum intensity projections of deconvolved z-stacks are shown. Bar, 1 μm. [V] Caf4p-HTM and Mdv1p-HTM were analyzed by subcellular fractionation. The total cell lysate (Total), high-speed supernatant (Cyto), and mitochondrial pellet (Mito) were analyzed by Western blot with an anti-Myc antibody. PGK (3-phosphoglycerate kinase) is a cytosolic marker, and porin is a mitochondrial outer membrane marker.
fusely cytosolic but also retains some localization to mitochondria (Tieu and Nunnari, 2000; Tieu et al., 2002). Together, these data indicate that the normal mitochondrial localization of both Caf4p and Mdv1p depends largely on Fis1p, although some low levels of residual localization can occur in the absence of Fis1p.

We also evaluated the localization of Caf4p-HTM by subcellular fractionation. We found a significant portion of both Caf4p and Mdv1p in the mitochondrial pellet (Fig. 7 V). Mdv1p had previously been shown to be present in mitochondrial fractions (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). However, in fis1Δ yeast both proteins behave as cytosolic proteins (Fig. 7 V). These data support our immunofluorescence studies and confirm that Mdv1p and Caf4p localize to mitochondria through their association with Fis1p.

Caf4p recruits Dnm1p-GFP to mitochondria

To understand the mechanism of mitochondrial fission, it is crucial to elucidate how Dnm1p is recruited to mitochondria. Given that Mdv1p associates with both Fis1p and Dnm1p, it is puzzling that Dnm1p assembly on mitochondria shows little or no dependence on Mdv1p (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Tieu et al., 2002; Cerveny and Jensen, 2003). With the identification of Caf4p as a component of the fission machinery, we reexamined this issue. We constructed a fully functional Dnm1p-GFP allele and analyzed its localization pattern using deconvolution microscopy (Table II). Similar to previous reports (Otsuga et al., 1998), Dnm1p-GFP is found predominantly in puncta associated with mitochondria (average 16.9 mitochondrial vs. 3.3 cytosolic puncta per cell) (Table II and Fig. 8, A–C). Deletion of CAF4 or MDV1 alone had little effect on this localization (15.4 mitochondrial vs. 5.2 cytosolic and 13.7 mitochondrial vs. 5.1 cytosolic per cell, respectively; Table II and Fig. 8, D–I). In all these strains, the Dnm1p puncta are relatively uniform in size and intensity.

In contrast, fis1Δ mutants showed dramatic defects, with the majority of the puncta now cytosolic (4.9 mitochondrial vs. 9.6 cytosolic) (Table II and Fig. 8, J–L). As has been previously noted, a small fraction of Dnm1p still localizes to mitochondria in fis1Δ cells (Tieu et al., 2002; Cerveny and Jensen, 2003), suggesting that Dnm1p may be recruited by a second pathway, perhaps through an intrinsic affinity for mitochondrial lipids or an unidentified mitochondrial binding partner. Importantly, a similar defect in Dnm1p localization was found in mdv1Δ caf4Δ cells (4.8 mitochondrial vs. 10.4 cytosolic per cell) (Table II and Fig. 8, M–O). In both fis1Δ and mdv1Δ caf4Δ cells, Dnm1p-GFP forms a few large aggregates and numerous less intense puncta. Similar results were obtained using immunofluorescence against a Dnm1p-HTM protein (unpublished data). These data clearly demonstrate that either Caf4p or Mdv1p is sufficient for effective recruitment of Dnm1p to mitochondria, and that Caf4p is essential for Mdv1p-independent recruitment of Dnm1p by Fis1p.

Discussion

CAF4 and MDV1 perform similar functions in mitochondrial fission

By applying affinity purification and mass spectrometry to Fis1p, we have identified Caf4p as a novel component of the mitochondrial fission machinery. Our biochemical and genetic characterization indicate that CAF4 functions in the same manner as MDV1 in mitochondrial fission. Biochemically, both proteins interact with Fis1p and Dnm1p. Caf4p and Mdv1p share a common domain architecture comprised of an NTE, a central CC, and a COOH-terminal WD40 repeat. The NH2-terminal regions mediate oligomerization and association with Fis1p, whereas the COOH-terminal WD40 regions mediate interactions with Dnm1p. In addition, both Caf4p and Mdv1p localize to mitochondria in a Fis1p-dependent manner.
Genetically, both MDV1 and CAF4 act positively in the mitochondrial fission pathway. mdv1Δ cells are dramatically compromised for mitochondrial fission, but a residual level of fission is mediated by CAF4. This residual fission activity is revealed by the observation that mdv1Δ yeast have a less severe mitochondrial morphology defect compared with fis1Δ or dnm1Δ yeast. In contrast, mdv1Δ caf4Δ yeast display predominantly collapsed mitochondrial nets, identical to those seen in fis1Δ and dnm1Δ cells. Time-lapse imaging of mitochondria in mdv1Δ cells indeed reveals a residual level of fission that is absent from mdv1Δ caf4Δ cells. These results directly support our conclusion that the morphology differences between mdv1Δ cells versus mdv1Δ caf4Δ, fis1Δ, and dnm1Δ cells are primarily due to differences in fission rates. It is also possible that the proposed role of Dnm1p in cortical distribution of mitochondria may contribute in part to the morphological differences (Otsuga et al., 1998). The mdv1Δ mutation acts as a weak suppressor of the glycerol growth defect in fzo1Δ cells. The mdv1Δ caf4Δ double mutation suppresses this phenotype much more efficiently. Based on these physical interaction and genetic data, we conclude that Caf4p likely acts in a similar manner to Mdv1p to promote mitochondrial fission.

Why are there two proteins that appear to perform similar and partially redundant roles in mitochondrial fission? This question is particularly intriguing because caf4Δ yeast have normal mitochondrial morphology, indicating that disruption of Caf4p does not cause a major loss of mitochondrial fission. First, CAF4 may play a more important role in mitochondrial fission under conditions not yet tested. Second, the presence of two proteins mediating interactions between Fis1p and Dnm1p would increase the ability of cells to accurately regulate the rate of mitochondrial fission. The heterotypic and homotypic interactions between Caf4p and Mdv1p may provide an additional layer of regulation. Finally, Caf4p may have an additional function in another pathway. Previous two-hybrid studies have implicated Caf4p in the CCR4- NOT complex, which is thought to be involved in regulation of transcription and/or mRNA processing (Liu et al., 2001).

A revised model for mitochondrial fission

The current models for mitochondrial fission propose that Mdv1p acts late in the fission pathway. One model proposes a two-step pathway in which Fis1p first recruits Dnm1p, in an Mdv1p-independent manner. Mdv1p then acts as a molecular adaptor at a post-recruitment step, along with Fis1p, to promote fission by Dnm1p (Shaw and Nunnari, 2002; Tieu et al., 2002; Osteryoung and Nunnari, 2003). A second model also proposes that Mdv1p acts after Dnm1p recruitment to organize an active fission complex (Cerveny and Jensen, 2003).

Our study reveals a new role for Mdv1p and Caf4p early in mitochondrial fission. Fis1p recruits Dnm1p to mitochondrial fission complexes through Mdv1p or Caf4p, which act as molecular adaptors. This revised model is strongly supported by our demonstration that Dnm1p recruitment in mdv1Δ yeast depends on Caf4p function. In the absence of both Mdv1p and Caf4p, Fis1p is unable to recruit Dnm1p.

Although Mdv1p and Caf4p clearly act early in the fission pathway, there is evidence that at least Mdv1p has a subsequent role in the activation of fission, as previously proposed (Shaw and Nunnari, 2002; Tieu et al., 2002; Cerveny and Jensen, 2003). In caf4Δ cells, Mdv1p recruits Dnm1p to fission complexes, and fission occurs in apparently normal levels. However, in mdv1Δ cells, Caf4p is similarly able to recruit Dnm1p to fission complexes, but mitochondrial fission is severely compromised. Therefore, Mdv1p and Caf4p can independently recruit Dnm1p, but complexes recruited by Mdv1p appear to be more highly active. These observations suggest that Dnm1p recruitment by itself is insufficient for fission to occur. Indeed, studies of Dnm1p dynamics indicate that most Dnm1p puncta do not result in fission (Legesse-Miller et al., 2003). Our identification of Caf4p as part of the fission machinery clarifies the early steps in mitochondrial fission. Future studies will need to define the additional steps beyond Dnm1p recruitment necessary for fission.

Materials and methods

Media and yeast genetic techniques

Yeast strains are listed in Table S1. Standard genetic techniques and yeast media were used. SC and YP media supplemented with either 2% dextrose, 3% glycerol, 2% raffinose, or 2% galactose were prepared as described previously (Guthrie and Fink, 1991). YG12 and DCY1557 are in the w303 background. All other strains are in the S288C background. fis1::KanMX6, mdv1::KanMX6, caf4::KanMX6, and dnm1::KanMX6 are derived from the MATa deletion library (Open Biosystems).

Plasmid construction

The MTH cassette was generated as follows. Primers Eg258 [see Table S3, available at http://www.jcb.org/cgi/content/full/jcb.200503148/DC1] and Eg259 were used to amplify URA3 from pRS416 (Strategene). Eg260 and Eg4, an FZO1 reverse primer, were used to amplify a TEV/His6 module from EG704 [pRS414 + 9XMyr/TEV/His6/FZO1]. The 3′ end of the URA3 product overlaps by 18 bp with the 5′ end of the TEV/His6 product. This overlap allows them to anneal together and be amplified in a second PCR with the primers Eg258 and Eg4. The URA3/TEV/His6 product was cloned into pRS403 as an EcoRV/Sall fragment (which removes all FZO1 sequence), resulting in EG928. 9XMyr/TEV was amplified with Eg256 and Eg260 from EG704 and fused to the 5′ end of the URA3 [Eg258/259 product] by mixing and amplifying with Eg256 and Eg259. The resulting product was cloned into EG928 as an EcoRV/EcoRI fragment, yielding EG940 [pRS403 + 9XMyr/TEV/URA3/TEV/His6]. EG940 was converted to pRS403 + 3XMyr/TEV/URA3/TEV/His6 by digesting with XbaI, yielding EG957.

To construct HA-tagged versions of CAF4 and CAF4 fragments, CAF4 sequences were PCR amplified from end3Δ genomic DNA (Open Biosystems). First, the CAF4 3′ untranslated region (UTR) was amplified with the primers Eg313 and Eg314 and cloned as a KpnI/Sall fragment into pRS416, resulting in pRS416 + CAF4 3′ UTR. 3XHA was amplified with Eg327 and Eg328 and cloned as a Sall/Xhol fragment into the Sall site to generate pRS416 + 3XHA/CAF4 3′ UTR. The CAF4 5′ UTR was cloned as a SacI/SpeI fragment using Eg312 and Eg317, resulting in pRS416 + CAF4 5′ UTR/3XHA/3′ UTR. Full-length CAF4 was amplified.

Table II. Quantification of Dnm1-GFP puncta localization

<table>
<thead>
<tr>
<th></th>
<th>Mitochondrial</th>
<th>Cytosolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>16.9 (± 5.5)</td>
<td>3.1 (± 2.1)</td>
</tr>
<tr>
<td>caf4Δ</td>
<td>15.4 (± 5.2)</td>
<td>5.2 (± 2.6)</td>
</tr>
<tr>
<td>mdv1Δ</td>
<td>13.7 (± 5.0)</td>
<td>5.1 (± 3.0)</td>
</tr>
<tr>
<td>fis1Δ</td>
<td>4.9 (± 2.7)</td>
<td>9.6 (± 4.3)</td>
</tr>
<tr>
<td>mdv1Δ caf4Δ</td>
<td>4.8 (± 2.5)</td>
<td>10.4 (± 3.9)</td>
</tr>
</tbody>
</table>

Dnm1p puncta were scored for colocalization with mitochondrial localized DiRed in deconvolved images. For each genotype, 140 budded cells were analyzed by scoring Dnm1p-GFP spots in both the mother and bud, and the average is presented with the SD in parentheses.
with Eg316 and Eg315 and cloned as a Spel/Xhol fragment into the Spel/Sall sites, resulting in EG1041. Caf4p N (residues 1–274) and C (residues 275–659) were amplified with Eg316/Eg315 and Eg322 and cloned as a SpeI/XhoI fragment into the pRS416, yielding pRS405 and pRS406 (Mumberg et al., 1994) containing a start codon inserted between the XbaI and EcoRI sites. The galoactose-inducible Caf4p expression vectors EG1133 (Caf4p-HA), EG1135 (Caf4p-HA, residues 251–659), and EG1136 (Caf4p-HA, residues 1–659) were amplified with Eg316/Eg353 and Eg315/Eg10. mito-GFP was integrated to the GAL1 promoter in the pYES-mtGFP (Westermann and Neupert, 2000) to generate M3TH-GFP. Yeast strain construction

EG686, replacing GFP with DsRed. OM45 was PCR amplified with primers Eg80 and Eg81. Both EG686 and EG687 were transformed into DCY1979 (wild-type) and DCY2305 (Mitofhis1 caf4pΔ) to generate EG823. EG823 was able to complement the mitochondrial morphology defects in mdv1Δ cells.

The galactose-inducible Caf4p expression vectors EG1133 (Caf4p-HA), EG1135 (Caf4p-HA, residues 251–659), and EG1136 (Caf4p-HA, residues 1–659) were amplified with Eg316/Eg353 and Eg315/Eg10. mito-GFP was integrated to the GAL1 promoter in the pYES-mtGFP (Westermann and Neupert, 2000) to generate M3TH-GFP. Yeast strain construction

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Differential centrifugation
Yeast strains CAF4-HTM (DCY2055), CAF4-HTM fis1Δ (DCY2094), MDV1-HTM (DCY2053), and MDV1-HTM fis1Δ (DCY2097) were grown in YPD and harvested at OD₆₀₀ ≈ 1.2. 100 OD units of cells were spheroplasted with zymolyase and lysed in a small clearance Dounce homogenizer (0.6 M sorbitol and 10 mM Tris, pH 7.4). The lysate was spun twice at 2,9 krpm for 5 min. An aliquot of the second supernatant was saved as the total lysate sample. The second supernatant was spun at 10 krpm for 10 min, and an aliquot of the supernatant was saved as the cytosol sample. The pellet was resuspended and spun again at 10 krpm for 10 min. An aliquot of the final pellet was saved as the mitochondrial pellet. Equal cell equivalents were loaded for Western blot analysis. The difference in porin intensity between the total and mitochondrial fractions most likely results from fewer obscuring proteins in the mitochondrial fraction.

Imaging
Images were acquired on a microscope (Axiovert 200M; Carl Zeiss Micro-Imaging, Inc.) using a 100× Plan-Apochromat NA 1.4, oil-immersion objective. Z-stack images (between 0.1- and 0.4-μm intervals for time-lapse images) were collected at RT with an ORCA-ER camera (Hamamatsu), controlled by AxioVision 4.2 software. Images were collected at either 30- or 40-s intervals for 30 min for time-lapse experiments. Iterative deconvolutions were performed with Axiovision 4.2 and maximum intensity projections were generated with AxioVision 4.2 for still images and Image J for time-lapse images. Fluorescent images in Figs. 2–5 were overlaid with differential interference contrast images [set at 50% opacity] in Adobe Photoshop CS.

Immunofluorescence
Cells were processed for immunofluorescence essentially as described previously (Guthrie and Fink, 1991) with the following modifications. Cultures were fixed for 15 min with 3.7% formaldehyde. Tween 20 (0.5%) was included in blocking buffer (PBS, 1% BSA) during a 15-min block step. Cells were stained with 9E10 hybridoma supernatant and a Cy3-conjugated anti–mouse secondary antibody. Washes after primary and secondary incubations were 5 min with blocking buffer, 5 min with blocking buffer containing 0.5% Tween 20, and two 5-min washes with blocking buffer. All incubations were performed at RT. GelMount (Biomeda) was used as mounting medium to preserve fluorescence.

Online supplemental material
Table S1 lists proteins identified in MudPIT experiments with M9TH-Fis1p. Table S2 shows yeast strains. Table S3 lists primer sequences. Videos 1 and 2 show mitochondrial fission in mdv1Δ yeast. Mitochondria were monitored by the mitochondrial outer membrane marker OM45-GFP. Arrows highlight a subset of fission events. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200503148/DC1.

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