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

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

Introduction

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

We and others have identified and characterized two additional proteins, Mdv1p and Fis1p, that act together with Dnm1p to facilitate fission (Fekkes et al., 2000; Mozdy et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Mdv1p is a predicted soluble protein containing at least three regions: an NH_2-terminal region of indeterminate structure, a predicted coiled-coil (C-C)* central domain, and a COOH-terminal seven-WD repeat domain. Cytology, genetics, and two-hybrid analyses indicate that Mdv1p interacts with Dnm1p in punctate structures to mediate mitochondrial fission (Fekkes et al., 2000; Tieu and Nunnari, 2000; Cerveny et al., 2001). Although Mdv1p is not required for the assembly of Dnm1p into punctate structures, Dnm1p-containing structures lacking Mdv1p are not able to complete division,

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Key words: mitochondria; membranes; fission; dynamin-related GTPase; WD repeat

*Abbreviations used in this paper: AD, activating domain; BD, binding domain; C-C, coiled coil; DSP, dithiobis(succinimidylpropionate); MBP, maltose-binding protein; NTE, NH_2-terminal extension; SB, sorbitol buffer.

indicating that Mdv1p function is required at a late step in the fission pathway (Tieu and Nunnari, 2000).

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

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

Results and discussion

Dnm1p puncta assemble in *fis1-L80P* cells, but fission is impaired

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

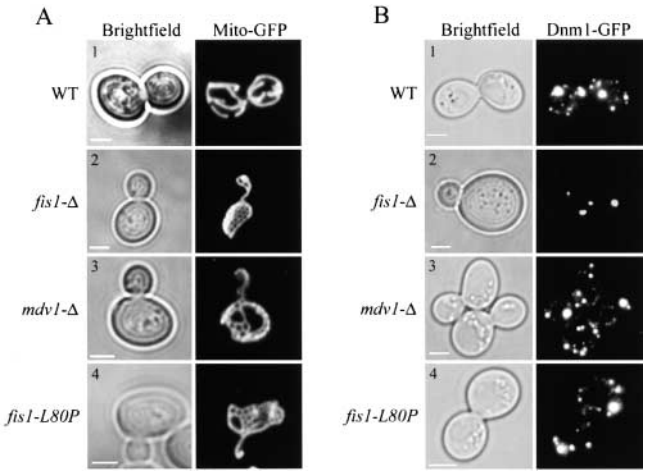

Figure 1. **The *fis1-L80P* mutation disrupts mitochondrial fission, but Dnm1p-containing puncta have wild-type characteristics.** Mito-GFP was used to visualize mitochondrial morphology. Dnm1p was visualized using a Dnm1p–GFP fusion protein. (A) Mitochondrial morphology of representative wild-type, gene deletion, and *fis1-L80P* cells. (B) Dnm1–GFPp localization pattern in *fis1-L80P* cells. Bars, 2 μm.

Examination of mitochondrial morphology in *fis1-L80P* cells with mitochondrial-targeted GFP revealed net-like mitochondrial structures, in contrast to the branched tubules seen in wild-type cells (Fig. 1 A; Table I). These structures were indistinguishable from those observed in *mdv1-Δ*, *fis1-Δ*, and *dnm1-Δ* cells and indicate that mitochondrial fission is impaired in *fis1-L80P* cells (Fig. 1 A). Furthermore, quantification of this mitochondrial morphology defect indicates that the frequency of net-like structures was almost as high as observed in *mdv1-Δ* and *fis1-Δ* cells, indicating that mitochondrial fission is severely defective in *fis1-L80P* cells (Table I).

To determine whether Dnm1p assembly is affected in *fis1-L80P* cells, we examined the steady-state localization pattern of Dnm1–GFP. Interestingly, the characteristics of Dnm1–GFP localization were not significantly altered as compared with wild-type cells (Fig. 1 B; Table I). The ability of Fis1-L80P protein to support the assembly of Dnm1p into punctate structures is in contrast to what is observed in *fis1-Δ* cells where Dnm1p assembly is aberrant, as indicated by the presence of fewer, brighter Dnm1p–GFP-labeled punctate structures (Fig. 1 B; Table I; Mozdy et al., 2000; Tieu and Nunnari, 2000).

Table I: **Quantification of mitochondrial morphology and Dnm1p-associated puncta**

	Mitochondrial morphology			Dnm1p localization	
Strain	**No. cells scored**	**Branched reticular**	**Nets**	**No. cells scored**	**No. Dnm1–GFPp puncta/cell**
		%	%		
WT	107	97	3	40	26 ± 7
fis1-Δ	103	1	99	30	6 ± 3
mdv1-Δ	99	0	100	30	23 ± 6
fis1-L80P	92	16	84	40	21 ± 4

These observations suggest that the fission defect observed in *fis1-L80P* cells is not the result of a defect in the assembly of Dnm1p-containing puncta, but rather occurs as a result of a defect at a later step in the division pathway. Significantly, the phenotypic characteristics of *fis1-L80P* cells are similar to those observed in *mdv1-Δ* cells (Fig. 1, A and B; Tieu and Nunnari, 2000). Together with our previous observation that in *dnm1-Δ* cells, Mdv1p remains associated with mitochondria in a Fis1p-dependent manner, these results support our hypothesis that Fis1p functions with Mdv1p to regulate fission at a post-Dnm1p assembly step.

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

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

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

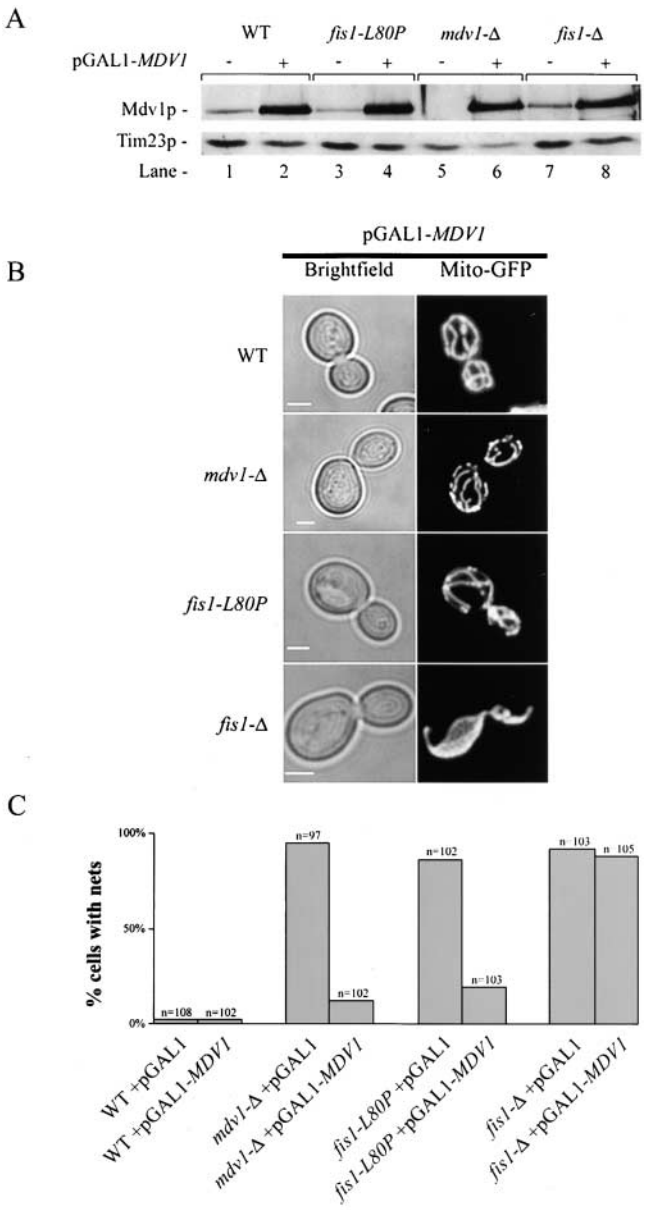

Figure 2. **Overexpression of *MDV1* suppresses the mitochondrial fission defect in *fis1-L80P* cells.** All strains harboring either the empty pGAL1 vector or pGAL1-*MDV1* vectors were grown in SRaf media to early log, subcultured into SGal media, and grown for an additional 12 h and analyzed as described.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

Mdv1p and Fis1p are in a complex

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

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

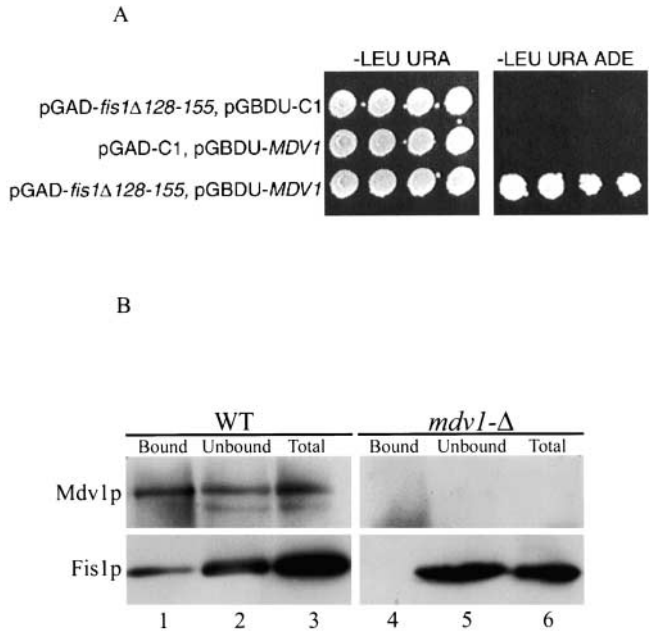

Figure 3. **Fis1p interacts with Mdv1p by two-hybrid and coimmunoprecipitation analyses.** (A) Two-hybrid analysis of *MDV1* and *FIS1*. Interaction between AD *fis1Δ128–155* and BD *MDV1* two-hybrid vectors was assessed by growth of indicated transformants on SD media as described. (B) Immunoprecipitation with anti-Mdv1p antibodies from DSP–cross-linked cells. In vivo, DSP–cross-linked extracts from wild-type and *mdv1-Δ* cells were immunoprecipitated with anti-Mdv1p antibodies and fractions were analyzed by SDS-PAGE and Western blotting as described in the Materials and methods.

immunoprecipitations with anti-Mdv1p from isolated detergent-solubilized mitochondria and whole cell extracts. We were unable to detect a Mdv1p–Fis1p complex in cell extracts, suggesting that a Mdv1p–Fis1p interaction might be labile in vitro. To overcome the possible instability associated with the Mdv1p–Fis1p interaction, proteins were cross-linked in vivo with the bifunctional, reversible cross-linker dithiobis(succinimidylpropionate) (DSP), and extracted under denaturing conditions before immunoprecipitation with antibodies. After immunoprecipitation, cross-links were reversed with a reducing agent and precipitates were analyzed by SDS-PAGE and Western blotting with anti-Mdv1p and Fis1p antibodies.

Western blot analysis of fractions from the anti-Mdv1p immunoprecipitation revealed that a significant fraction of Mdv1p from wild-type cells was present in the precipitate (Fig. 3 B, lanes 1–3). Significantly, we observed that a fraction of Fis1p from cross-linked extracts was reproducibly coimmunoprecipitated by anti-Mdv1p antibodies (Fig. 3 B, lanes 1–3). As a control for specificity, anti-Mdv1p antibodies were used to perform immunoprecipitations from DSP–cross-linked *mdv1-Δ* cell extracts (Fig. 3 B, lanes 4–6). Under these conditions, Fis1p was not observed in precipitates, indicating that the coimmunoprecipitation of Fis1p with anti-Mdv1p antibodies specifically is dependent on Mdv1p (Fig. 3, lanes 4–6). The Mdv1p–Fis1p interaction could also be detected by coimmunoprecipitation in extracts prepared from isolated mitochondria after DSP cross-linking (unpublished data). Thus, both two-hybrid and coimmunoprecipitation data indicate that Mdv1p and Fis1p interact within a complex in cells.

The Mdv1p–Fis1p interaction is abolished in *fis1-L80P* cells

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

We first examined the stability and intracellular localization of Fis1p and Mdv1p in *fis1-L80P* cells. Wild-type and *fis1-L80P* cell extracts were fractionated by differential centrifugation, and analyzed by SDS-PAGE and Western blotting. Consistent with a mitochondrial localization, in extracts from both wild-type and *fis1-L80P* cells, the majority of Mdv1p and Fis1p cofractionated with porin, the mitochondrial marker, in the mitochondrial-enriched pellet fraction (Fig. 4 A, compare lanes 1–3 with 4–6). In addition, levels of Mdv1p and Fis1p were similar in *fis1-L80P* and wild-type cell extracts (Fig. 4 A, compare lanes 1–3 with 4–6). These results indicate that both Fis1p and Mdv1p are expressed stably and localized correctly to mitochondria in *fis1-L80P* cells. Thus, the fission defect ob-

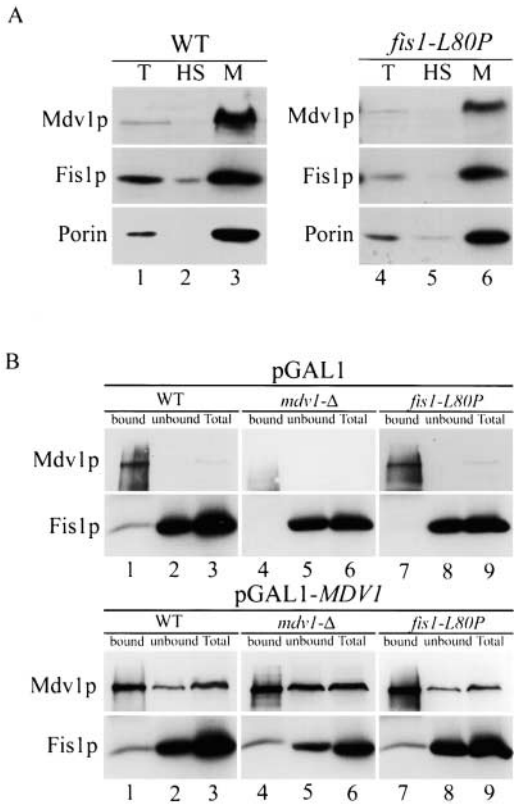

Figure 4. **The Fis1p–Mdv1p interaction is disrupted in *fis1-L80P* cells and restored upon overexpression of Mdv1p.** (A) Fractionation of cell extracts from wild-type and *fis1-L80P* cells by differential centrifugation. Fractions were analyzed by SDS-PAGE and Western blotting as described. (B) In vivo, DSP–cross-linked extracts from cells harboring either pGAL1 or pGAL1-*MDV1*, grown in SGal media, were immunoprecipitated with anti-Mdv1p antibodies and analyzed by SDS-PAGE and Western blotting.

served in *fis1-L80P* cells is not simply the result of Fis1p and/or Mdv1p instability.

To test whether the Mdv1p–Fis1p interaction is altered in *fis1-L80P* cells, we determined whether Fis1p coimmunoprecipitated with Mdv1p under conditions where a complex was detected in wild-type cells (Fig. 3). Interestingly, when Mdv1p was immunoprecipitated from DSP–cross-linked extract from *fis1-L80P* cells with anti-Mdv1p antibodies, we failed to detect Fis1p in the precipitates, in contrast to wild-type cells (Fig. 4 B, pGAL1). These results suggest that a complex containing Mdv1p and Fis1-L80Pp fails to form, or that interactions within the complex are weakened and thus harder to detect. Consistent with the latter possibility is our observation that overexpression of Mdv1p in *fis1-L80P* cells suppresses the mitochondrial fission defect. Thus, we tested whether increasing the amount of Mdv1p in the *fis1-L80P* cells could, by mass action, restore the Mdv1p–Fis1p interaction observed by coimmunoprecipitation with anti-Mdv1p antibodies.

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

Mdv1p functions as a molecular adaptor in fission

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

Mdv1p contains at least three distinct regions: a novel NH_2-terminal extension region (NTE), a middle region predicted to form a C-C structure, and a COOH-terminal region that contains seven WD repeats predicted to form a seven-bladed propeller structure (WD) (Fig. 5 A; Tieu and Nunnari, 2000). To analyze the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p, we constructed GAL1-regulated GFP fusions to each of these putative domains and examined their localization patterns after expression in wild-type, *mdv1-Δ*, *fis1-Δ*, *dnm1-Δ*, and *fis1-Δ dnm1-Δ* cells. We also examined mitochondrial morphology, particularly in wild-type and *mdv1-Δ* cells, to determine whether expression of Mdv1p domains produced dominant negative phenotypes or could complement the loss of *MDV1* function, respectively.

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

Mitochondrial net-like structures were observed in *mdv1-Δ* strains expressing each Mdv1p region alone, indicating that no single region was sufficient for wild-type levels of mitochondrial fission (Fig. 5 B, panels 2, 6, 10, and 14; 100%, $n = 50$ in all strains). However, in wild-type, *mdv1-Δ*, and *fis1-Δ* cells expressing a GFP-tagged version of WD region of Mdv1p, GFP fluorescence was observed in punctate structures, primarily associated with mitochondria (Fig. 5 B, panels 9–12). The localization of GFP–WD to punctate structures was not observed in *dnm1-Δ* cells, indicating that Dnm1p is required for their formation (Fig. 5 B, panel 12). In addition, punctate structures labeled by GFP–WD also contained Dnm1p, as assessed by colocalization of Dnm1–dsRed in wild-type cells (Fig. 5 C). Taken together, these observations suggest that the WD region is sufficient to interact with Dnm1p.

GFP–NTE labeled both mitochondria and punctate structures in wild-type cells (Fig. 5 B, panels 5–8). Unlike GFP–WD, however, the punctate structures labeled by GFP–NTE probably do not reflect an interaction of NTE with Dnm1p and may be the result GFP–NTE self-aggregation because they also were observed in *dnm1-Δ* and *dnm1-Δ fis1-Δ* cells (Fig. 5 B, panel 12; unpublished data). In addition, GFP–NTE-labeled punctate structures were observed localized at the cell cortex, not associated with mitochondria. The dispersive mitochondrial labeling pattern observed for GFP–NTE in wild-type cells was not observed in *fis1-Δ*, suggesting that the NTE interacts specifically with Fis1p in cells (Fig. 5 B,

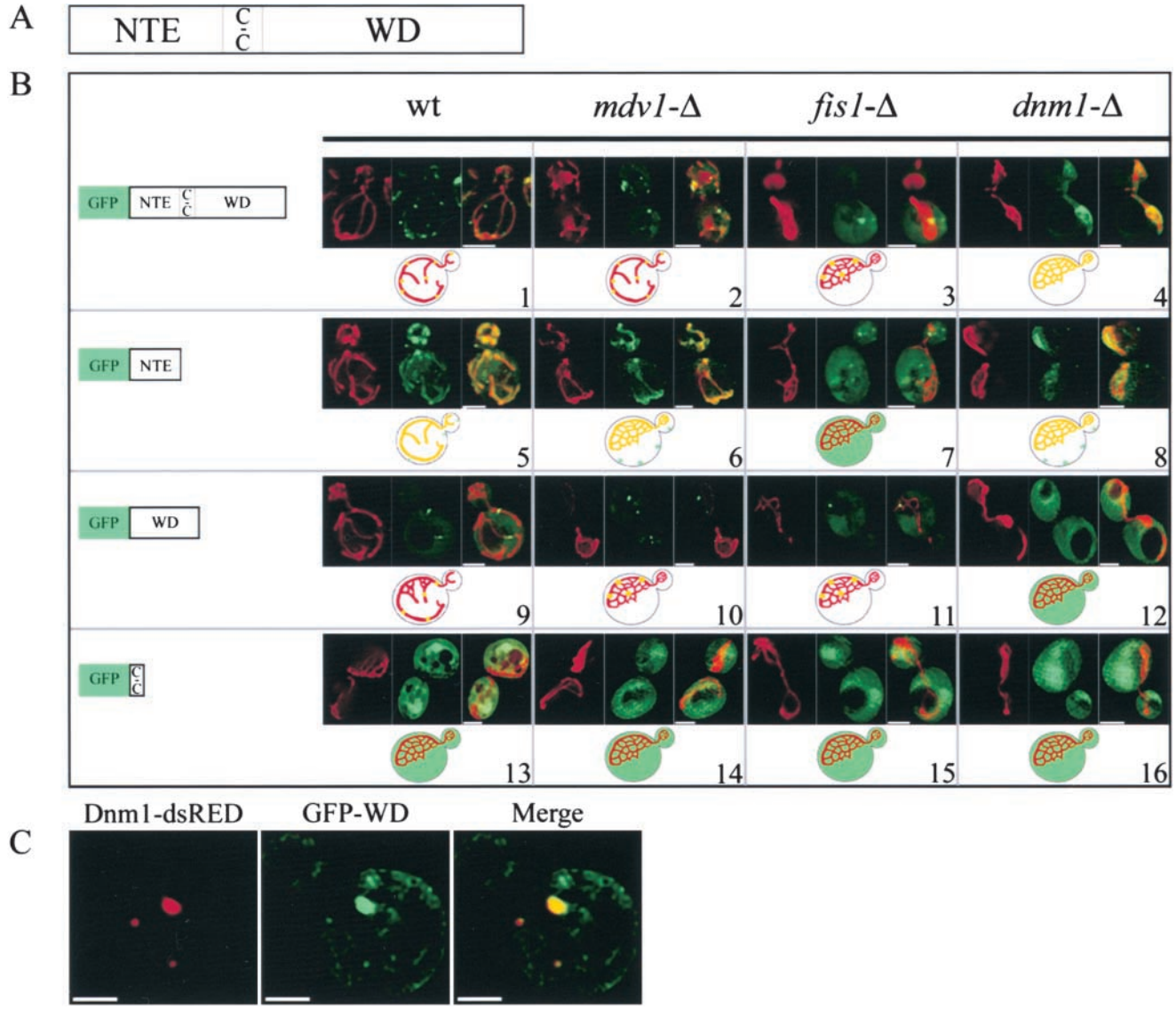

Figure 5. **Mdv1p functions as a molecular adaptor during mitochondrial fission.** (A) Predicted structural domains of Mdv1p (NTE, C-C, and WD [seven-WD repeat domain]). (B) Dual localization of mitochondria labeled with MitoTracker (in red, left) and GFP-tagged Mdv1p regions (in green, middle) in cells. Overlay (right image) was obtained by merging the signals from MitoTracker and GFP-tagged Mdv1p regions. Indicated strains were grown in SRaf, subcultured in SGal, supplemented with 1% dextrose to partially repress galactose-induced overexpression of Mdv1p domains for optimal visualization, and imaged by deconvolution microscopy. Dextrose was omitted for the cytological analysis of Mdv1p regions in *fis1*-Δ cells because higher expression levels were required to detect interactions with Dnm1p-containing structures. In cells, schematically represented, GFP-tagged Mdv1p regions and mitochondria are depicted in green and red, respectively. (C) GFP–WD and Dnm1–dsRed colocalize in punctate structures in wild-type cells. Cells were grown and analyzed as described in B. Bars, 2 μm.

panel 7). Consistent with this interpretation, GFP–NTE also was observed to be associated with mitochondria in *dnm1-Δ* cells (Fig. 5 B, panel 8). In contrast, GFP–WD was observed in a diffuse, cytosolic pattern in the majority of *dnm1-Δ* cells (Fig. 5 B, panel 12). Thus, the cytological analysis of Mdv1p domains suggests that the WD and NTE regions are each sufficient to interact with Dnm1p and Fis1p, respectively.

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

To independently test our cytological observations, we also examined the structural features of Mdv1p required for its interactions with Dnm1p and Fis1p using the two-hybrid assay. We tested both the full-length Mdv1p and Dnm1p protein fusions and Fis1Δ128–155p with regions of Mdv1p to determine protein–protein interactions. An interaction was indicated by cells growing on media lacking adenine (indicated by + in Table II).

As previously shown, full-length Mdv1p was observed to interact with both Fis1-Δ128–155p and Dnm1p in this as-

Table II. **Two-hybrid interactions between Mdv1p and Mdv1p domains, Fis1Δ128–155p and Dnm1p**

	Fis1Δ128–155p	Dnm1p	Mdv1p
Mdv1p	+	+	+
WD	−	+	−
NTE/C-C	+[a]	−	+
C-C	−	−	+

Five colonies for each construct pair were tested. All constructs paired with empty BD and AD plasmids did not grow on adenine$^-$ selective medium. −, no growth on selective medium; +, growth on selective medium.
[a]Growth observed only for one of the reciprocal AD and BD plasmid pairs.

say (Fig. 3 A; Tieu and Nunnari, 2000). In addition, we observed that full-length Mdv1p interacted with full-length Mdv1p in this assay, suggesting that Mdv1p is able to oligomerize. The WD region was observed to interact with Dnm1p exclusively, consistent with our cytological observations using GFP-tagged WD. The NTE region was expressed as a BD or AD fusion only when the C-C region was included (NTE/C-C; unpublished data) and specifically displayed an interaction with Fis1Δ128–155p, consistent with our cytological analysis of the NTE region alone.

We also observed an interaction of NTE/C-C with full-length Mdv1p by two-hybrid analysis (Table II). Indeed, two-hybrid analysis indicates that the C-C region alone was sufficient to specifically interact with Mdv1p, suggesting that Mdv1p forms a higher order homo-oligomeric structure via the predicted C-C region (Table II). Analysis of the primary structure of Mdv1p by the MultiCoil algorithm indicates that Mdv1p has a high probability of forming a parallel dimeric C-C structure (Wolf et al., 1997). It is possible, therefore, that the C-C region exerts its dominant negative effect on mitochondrial fission in wild-type cells by preventing the formation of an Mdv1p dimer (Fig. 5 B, panels 13–16). The localization of the GFP-tagged C-C region to the cytosol in cells further suggests that full-length Mdv1p interacts with both Dnm1p and Fis1p as a homodimeric or higher order complex.

A molecular model for mitochondrial fission

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

Materials and methods

Media and yeast genetic techniques

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

Strains, plasmid construction, and yeast two-hybrid analysis

The *fis1-L80P* allele was isolated as an extragenic suppressor of *fzo1-1* cells as described and identified by analyzing linkage from a cross to an *fzo1-1 fis1-Δ* strain (Tieu and Nunnari, 2000). The mutation was identified by amplifying the *FIS1* locus in *fis1-L80P* cells by PCR using Vent polymerase (New England Biolabs, Inc.) and sequencing the products directly (Davis Sequencing, University of California, Davis).

A plasmid containing GAL1-regulated *MDV1* was constructed by introducing EcoRI and BamHI sites by PCR amplification of the *MDV1* ORF, followed by subcloning into p416GALL (American Type Culture Collection [ATCC]), generating the pGAL1-*MDV1* plasmid. The GFP-tagged Mdv1p domains were constructed by PCR amplifying S65T GFP lacking the 3′ terminator codon using oligonucleotides engineered with 5′ SpeI and 3′ XbaI sites. The *MDV1* ORF and nucleotide regions 1–723, 1188–2142, and 723–903 were amplified lacking a 5′ initiator codon using 5′ oligonucleotides engineered with an XbaI site and 3′ oligonucleotides with the terminator codon and a SpeI site. The amplified products were ligated in frame with the S65T GFP into p416GAL1 (ATCC), yielding plasmids pGAL1-GFP–*MDV1*, pGAL1-GFP–*MDV1*(NTE), pGAL1-GFP–*MDV1*(WD), and pGAL1-GFP–*MDV1*(C-C), respectively.

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

Table III. **Yeast strains**

Strains	Genotype	Source
JSY1826	*leu2Δ1, his3Δ200, trp1Δ63, ura3-52, Mata*	Mozdy et al., 2000
JSY2977	*leu2Δ1, his3Δ200, trp1Δ63, ura3-52, fzo1-1, Mata*	Mozdy et al., 2000
JSY1371	*leu2Δ1, his3Δ200, ura3-52, dnm1-Δ::HIS3, Matα*	Otsuga et al., 1998
JNY854	JSY1826, except *mdv1-Δ::*his5+, *Mata*	Tieu and Nunnari, 2000
JNY855	JSY1826, except *fis1-Δ::*his5+, *Mata*	This study
JNY866	JSY1826, except *fis1-L80P, Mata*	This study
JNY159	PJ69-4A	James et al., 1996

903 with a 5′ ATG initiator. DNA encoding all four regions of *MDV1* were subcloned into both pGAD and pGBDU plasmids. All 3′ primers contained TGA stop codons. pGAD-*fis1-Δ128–155* and pGAD-*DNM1* were gifts from J. Shaw and A. Mozdy, University of Utah, Salt Lake City, UT. For two-hybrid analysis, plasmids constructed as described above from pGBDU and pGAD were cotransformed into the yeast strain PJ69-4A and tested for interactions as previously described (James et al., 1996).

For the production of anti-Fis1p antibodies, the *FIS1* PCR product was amplified from the 5′ ATG initiator codon to nucleotide 384 (*FIS1* lacking the transmembrane and intermembrane space regions) and subcloned in frame with maltose-binding protein (MBP) coding sequence into pMAL-C2 (New England Biolabs, Inc.) using a 5′ BamHI site and a 3′ HindIII site. All plasmid constructs were analyzed by sequencing and contained no additional mutations.

Cytological analysis

Mitochondrial morphology was analyzed and quantified using MitoTracker CMXR (Molecular Probes) or mito-GFP expressed from the plasmid pYX232 (provided by B. Westermann, Ludwig Maximilians Universitaet, Muenchen, Germany) as previously described (Tieu and Nunnari, 2000). Dnm1p was visualized and quantified by transforming cells with pHS20 (*DNM1*–GFP) (gift from R. Jensen, John Hopkins University, Baltimore, Maryland) or pECJN233 (*DNM1*–dsRED) as previously described (Tieu and Nunnari, 2000). pGAL1 plasmids containing GFP-tagged *MDV1* regions were transformed into JSY1826, JNY854, JNY855, and JSY1371. Transformants were grown in SD, subcultured into SRaf media, further subcultured into SGal, supplemented with 1% dextrose to induce expression of GFP-tagged Mdv1p domains, and imaged after 12 h of logarithmic growth. All samples were imaged using either a Leica confocal or DeltaVision microscope with either a 100× 1.4NA or 60× 1.4NA objective.

Biochemical analyses

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

Cell extracts were fractionated by differential centrifugation and analyzed by SDS-PAGE and Western blotting as previously described (Tieu and Nunnari, 2000). Immunoprecipitation of Mdv1p or Fis1p was performed from yeast cells grown in rich or SD media to a density of 1 OD_{600}. After washing in water, 10 OD_{600} equivalents of cells were resuspended in 0.1 ml of sorbitol buffer (SB; 1.2 M sorbitol, 10 mM Hepes, 1 mM $MgCl_2$) digested with 10 μl of 10 mg/ml yeast lytic enzyme (80,000 U/g; ICN Biomedicals) for 15 min at 30°C, and washed twice with 10 volumes of cold SB. After treatment with lytic enzyme, cells were resuspended in 0.92 ml of cold SB, and 0.08 ml of 18 mM DSP (Pierce Chemical Co.) in DMSO was added and cells were incubated for 2 h on ice to cross-link proteins. Cross-linking was terminated by quenching with 0.15 ml of 1 M glycine, pH 8.0, and cells were washed twice with two volumes of cold SB. Cross-linked cells were resuspended in 0.6 ml of 20 mM Tris, pH 8.0, 50 mM ammonia acetate, 2 mM EDTA, and transferred to Eppendorf tubes containing 1 ml of glass beads (Sigma-Aldrich) and 0.60 ml of 20% TCA. The cells were lysed by bead beating with three cycles of 30-s intervals with cooling on ice between cycles. The cell lysate was removed, collected by centrifugation, resuspended in 120 μl of buffer containing 3% SDS, 100 mM Tris, pH 11, and incubated for 5 min at 65°C and 100°C for 5 min. Insoluble material was pelleted by centrifugation. To 40 μl of the SDS-solubilized sample, 560 μl of solution containing 13.3 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.02% NaN_3, 6 μl trasylol, and 6 μl protease inhibitor cocktail (Sigma-Aldrich) was added. The renatured sample was incubated with 50 μl protein A agarose beads equilibrated with buffer containing 0.2% SDS, 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 0.02% NaN_3 (IPS) at 4°C with gentle shaking for 15 min. The cleared supernatant was removed to a new eppendorf containing 20 μl of IPS-equilibrated anti-Mdv1p–protein A cross-linked beads, and incubated at 4°C with gentle shaking for 45 min. The cross-linked anti-Mdv1p–protein A agarose beads were prepared as previously described (Harlow and Lane, 1998). Specifically, a total of 6 mg of crude anti-Mdv1p antibodies were cross-linked to 2.0 ml of protein A agarose beads (Santa Cruz Biotechnology, Inc.) with 20 mM of the water-soluble cross-linker dimethylpimelimidate. 25 μl of anti-Mdv1p–protein A cross-linked beads (antibody excess) were used per immunoprecipitation with 10 OD_{600} equivalent of cells prepared as described above. The protein-bound resin was washed five times with 1 ml of cold PBS, resuspended with 30 μl SDS-PAGE loading buffer, boiled for 5 min, and subjected to SDS-PAGE and Western blotting.

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