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 NH₂-terminal half and with Dnm1p through its COOH-terminal WD40 domain. We demonstrate that mdv1Δ yeast contain residual mitochondrial fission due to the redundant activity of Caf4p. Moreover, recruitment of Dnm1p to mitochondria is disrupted in mdv1Δ caf4Δ 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 fis1Δ 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 NH₂-terminal half and with Dnm1p through its COOH-terminal WD40 domain. However, Mdv1p appears dispensable for Dnm1p assembly on mitochondria because mdv1Δ 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 9X Myc/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 9X Myc/TEV/His8 tag (MfTH-Fis1p) from the endogenous locus.

Tandem affinity-purified MfTH-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 MfTH-FIS1 (3XMy/TEV/His8-FIS1) and deleted for CAF4 or MDV1, respectively. When MfTH-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-
ouslly reported (Tieu et al., 2002; Cerveny and Jensen, 2003). The WD40 domain of both Mdv1p and Caf4p interacted strongly with Dnm1p. However, full-length Mdv1p interacted more weakly and an interaction between full-length Caf4p and Dnm1p was not detected. These results suggest that the interaction of the WD40 domain with Dnm1p is regulated and may be inhibited by the NH2-terminal region of Caf4p and Mdv1p.

We also detected homotypic and heterotypic interactions between Caf4p and Mdv1p. Approximately 5% of Caf4p-HA and Caf4p-N-HA (residues 1–774), coimmunoprecipitate with full-length Caf4p-HTM (Fig. 2 B, lanes 13–15). A similar level of Mdv1p-HA and Mdv1p-N-HA (residues 1–300), but not Mdv1-C-HA (residues 301–774), coimmunoprecipitated with Mdv1p-HTM (Fig. 2 B, lanes 16–18). When Caf4p-HTM was precipitated, ~1% of Mdv1p-HA and Mdv1p-N-HA, but not Mdv1p-C-HA, coprecipitated (Fig. 2 B, lanes 10–12). Similarly, when Mdv1p-HTM was precipitated, ~1% of Caf4p-HA and Caf4p-N-HA, but not Caf4p-C-HA, coprecipitated (Fig. 2 B, lanes 7–9). Moreover, the NTE/CC regions of Caf4p and Mdv1p interact in the two-hybrid assay (Table I). Therefore, Caf4p interacts with all three members of the fission pathway, with the NH2-terminal region mediating interactions with Fis1p, Mdv1p, and homotypic interactions with Caf4p.

**Caf4p is involved in mitochondrial division**

Given that Caf4p interacts with Fis1p, Mdv1p, and Dnm1p, we hypothesized that Caf4p, like Mdv1p, is a component of the mitochondrial division apparatus. *caf4Δ* yeast, however, display normal mitochondrial morphology, with tubular mitochondria evenly dispersed around the cell cortex (Fig. 3). Wild-type mitochondrial morphology was also observed at elevated temperatures and on carbon sources other than dextrose (glycerol or galactose; unpublished data). This observation is not surprising, given that *CAF4* was not identified in a genome-wide screen of deletion strains for mitochondrial morphology mutants (Dimmer et al., 2002).

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

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### Table I. Caf4p and Mdv1p interact with Dnm1p and Fis1p in a yeast two-hybrid assay

| Genotype   | Wild-type | Collapsed net | Spread net |
|------------|-----------|---------------|------------|
| Wild-type  | 100       | 0             | 0          |
| caf4Δ      | 100       | 0             | 0          |
| mdv1Δ      | 0         | 45.5          | 54.5       |
| dnm1Δ      | 0         | 90            | 10         |
| fis1Δ      | 0         | 92.5          | 7.5        |
| mdv1Δ caf4Δ| 0         | 91.5          | 8.5        |
| mdv1Δ dnm1Δ| 0         | 91.5          | 8.5        |

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**Figure 3.** *CAF4 regulates mitochondrial morphology.* Strains expressing mitochondrially 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.
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
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 the 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 fzo1Δ cells (Blazard 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 \( fzo1 \Delta \) cells less efficiently than deletion of \( DNM1 \) (Cerveny et al., 2001). To further test our hypothesis that \( mdv1 \Delta \) cells have only a partial loss of mitochondrial fission, we compared the efficiencies with which the \( mdv1 \Delta \) and \( dnm1 \Delta \) mutations suppress the glycerol growth defect of \( fzo1 \Delta \) 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 \( fzo1 \Delta \) spores grew on glycerol plates. Of 17 \( mdv1 \Delta fzo1 \Delta \) 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 \( dnm1 \Delta fzo1 \Delta \) spores grew robustly on glycerol plates, with between 20 and 50% cell survival. Most importantly, the triple mutant \( mdv1 \Delta caf4 \Delta fzo1 \Delta \) spores grew much more robustly than the \( mdv1 \Delta fzo1 \Delta \) spores, with all spores growing on glycerol and the majority between 20 and 50% cell survival. The markedly enhanced bypass suppression of \( fzo1 \Delta \) by \( mdv1 \Delta caf4 \Delta \) double mutations compared with the \( mdv1 \Delta \) mutation provides genetic evidence that \( mdv1 \Delta \) cells retain residual mitochondrial fission due to the activity of Caf4p.

\textbf{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 mitochondrial markers (Fig. 7). We analyzed by Western blot with an anti-Myc antibody in wild-type (left) and \( fzo1 \Delta \) (right) yeast. PGK (3-phosphoglycerate kinase) is a mitochondrial marker, and porin is a mitochondrial outer membrane marker.

\begin{figure}[h]
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\caption{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–U) and \( fzo1 \Delta \) 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 \( fzo1 \Delta \) cells. Cells were grown in YP dextrose (A–I) or YP galactose (M–U). Representative maximum intensity projections of deconvolved z-stacks are shown. Bar, 1 \( \mu \)m. (V) Caf4p-HTM and Mdv1p-HTM were analyzed by subcellular fractionation. The total cell lysate (Total), high-speed supernatant (Cytosol), and mitochondrial pellet (Mitochondria) were analyzed by Western blot with an anti-Myc antibody in wild-type (left) and \( fzo1 \Delta \) (right) yeast.}
\end{figure}
fusely cytosolic but also retains some localization to mito-
chondria (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 mito-
chondrial 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 as-
sociation 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 compo-
nent of the fission machinery, we reexamined this issue. We con-
structed 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 mito-
chondria (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 mito-
chondrial vs. 5.2 cytosolic and 13.7 mitochondrial vs. 5.1 cy-
tosolic 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 mitochon-
drial vs. 9.6 cytosolic) (Table II and Fig. 8, J–L). As has
been previously noted, a small fraction of Dnm1p still local-
izes to mitochondria in fis1Δ cells (Tieu et al., 2002; Cerveny
and Jensen, 2003), suggesting that Dnm1p may be re-
cruited by a second pathway, perhaps through an intrinsic
affinity for mitochondrial lipids or an unidentified mitochon-
drial binding partner. Importantly, a similar defect in Dnm1p
localization was found in mdv1Δ caf4Δ cells (4.8 mitochon-
drial 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 man-
ner 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.
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 at 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). YJG12 and DCY1557 are in the w303 background. All other strains are in the S288C background. fis1Δ::KanMX6, mdv1Δ::KanMX6, caf4Δ::KanMX6, and dnm1::KanMX6 are derived from the MATa deletion library (Open Biosystems).

Plasmid construction

The M9TH 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 (Stratagene). Eg260 and Eg4, an FZO1 reverse primer, were used to amplify a TEV/His6 module from EG704 (pRS414 + 9XMyc/TEV/His6/ZFO1). 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. 9XMyc/TEV was amplified with Eg256 and Eg260 from EG704 and fused to the 5′ end of 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 + 9XMyc/TEV/URA3/His6). EG940 was converted to pRS403 + 3×Myc/TEV/URA3/TEV/His6 by digesting with XbaI, yielding EG957.

To construct HA-tagged versions of Caf4p and Caf4p fragments, HA sequences were PCR amplified from end3Δ genomic DNA (Open Biosystems). First, the Caf4p 3′ untranslated region (UTR) was amplified with the primers Eg313 and Eg314 and cloned as a KpnI/SalI fragment into pRS416, resulting in pRS416 + Caf4p 3′ UTR. 3XHA was amplified with Eg327 and Eg328 and cloned as a Xhol fragment into the Sall site to generate pRS416 + 3XHA/Caf4p 3′ UTR. The Caf4p 5′ UTR was cloned as a SacI/SpeI fragment using Eg312 and Eg317, resulting in pRS416 + Caf4p 5′ UTR/3XHA/3′ UTR. Full-length Caf4p was amplified

Table II. Quantification of Dnm1-GFP puncta localization

|          | Mitochondrial | Cytosolic |
|----------|---------------|-----------|
| Wild-type| 16.9 (± 5.5)  | 3.3 (± 2.1) |
| caf4Δ   | 15.4 (± 5.2)  | 5.2 (± 2.6) |
| mdv1Δ   | 13.7 (± 5.0)  | 5.1 (± 3.0) |
| fis1Δ   | 4.9 (± 2.7)   | 9.6 (± 4.3) |
| mdv1Δ caf4Δ | 4.8 (± 2.5) | 10.4 (± 3.9) |

Dnm1p puncta were scored for colocalization with mitochondrially localized DsRed 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/XhoI fragment into the Spel/Sall sites, resulting in EG1041. CAF4 N (residues 1–274) and C (residues 275–659) were amplified with Eg316/Eg353 and Eg315/Eg352, respectively, and cloned as SacII/SpeI fragments, resulting in EG1043 and EG1044. Four independent cloned encoded glutamine at residue 110 and arginine at residue 111. Full-length CAF4-HA was able to complement caf4 in caf4Δ mvd1Δ yeast, indicating that it was functional.

To construct HA-tagged versions of MDV1 and MDV1 fragments, MDV1 sequences were amplified by PCR from end3α genomic DNA. First, the MDV1 3′ UTR was amplified with the primers Eg322 and Eg324 and cloned as a SacII/SalI fragment into the pKD4 vector, resulting in M3TH-ΔMDV1 (Mumberg et al., 1994) containing a start codon inserted between the XbaI and EcoRI sites. The galactose-inducible CAF4p expression vectors EG1133 (CaF4p-HA), EG1135 (CaF4p-HA, residues 251–659), and EG1136 (CaF4p-HA, residues 1–250) were generated by replacing the CAF4 5′ UTR in EG1041, EG1043, and EG1045 with a SacI/Clal Gall promoter fragment from pGALL (Mumberg et al., 1994) containing a starting codon inserted between the XbaI and EcoRI sites. The galactose-inducible CAF4p expression vectors EG1133 (CaF4p-HA), EG1135 (CaF4p-HA, residues 251–659), and EG1136 (CaF4p-HA, residues 1–250) were generated by replacing the CAF4 5′ UTR in EG1041, EG1043, and EG1045 with a SacI/Clal Gall promoter fragment from pGALL (Mumberg et al., 1994) containing a starting codon inserted between the XbaI and EcoRI sites.

Yeast strain construction

An MTH/FIS1 strain was generated by amplifying the 5′MYC/TEV-URA3/TEV/Hisα cassette from EG943 (pRS403-5′MYC/TEV/URA3/TEV/Hisα) with the FIS1-targeting primers Eg261 and Eg262 and transforming YG12. URA3 transformants were screened by PCR for correct integration (2 out of 8 positive), grown overnight in YPD to allow for loss of URA3, and plated on 5-FOA plates. Genes were cloned by Western blotting for expression of MTH/FIS1p (9 out of 16 positive). This strain displayed wild-type morphology in 64% of cells and moderate defects in the remaining cells. The same strategy was used to generate Mth/FIS1 from the pRS403-3′MYC/TEV/URA3/TEV/Hisα template (EG597) for subsequent experiments in the S288C background. This strain (DCY2192) displayed wild-type morphology in 89% of cells and wild defects in the remaining cells. DCY2192 was crossed to mvd1Δ and caf4Δ strains (Open Biosystems MATα deletion library) and sporulated to generate Mth/FIS1 mvd1Δ (DCY2302) and Mth/FIS1 caf4Δ (DCY2303).

Zo1::His5 was transformed by transformation with a HIS5 plasmid (Sacchary:clonyer) fragment amplified with the FZO1 targeting primers Eg9 and Eg10: mto-HisG was integrated to the leu2a locus by transformation with Nit1-digested EG686 (pRS403 + GPD/mto-HisG). mto-DisRed was integrated to the leu2a locus by transformation with Hpal-digested EG823 (pRS403 + GPD/mto-DisRed). mzd1::His5 was transformed by transformation with a HIS5 (S. kluveri) fragment amplified with the D6N1 targeting primers Eg57 and Eg58. Chromosomal CAF4/HAM1 was generated by transformation of DCY1979 with Hisα/2TEV/9XMYC/Hisα cassette (Seol et al., 1999) amplified with the CAF4 targeting primers Eg28 and Eg285. Chromosomal MDV1-HTM was generated by transformation of DCY1979 with a Hisα/2TEV/9XMYC/Hisα cassette (Seol et al., 1999) amplified with the MDV1 targeting primers Eg80 and Eg81. Both CAF4/HAM1 and MDV1-HTM are functional because 70% of CAF4/HAM1 mvd1Δ yeast displayed spread mitochondrial nets and 98% of MDV1-HTM yeast cells display wild-type mitochondrial morphology.

DNN1::GFP was generated by amplifying GFP-His5 from pKT128 (Sheff and Thorn, 2004) with Eg342 and Eg343. This product was transformed into DCY1626 (wild-type yeast with mto-DisRed) to generate DCY2370. DCY2370 was crossed to fis1Δ and mvd1Δ caf4Δ strains to generate DCY2404 [DNM1-GFP fis1Δ], DCY2414 [DNM1-GFP caf4Δ], DCY2417 [DNM1-GFP mvd1Δ], and DCY2418 [DNM1-GFP mvd1Δ caf4Δ].

Tandem affinity purification MudPIT Pellets from 21 cultures (OD600 = 1.5) grown in YPD were prepared essentially as described previously for HPM tag Dual-Step affinity purification (Graumann et al., 2004), with the following modifications. Fungal protease inhibitors were used (Sigma-Aldrich) and lysates were cleared at 20 kg for 15 min. Cleavage from 9E10N beads was performed with GST-TEV protease for 3 h at RT. The second affinity step was performed with 40 μM Magne-His beads (Promega). Samples were proteolytically digested and immobilized by multimeric gel chromatography in-line with a Deca XP ion trap mass spectrometer (ThermoElectron) as described previously (Mayor et al., 2005). Samples were released stepwise from the strong cation exchanger phase of the triphasic capillary columns as reported previously (Graumann et al., 2004).

Immunoprecipitation CAF4-HA (EG1041), CAF4-HA residues 1–274 (EG1043), and CAF4-HA residues 275–659 (EG1045) were expressed in strains DCY1979 (wild-type) and DCY2305 (Mth/FIS1 caf4Δ). MDV1-HA (EG1047), MDV1-HA residues 301–100 (EG1049), and MDV1-HA residues 301–714 (EG1051) were expressed in DCY1979 or DCY2302 (Mth/FIS1 caf4Δ). Cultures were grown in selective SD media and harvested at OD600 ~0.8. 20 OD600 units of cells were lysed with glass beads (40 s with a vortex mixer, 4 times) in 500 μl ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100) in the presence of Fungal protease inhibitors (Sigma-Aldrich). Lysates were cleared by centrifuging 5 min at 5 krpm and 15 min at 14 krpm. At this point, a total lysate sample was taken. 400 μl of cleared lysate was mixed with a 20-μl bead volume of 9E10-conjugated protein A–Sepharose beads (Sigma-Aldrich) for 90 min. Beads were washed four times with 1 ml washes of lysis buffer. Precipitate was eluted with 100 μl SDS buffer at 95°C for 5 min. SDS-PAGE Western blotting was performed with 9E10 hybridoma supernatant (anti-Myc) or 12CA5 ascites fluid (anti-HA).

Yeast two-hybrid assay pGAD vectors were transformed into PJ69-4a, pGBD vectors were transformed into PJ69-4a (James et al., 1996). Indicated vectors were mated on YPD plates using two transformants for each vector (totaling four matings per combination). Diploids were selected by replica plating to SD-leu-ura plates. Interactions were assayed by replica plating to SD-leu-ura-lys-ade and incubating for 4 d at 30°C.

Mitochondrial morphology analysis Mitochondrionally targeted GFP-mto-HisG was used to monitor mitochondrial morphology. DCY1979 (wild-type), DCY1945 (caf4Δ), DCY1984 (caf4Δ mvd1Δ), DCY2009 (fis1Δ), DCY2128 (mvd1Δ), DCY2155 (mvd1Δ dnm1Δ), and DCY2312 (dnm1Δ) were grown overnight, diluted 1:20 into fresh medium, and grown for 4 h at 30°C. Cultures were fixed by adjusting cultures to 3.7% formaldehyde and incubated 10 min at 30°C. Cells were washed 3 times with 1 ml PBS and scored for mitochondrial morphology. For CAF4 overexpression studies, plasmids p416 Goll/CAF4-HA (EG1133), p416 Goll/CAF4-HA residues 251–659 (EG1135), or p416 Goll/CAF4-HA residues 1–250 (EG1137) were transformed into DCY1979. Cultures were grown overnight in selective SRAff and diluted 1:20 in fresh YPD or YPGal and grown 3 h at 30°C. Samples were taken for Western analysis and the remaining culture was fixed as described above.

For latrunculin A treatment, overnight YPD cultures were diluted 1:20 in fresh YPD and grown for 3 h. Cultures were then treated for 1 h at 30°C with 200 μM latrunculin A in or with an equivalent amount of vehicle (DMSO). Cultures were then fixed as described above. For time-lapse imaging, overnight SGal cultures were diluted 1:20 in fresh YPGal and grown for 3 h. Cells were pelleted, resuspended in fresh media, and embedded in 1% low melting point agarose containing 200 μM latrunculin A.

Bypass suppression assay DCY2002 and DCY2343 were sporulated and dissected onto YPD plates. Spores were picked, grown overnight in 3 ml YPD at 30°C, pelleted, and resuspended to OD600 ~1.0 in YP. 3 μl of 1:5 serial dilutions were spotted on YPD and YPGlycerol and grown at 30°C for 2 and 4 d, respectively, to determine the fraction of cells that grow on glycerol. Genotypes were determined by PCR.
Differential centrifugation

Yeast strains CAFA4HTM (DCY2055), CAFA4HTM 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 2,900 rpm for 5 min. An aliquot of the supernatant was saved as the total lysate sample. The second supernatant was spun at 10 rpm for 10 min, and an aliquot of the supernatant was saved as the cytosol sample. The pellet was resuspended and spun again at 10 rpm 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 protein 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 MicroImaging, Inc.) using a 100× Plan-Neofluar, NA 1.4, oil-immersion objective. Z-stack images (between 0.1- and 0.4-μm intervals for time-lapse images and between 0.3- 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. 3–5 were overlaid with differential interference contrast images (set at 50% opacity) in Adobe Photoshop CS.

Immunochemistry

Cells were processed for immunofluorescence essentially as described previously (Guthrie and Fink, 1991) with the following modifications. Cultures were fixed for 15 min with 2.7% formaldehyde. Mounting medium 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 mouse secondary antibody. Washes after primary and secondary incubations were performed at RT. GelMount (Biomeda) was used as a mounting medium to preserve fluorescence.

Online supplemental material

Table S1 lists proteins identified in MudPIT experiments with MtThFis1p. Table S2 shows yeast strains. Table S3 lists primer sequences. Videos 1 and 2 show mitochondrial fission in mtΔ yeast. Mitochondria were monitored by the mitochondrial outer membrane marker OMG45-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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Dnm1p and Fis1p to regulate division of mitochondria. Mol. Biol. Cell. 14:4126–4139.

Cerveny, K.L., J.M. McCaffrey, and R.E. Jensen. 2001. Division of mitochondrial fission requires a novel Dnm1-interacting protein, Net2p. Mol. Biol. Cell. 12:309–321.

Denis, C.L., and J. Chen. 2003. The CCR4-NOT complex plays diverse roles in mRNA metabolism. J. Cell. Biol. 157:1539–1549.

Dimmer, K.S., S. Fritz, F. Fuchs, M. Messerschmitt, N. Weinbach, W. Neupert, and B. Westermann. 2002. Genetic basis of mitochondrial function and morphology in Saccharomyces cerevisiae. Mol. Biol. Cell. 13:847–853.

Fannjiang, Y., W.-C. Chung, S.J. Lee, B. Qi, J. Pevsner, J.M. McCaffrey, R.B. Hill, G. Basanze, and J.M. Hardwick. 2004. Mitochondrial fission protein Msms regulates programmed cell death in yeast. Genes Dev. 18:2785–2797.

Fekkes, P.K.A. Shepard, and M.P. Yaffe. 2000. Gug3p, an outer membrane protein required for fission of mitochondrial tubules. J. Cell Biol. 151:335–340.

Frank, S., B. Gaume, E.S. Bergmann-Leitner, W.W. Leitner, E.G. Robert, F. Catez, C.L. Smith, and R.J. Youle. 2001. The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell. 1:515–525.

Graumann, J., L.A. Dunipace, J.H. Seol, W.H. McDonald, J.R. Yates III, B.J. Wold, and R.J. Deshaies. 2004. Applicability of tandem affinity purification MudPIT to pathway proteomics in yeast. Mol. Cell. Proteomics. 3:226–237.

Guthrie, C., and G. Fink. 1991. Guide to Yeast Genetics and Molecular Biology. Academic Press, San Diego, CA. 933 pp.

Huh, W.K., J.V. Falvo, L.C. Gerke, A.S. Carroll, R.W. Howe, J.S. Weissman, and E.K. O’Shea. 2003. Global analysis of protein localization in budding yeast. Nature. 425:686–911.

Jagasia, R., P. Grote, B. Westermann, and B. Conradt. 2005. DpR1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature. 433:754–760.

James, P., J. Halliday, and E.A. Craig. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics. 144:1425–1436.

Jensen, R.E., A.E. Hobbs, K.L. Cerveny, and H. Sesaki. 2000. Yeast mitochondrial dynamics: fusion, division, segregation, and shape. Microsc. Res. Tech. 51:573–583.

Legesse-Miller, A., R.H. Massol, and T. Kirchhausen. 2003. Constriction and Dnm1p recruitment are distinct processes in mitochondrial fission. Mol. Biol. Cell. 14:1953–1963.

Link, A.J., E.J. Eng, D.M. Schiltz, E. Carmack, G.J. Mize, D.R. Morris, B.M. Garvik, and J.R. Yates III. 1999. Direct analysis of protein complex using mass spectrometry. Nat. Biotechnol. 17:676–682.

Liu, H.Y., Y.C. Chiang, J. Pan, J. Chen, C. Salvador, D.C. Audino, V. Badarinarayana, V. Palaniswamy, B. Anderson, and C.L. Denis. 2001. Characterization of CAF4 and CAF16 reveals a functional connection between the CCR4-NOT complex and a subset of SRB proteins of the RNA polymerase II holenzyme. J. Biol. Chem. 276:7541–7548.

Mayor, T., J.R. Lipford, J. Graumann, G.T. Smith, and R.J. Deshaies. 2005. Analysis of polyubiquitin conjugates reveals that the rpn10 substrate receptor contributes to the turnover of multiple proteasome targets. Mol. Cell. Proteomics. 4:741–751.

Moody, A.D.M., J.M. McCaffrey, and J.M. Shaw. 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral mitochondrial membrane protein Fis1p. J. Cell Biol. 151:367–380.

Mumberg, D., R. Muller, and M. Funk. 1994. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. 22:5767–5768.

Mumberg, D., R. Muller, and M. Funk. 1995. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene. 156:119–122.

Osteryoung, K.W., and J. Nunnari. 2003. The division of endosymbiotic organelles. Science. 302:1698–1704.

Otsuga, D., B.R. Keegan, E. 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. 143:333–341.

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.

Seol, J.H., B.M. Feldman, W. Zachariae, A. Shevchenko, C.C. Correll, S. Lyapina, Y. Chi, M. Galova, J. Claypool, S. Sandmeyer, et al. 1999. Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. Genes Dev. 13:1614–1626.

Sesaki, H., and R.E. Jensen. 1999. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. J. Cell Biol. 147:699–706.
Shaw, J.M., and J. Nunnari. 2002. Mitochondrial dynamics and division in budding yeast. Trends Cell Biol. 12:178–184.

Sheff, M.A., and K.S. Thorn. 2004. Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae. Yeast. 21:661–670.

Sickmann, A., J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H.E. Meyer, B. Schönfisch, I. Perschil, A. Chacinska, B. Guiard, et al. 2003. The proteome of Saccharomyces cerevisiae mitochondria. Proc. Natl. Acad. Sci. USA. 100:13207–13212.

Smirnova, E., L. Griparic, D.L. Shurland, and A.M. van der Bliek. 2001. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol. Biol. Cell. 12:2245–2256.

Tieu, Q., and J. Nunnari. 2000. Mdv1p is a WD repeat protein that interacts with the dynamin-related GTPase, Dnm1p, to trigger mitochondrial division. J. Cell Biol. 151:353–366.

Tieu, Q., V. Okreglak, K. Naylor, and J. Nunnari. 2002. The WD repeat protein, Mdv1p, functions as a molecular adaptor by interacting with Dnm1p and Fis1p during mitochondrial fission. J. Cell Biol. 158:445–452.

Westermann, B., and W. Neupert. 2000. Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in Saccharomyces cerevisiae. Yeast. 16:1421–1427.

Wolf, E., P.S. Kim, and B. Berger. 1997. MultiCoil: a program for predicting two- and three-stranded coiled coils. Protein Sci. 6:1179–1189.