Molecular Biology of the Cell
Vol. 21, 2443–2452, July 15, 2010

Mdm36 Is a Mitochondrial Fission-promoting Protein in *Saccharomyces cerevisiae*

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Submitted February 4, 2010; Revised May 14, 2010; Accepted May 17, 2010
Monitoring Editor: Gero Steinberg

The division of mitochondrial membranes is a complex process mediated by the dynamin-related protein Dnm1 in yeast, acting in concert with several cofactors. We have identified Mdm36 as a mitochondria-associated protein required for efficient mitochondrial division. Δ*mdm36* mutants contain highly interconnected mitochondrial networks that strikingly resemble known fission mutants. Furthermore, mitochondrial fission induced by depolymerization of the actin cytoskeleton is blocked in Δ*mdm36* mutants, and the number of Dnm1 clusters on mitochondrial tips is reduced. Double mutant analyses indicate that Mdm36 acts antagonistically to fusion-promoting components, such as Fzo1 and Mdm30. The cell cortex-associated protein Num1 was shown previously to interact with Dnm1 and promote mitochondrial fission. We observed that mitochondria are highly motile and that their localization is not restricted to the cell periphery in Δ*mdm36* and Δ*num1* mutants. Intriguingly, colocalization of Num1 and Dnm1 is abolished in the absence of Mdm36. These data suggest that Mdm36 is required for mitochondrial division by facilitating the formation of protein complexes containing Dnm1 and Num1 at the cell cortex. We propose a model that Mdm36-dependent formation of cell cortex anchors is required for the generation of tension on mitochondrial membranes to promote mitochondrial fission by Dnm1.

INTRODUCTION

Mitochondria are highly dynamic organelles of eukaryotic cells. In many cell types, they continuously move along cytoskeletal tracks and frequently fuse and divide (Okamoto and Shaw, 2005; Dimmer and Scorrano, 2006; Detmer and Chan, 2007; Hoppins *et al*., 2007; Westermann, 2008). This dynamic behavior is important for several cellular processes. For example, mitochondrial fusion allows the cell to build large interconnected mitochondrial networks that facilitate efficient dissipation of energy in the cell (Skulachev, 2001). Furthermore, these extended networks allow transmission of calcium signals (Szabadkai *et al*., 2006), and they are crucial for certain developmental processes (Hales and Fuller, 1997; Chen *et al*., 2003). Fusion also serves to mix and unify the mitochondrial compartment, an activity that is thought to counteract the accumulation of organellar damages during aging (Sato *et al*., 2006). In contrast, fission generates numerous morphologically and functionally distinct organelles. Fragmentation of the mitochondrial network is an important step in the programmed cell death pathway (Youle and Karbowski, 2005; Parone and Martinou, 2006; Suen *et al*., 2008), and it is critical for cell differentiation processes, including formation of synapses and dendritic spines in neurons (Li *et al*., 2004) and embryonic development (Labrousse *et al*., 1999). Because mitochondria are propagated by growth and division of preexisting organelles, mitochondrial inheritance depends on mitochondrial division during cytokinesis (Warren and Wickner, 1996).

The core machinery mediating mitochondrial fusion and fission consists of three large GTPases that have been evolutionarily conserved throughout the fungal and animal kingdoms: Fzo1/mitofusin family members are outer membrane proteins essential for fusion (Hales and Fuller, 1997; Hermann *et al*., 1998; Rapaport *et al*., 1998; Santel and Fuller, 2001), Mgm1/OPA1 are dynamin-related proteins located in the intermembrane space where they mediate inner membrane fusion and cristae maintenance (Wong *et al*., 2000; Cipolat *et al*., 2004; Meeusen *et al*., 2006; Song *et al*., 2009), and Dnm1/DRP1 are dynamin-related proteins that assemble on the mitochondrial surface to mediate organelle division (Otsuga *et al*., 1998; Smirnowa *et al*., 1998; Bleazard *et al*., 1999; Labrousse *et al*., 1999). Although the function of these large GTPases has been highly conserved from yeast to humans, different accessory proteins exist that assemble and regulate the fusion and fission machineries in different organisms (Cerveny *et al*., 2007b).

The availability of various genetic, cytological, and biochemical assays has made baker's yeast, *Saccharomyces cerevisiae*, a prime model organism to dissect the fusion and fission machineries at the molecular level (Jensen *et al*., 2000; Shaw and Nunnari, 2002; Okamoto and Shaw, 2005; Merz *et al*., 2007). In yeast, the membrane fusion activities of Fzo1 in the outer and Mgm1 in the inner membrane are coordinated by the outer membrane protein Ugo1 (Sesaki and Jensen, 2001; Wong *et al*., 2003; Sesaki and Jensen, 2004). Fis1 is an outer membrane protein (Mozdy *et al*., 2000) that together with the soluble adapter proteins Mdv1 or Caf4 (Tieu and Nunnari, 2000; Griffin *et al*., 2005) recruits and assembles Dnm1 on the mitochondrial surface. The Dnm1/Fis1/Mdv1 machinery is thought to be of major importance for mito-

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10–02–0096) on May 26, 2010.

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Abbreviations used: SD, synthetic dextrose medium.

chondrial division, whereas the role of the Mdv1 paralogue Caf4 is still unclear (Lackner *et al*., 2009; Lackner and Nunnari, 2009). Other factors known to be involved in mitochondrial fusion and fission include Mdm30, an F-box protein regulating the turnover of Fzo1 (Fritz *et al*., 2003); Pcp1, a protease required for Mgm1 processing in the inner membrane (Herlan *et al*., 2003; McQuibban *et al*., 2003; Sesaki *et al*., 2003); and Num1, a cell cortex-associated protein that supports mitochondrial fission by a yet unknown mechanism (Cerveny *et al*., 2007a). Given the complexity of coordinated fusion and fission of a double membrane-bounded organelle, it seems likely that additional, yet unknown components are involved.

In a systematic screen of the yeast deletion library, we identified 10 previously uncharacterized open reading frames (ORFs) encoding proteins essential for *m*itochondrial *d*istribution and *m*orphology, *MDM30* through *MDM39* (Dimmer *et al*., 2002). Although a specific role in mitochondrial morphogenesis could be demonstrated for most of these genes in subsequent studies (Fritz *et al*., 2003; Herlan *et al*., 2003; Messerschmitt *et al*., 2003; Youngman *et al*., 2004; Dimmer *et al*., 2005; Nowikovsky *et al*., 2007; Longen *et al*., 2009), almost nothing is known about *MDM36*. Here, we report a detailed functional analysis of the Δ*mdm36* mutant and provide evidence that Mdm36 is a novel protein involved in mitochondrial fission in yeast.

MATERIALS AND METHODS

Plasmids

Standard procedures were used for cloning and amplification of plasmids. Plasmids pVT100U-mtGFP and pYX113-mtGFP (Westermann and Neupert, 2000) were used for expression of mitochondria-targeted green fluorescent protein GFP (mtGFP), plasmids pRS416-GAL1+PrFoATP9-RFP (Mozdy *et al*., 2000) and pVT100U-mtRFP (Dürr, Universität Bayreuth, unpublished) were used for expression of mitochondria-targeted red fluorescent protein (mtRFP), plasmid pWP1055 (Prinz *et al*., 2000) was used for expression of endoplasmic reticulum (ER)-targeted GFP, and plasmid pHS20 (Sesaki and Jensen, 1999) was used for expression of Dnm1-GFP in Num1-RFP–expressing strains. Plasmid pGEM3-*MDM36* was constructed by polymerase chain reaction (PCR) amplification of the *MDM36* ORF by using oligonucleotides 5′-AAA GAG CTC GAT GAA AAC GGT ACA GTA AAG CC and 5′-AAA GGT ACC TCA AGT ATT TTG TGA AGA AGG TTG and cloning into the SacI and KpnI sites of vector pGEM3 (Promega, Madison, WI). Plasmid pBG1805-*MDM36* (Gelperin *et al*., 2005) was used for expression of epitope-tagged (hemagglutinin) Mdm36 (Mdm36-HA) under control of the *GAL1* promoter.

Yeast Strains

Growth and manipulation of yeast strains was according to standard procedures (Burke *et al*., 2000). If not indicated otherwise, yeast strains were isogenic to BY4741, BY4742, and BY4743 (Brachmann *et al*., 1998; see Figures 1A, 2B, 3–5, and 6, A and B; Supplemental Figures S1 and S3; Supplemental Videos 1–12; and Supplemental Table S1). Δ*dnm1*, Δ*mdm30*, Δ*mdm36* and Δ*num1* mutants were taken from the *MAT*α yeast deletion collection (Giaever *et al*., 2002) or obtained from Euroscarf (Frankfurt, Germany). Double deletion mutants were constructed by mating, sporulation, and tetrad dissection. A yeast strain expressing Dnm1-GFP from its normal chromosomal locus (*DNM1:GFP*) has been described previously (Schauss *et al*., 2006). The *MDM36* gene was deleted in the Dnm1-GFP–expressing strain by replacing the coding region by a *HIS3MX6* cassette (Wach *et al*., 1997), and deletion strains carrying the *DNM1:GFP* allele and a deletion of the *NUM1* gene or a Δ*mdm36* Δ*num1* double deletion were created by mating, sporulation, and tetrad dissection. Δ*fzo1* (*fzo1::kanMX4*) (Rapaport *et al*., 1998) and Δ*mdm36* (*mdm36::HIS3MX6*) (Dimmer, unpublished) single and double mutants and strains used for electron microscopy were isogenic to YPH499, YPH500, and YPH501 (Sikorski and Hieter, 1989; see Figures 1B and 2A and Supplemental Figure S2). Δ*fzo1* Δ*mdm36* double deletion mutants were constructed by mating, sporulation, and tetrad dissection or sporulation and tetrad dissection of a heterozygous diploid strain carrying a wild-type *FZO1* allele on plasmid pRS416-*FZO1* (Fritz *et al*., 2001) and subsequent chase of the plasmid by growth on 5-fluoroorotic acid. Strain RJ2194 (Cerveny *et al*., 2007a) carries a deletion of the *DNM1* gene and expresses Num1-RFP from its normal chromosomal locus. A Δ*mdm36* deletion was constructed in this background by replacing the *MDM36* coding region by a *natNT2* cassette (Janke *et al*., 2004; see Figure 6C and Supplemental Figure S4).

Staining of Cellular Structures

Mitochondria, ER, and Dnm1 were visualized in strains transformed with plasmids mentioned above. If not indicated otherwise, living cells embedded in 0.5% low melting point agarose were observed by fluorescence microscopy. In some experiments, cells were fixed by incubation in 4% formaldehyde for 30 min at room temperature and washed two times with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 19 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4). The assay of mitochondrial fusion in vivo was performed essentially as described previously (Fritz *et al*., 2003) with the exception that haploid cells were precultured in YPGal medium. Staining of the vacuole with 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (Invitrogen, Carlsbad, CA) was according to the manufacturer's instructions. Staining of the actin cytoskeleton with rhodamine-phalloidin (Invitrogen) was according to published procedures (Amberg, 1998). For depolymerization of the actin cytoskeleton, cells were grown in 5 ml of YPD medium to mid-logarithmic growth phase (OD_{600} 0.2–0.5), harvested by centrifugation, and resuspended in 1 ml YPD. The actin cytoskeleton was depolymerized by the addition of 5 μl of latrunculin A (2 mM stock solution in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Latrunculin A-treated and mock-treated cells were further incubated for 1 h at 30°C. For staining of the cell wall, cells were harvested from a 10-ml log phase (OD_{600} 0.7–1.0) YPD culture, washed two times in PBS, and resuspended in 0.4 ml of PBS. Then, 8 mg of Sulfo-NHS-LC-Biotin (Roche Diagnostics, Mannheim, Germany) dissolved in 0.3 ml of PBS was added, and the sample was incubated for 15 min at room temperature to allow covalent attachment of biotin to the cell wall. Cells were washed three times in PBS and resuspended in 1 ml of YPD medium. After addition of 1–10 μg of streptavidin-Alexa Fluor 488 conjugate (Invitrogen), cells were incubated for 10–20 min at room temperature. Alternatively, in some experiments cells were washed in 10 mM HEPES/2% glucose buffer, pH 7.2, resuspended in HEPES/glucose buffer and stained with 25 μM calcofluor (Invitrogen) for 15 min at 30°C. Cells were fixed by addition of 4% formaldehyde and incubation for 30 min at room temperature. After two washes in PBS, cells were resuspended in 50–200 μl of PBS.

Microscopy

Epifluorescence microscopy was performed using an Axioplan 2 microscope (Carl Zeiss Lichtmikroskopie, Göttingen, Germany) equipped with a Plan-Neofluar 100×/1.30 Ph3 oil objective (Carl Zeiss Lichtmikroskopie). Images were recorded with an Evolution VF Mono Cooled monochrome camera (Intas, Göttingen, Germany) and processed with Image ProPlus 5.0 and Scope Pro4.5 software (Media Cybernetics, Silver Spring, MD). Confocal microscopy was performed using a TCS SP1 system (Leica Microsystems, Wetzlar, Germany) in combination with an inverted microscope equipped with a 100×/1.40 HCX PL APO oil objective, or by using an SP5 system (Leica Microsystems) in combination with an inverted microscope equipped with a 63×/1.30 GLYC 21°C UV glycerol objective. Electron microscopy and generation of three-dimensional (3D) models of serial ultrathin sections was performed as described previously (Dürr *et al*., 2006). CorelDRAW graphics suite, version 12.0 (Corel, Ottawa, ON, Canada) was used for mounting of the figures; image manipulations other than minor adjustments of brightness and contrast were not performed.

Binding of Mdm36 to Mitochondria In Vitro and Isolation of Subcellular Fractions

In vitro transcription and translation of ^{35}S-labeled Mdm36 was performed using plasmid pGEM3-*MDM36* and TNT SP6-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Mitochondria were isolated from yeast cells by differential centrifugation (Daum *et al*., 1982) and further purified by sucrose gradient centrifugation as described previously (Rowley *et al*., 1994), with the exception that an SW-40 rotor (Beckman Coulter, Fullerton, CA) was used for ultracentrifugation (40,000 rpm for 30 min at 4°C). To analyze binding of Mdm36 to mitochondria, 200 μg of mitochondria was resuspended in 200 μl of import buffer (50 mM HEPES/KOH, pH 7.2, 3% fatty acid-free bovine serum albumin, 0.5 M sorbitol, 80 mM KCl, 10 mM magnesium acetate, 2 mM $MnCl_2$, and 2 mM potassium phosphate, pH 7.2) and incubated for 5 min at 25°C in the presence of 5 mM NADH, 2.5 mM ATP, 10 mM phosphocreatine, and 100 μg/ml creatine kinase. Then, 20 μl in vitro-translated protein was added and incubated for 15 min at 25°C. Mitochondria were reisolated by centrifugation for 10 min at 4°C and 12,000 rpm in a microfuge, washed two times in SEM buffer (250 mM sucrose, 1 mM EDTA, and 10 mM MOPS/KOH, pH 7.2) and resuspended in 500 μl of SEM with 1 mM phenylmethylsulfonyl fluoride. Pretreatment of mitochondria with trypsin and sucrose gradient flotation was as described previously (Fuchs *et al*., 2002), with the exception that ultracentrifugation was performed at 25,000 rpm in an SW60 rotor (Beckman Coulter). Mitochondria were harvested, washed with SEM buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and blotting to nitrocellulose membranes. Mdm36 was detected by autoradiography using BAS-IP MS 2025 imaging

plates, FLA-7000 bio-imaging analyzer, and Multi Gauge, version 3.2 software (Fujifilm, Tokyo, Japan). Control proteins were detected by Western blotting using horseradish peroxidase-coupled secondary antibodies and chemoluminescence detection in a LAS-4000 luminescent image analyzer and Image Reader LAS-4000 software (Fujifilm). For the isolation of subcellular fractions, yeast cells were grown in media containing galactose as a carbon source. Mitochondria were isolated by differential centrifugation. Cytosol was prepared by ultracentrifugation of the postmitochondrial supernatant in a Ti60 rotor (37,000 rpm for 60 min at 4°C; Beckman Coulter). Total cell extract was prepared by vortexing spheroplasts in SDS-PAGE sample buffer and incubation for 5 min at 99°C. Detection of proteins by Western blotting was as described above.

RESULTS

Cells Lacking Mdm36 Contain Net-like Mitochondria

The *MDM36* gene (systematic name *YPR083w*) was discovered by screening 4794 yeast deletion mutants for strains with aberrant mitochondrial distribution and morphology (Dimmer *et al*., 2002). In this study, we reported that Δ*mdm36* mutant cells contained mitochondria that were aggregated at one side of the cell. *MDM36* encodes a protein of 579 amino acids with a molecular mass of 65 kDa. Homologues can be found in many other ascomycete fungi. The protein sequence contains a conserved predicted coiled coil domain between amino acid residues 405 and 450 (Lupas *et al*., 1991) and a serine-rich region of unknown function between residues 466 and 518. We could not identify any predicted transmembrane region, mitochondrial targeting sequence, or additional domains with known functions.

To further characterize the role of Mdm36 in mitochondrial morphogenesis, we carefully analyzed the mitochondrial phenotype. Wild-type and Δ*mdm36* cells expressing mitochondria-targeted GFP were grown to logarithmic growth phase in rich media containing fermentable (YPD) or nonfermentable (YPG) carbon sources and analyzed by fluorescence microscopy. Mitochondria resembling the branched tubular network characteristic for wild-type cells could never be found in Δ*mdm36* mutant cells (Figure 1A). Instead, mitochondria mostly appeared as large interconnected structures. Intriguingly, mitochondria formed extended and highly interconnected nets in ~10% of mutant cells (Figure 1A). These nets were also seen in high-resolution 3D models obtained by electron microscopic analysis of serial ultrathin sections (Figure 1B). They strikingly resemble net-like mitochondria that are characteristic for Δ*dnm1* (Otsuga *et al*., 1998; Bleazard *et al*., 1999), Δ*fis1* (Mozdy *et al*., 2000), and Δ*mdv1* (Tieu and Nunnari, 2000) mutants. Highly interconnected mitochondrial nets are thought to be formed by ongoing mitochondrial fusion that is unopposed by fission. Thus, the mitochondrial phenotype of Δ*mdm36* cells points to a possible role of Mdm36 in mitochondrial division.

To test whether deletion of the *MDM36* gene specifically affects mitochondria, we analyzed the morphology of vacuoles, ER, and actin filaments in wild-type and Δ*mdm36* cells by fluorescence microscopy. The observation that these structures looked normal (Supplemental Figure S1) and that Δ*mdm36* mitochondria did not show any ultrastructural abnormalities (Supplemental Figure S2) is compatible with a role of Mdm36 in mitochondrial division.

Mdm36 and Components of the Mitochondrial Fusion Machinery Regulate Mitochondrial Shape in an Antagonistic Manner

The mitochondrial fusion and fission machineries regulate mitochondrial shape in an antagonistic manner, i.e., fragmentation of the mitochondrial network in fusion-defective mutants can be rescued by deletion of genes encoding components essential for mitochondrial fission (Bleazard *et al*., 1999; Sesaki and Jensen, 1999). To test whether deletion of the *MDM36* gene blocks fragmentation of mitochondria in cells lacking the fusion component Fzo1, we generated Δ*mdm36* Δ*fzo1* double mutants. Because it has been reported that the order of gene deletion may be important (Sesaki and Jensen, 1999), we constructed double mutants both by sequential gene deletion and by sporulation and tetrad dissection of heterozygous diploid cells. In all cases we observed an intermediate mitochondrial phenotype. Although numerous mitochondrial fragments were scattered around the cytoplasm in Δ*fzo1* cells, mitochondria were mostly aggregated in restricted areas of the cell in Δ*mdm36* Δ*fzo1* double mutants (Figure 2A). This suggests that mitochondria are more interconnected in double mutants, although the ability to form wild type-like tubular mitochondria is not restored.

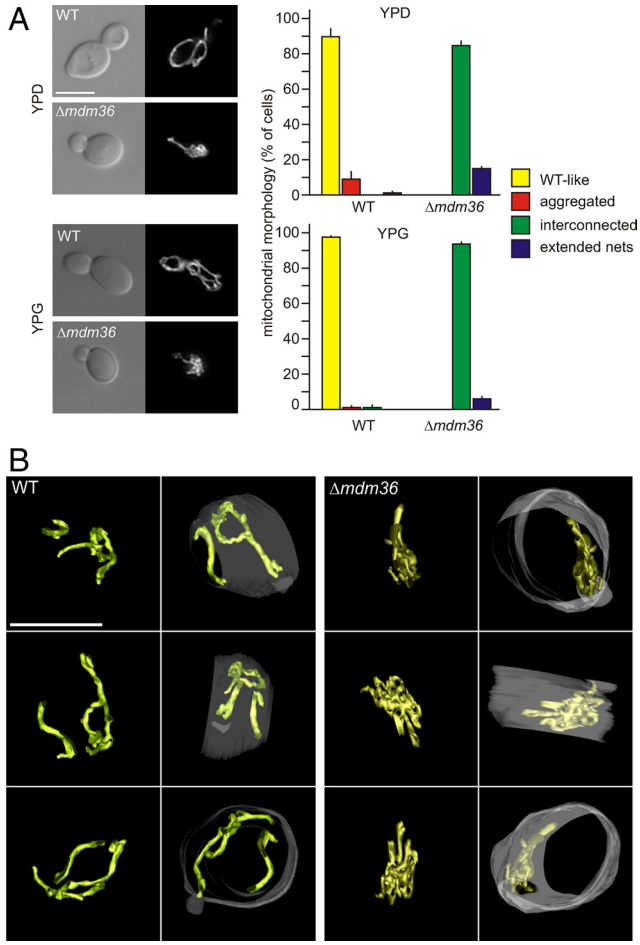

Figure 1. Δ*mdm36* cells contain interconnected and net-like mitochondria. (A) Yeast strains expressing mitochondria-targeted GFP were grown to logarithmic growth phase in glucose- (YPD) or glycerol (YPG)-containing media and analyzed by differential interference contrast and fluorescence microscopy. Left, representative cells. Bar, 5 μm. Right, quantification of mitochondrial phenotypes (error bars represent SDs for three independent experiments with 100 cells per strain and growth condition). (B) Yeast cells were grown to logarithmic growth phase in YPD and analyzed by transmission electron microscopy. The three-dimensional structure of mitochondria of representative cells was reconstructed from 35 consecutive serial ultrathin (70-nm) sections using IMOD 3.13.5 software (Kremer *et al*., 1996). Mitochondria are colored in yellow; images on the right hand side show the cell wall in addition. All images for wild type or Δ*mdm36*, respectively, show the same mitochondrion from different angles. Bar, 5 μm.

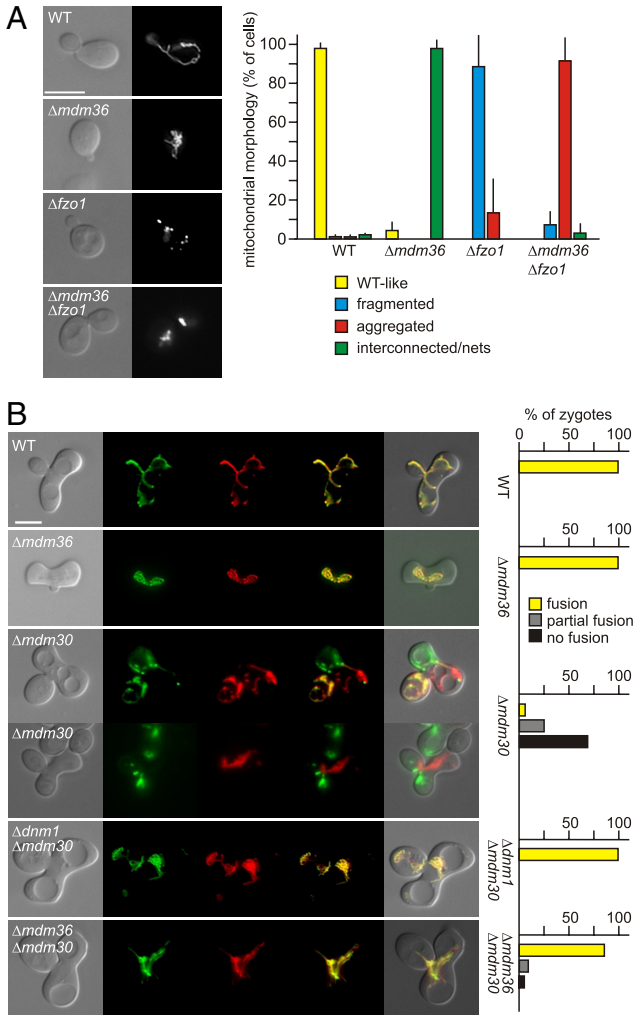

Figure 2. Mdm36 acts antagonistically to mitochondrial fusion components. (A) Yeast cells expressing mitochondria-targeted GFP were grown to logarithmic growth phase in YPD and analyzed by differential interference contrast (DIC) and fluorescence microscopy. Left, representative cells. Bar, 5 μm. Right, quantification of mitochondrial phenotypes (error bars represent SDs for three independent experiments with 100 cells per strain and growth condition). (B) Yeast cells of opposite mating types containing mitochondria preloaded either with GFP or RFP were mated, and zygotes were analyzed by DIC and fluorescence microscopy. For Δ*mdm30*, a representative zygote containing partially fused mitochondria, and a representative zygote containing nonfused mitochondria is shown. Images from left to right: DIC; green fluorescence; red fluorescence; merge of green and red fluorescence; merge of DIC, green and red fluorescence. Bar, 5 μm. Right, quantifications of 20–60 zygotes per strain.

Δ*fzo1* mutants are unable to maintain the mitochondrial genome as a secondary consequence of mitochondrial fragmentation (Hermann *et al*., 1998; Rapaport *et al*., 1998; Merz and Westermann, 2009). Although mitochondrial DNA can be stably maintained in Δ*dnm1* Δ*fzo1* double mutants (Bleazard *et al*., 1999; Sesaki and Jensen, 1999), we observed rapid loss of the mitochondrial genome in Δ*mdm36* Δ*fzo1* double mutants (our unpublished observations). Thus, deletion of the *MDM36* gene rescues the Δ*fzo1* mutant phenotype only partially.

We reasoned that full rescue of Δ*fzo1* would require a complete block of mitochondrial fission and that there might be some residual division activity in Δ*mdm36* cells. Possibly, antagonistic roles in fusion and fission might become more clearly apparent in mutants displaying a partial block of mitochondrial fusion. The *MDM30* gene encodes an F-box protein that regulates the turnover of Fzo1 (Fritz *et al*., 2003; Cohen *et al*., 2008). Mitochondria are fragmented in Δ*mdm30* cells but capable to maintain mitochondrial DNA. Accumulation of Fzo1 in Δ*mdm30* mutant cells results in a severe defect of mitochondrial fusion, as is indicated by defective mitochondrial content mixing in zygotes. Intriguingly, mitochondrial content mixing is restored in Δ*mdm30* Δ*dnm1* double mutants (Fritz *et al*., 2003). This can be explained by the presence of a greatly reduced, albeit not completely blocked, fusion activity in cells lacking Mdm30. If the mitochondrial network is fully interconnected in both mating partners, one single fusion event is sufficient to allow complete content mixing in the zygote, whereas several rounds of fusion are required in cells with intact fission machineries. To test whether similar effects can be observed in cells lacking Mdm36, we examined mitochondrial fusion by fluorescence microscopy of zygotes obtained by mating of haploid yeast cells preloaded with matrix-targeted GFP or RFP. As expected, complete mixing of mitochondrial contents was observed in wild-type and Δ*mdm36* zygotes (Figure 2B). Consistent with previous observations (Fritz *et al*., 2003) 69% of Δ*mdm30* zygotes showed a complete and 25% a partial block of fusion, whereas efficient content mixing could be observed in only 6% of Δ*mdm30* zygotes. Mitochondrial content mixing was fully restored by deletion of *DNM1*. Remarkably, also deletion of *MDM36* restored content mixing completely in 86% of zygotes lacking Mdm30. Content mixing occurred partially in 9% and failed in only 5% of Δ*mdm36* Δ*mdm30* double mutant zygotes (Figure 2B). This suggests that the mitochondrial matrix is highly interconnected in cells lacking Mdm36, because residual fusion activity in the absence of Mdm30 is sufficient to allow efficient intermixing of mitochondrial matrix contents in these cells. We conclude that Mdm36 acts antagonistically to fusion-promoting components.

Mdm36 Acts in the Mitochondrial Fission Pathway

Next, we asked whether *MDM36* genetically interacts with genes encoding components of the mitochondrial fission machinery. The Δ*mdm36* mutant does not show any detectable growth defect on fermentable or nonfermentable carbon sources. In this respect, it resembles Δ*dnm1*, Δ*fis1*, Δ*mdv1*, and Δ*caf4* mutants and also a Δ*mdm36* Δ*dnm1* double mutant grows like wild type (our unpublished observations). However, we noticed subtle but significant differences in the mitochondrial phenotypes of Δ*mdm36* and Δ*dnm1*. The majority of Δ*mdm36* cells contain interconnected mitochondria located in restricted areas of the cell, whereas only ~10% of the cells contain extended nets (Figure 3A). In contrast, extended mitochondrial nets are the predominant phenotype in Δ*dnm1* cells. Δ*mdm36* Δ*dnm1* double mutants are indiscernible of Δ*dnm1* (Figure 3A), suggesting that *dnm1* mutations are epistatic to *mdm36*. This is compatible with the view that Dnm1 is an essential mitochondrial division component, whereas Mdm36 might play an accessory role.

Num1 was first described as a cell cortex-associated protein required for control of nuclear migration by affecting the orientation of the mitotic spindle (Kormanec *et al*., 1991; Farkasovsky and Küntzel, 1995). During our screen of the deletion library, we observed that Δ*num1* mutants display highly aggregated mitochondria (Dimmer *et al*., 2002), and Cerveny *et al*. (2007a) showed that *num1* and *dnm1* genetically interact, that Δ*num1* mutants contain an interconnected mitochondrial network resembling Δ*dnm1*, and that subfrac-

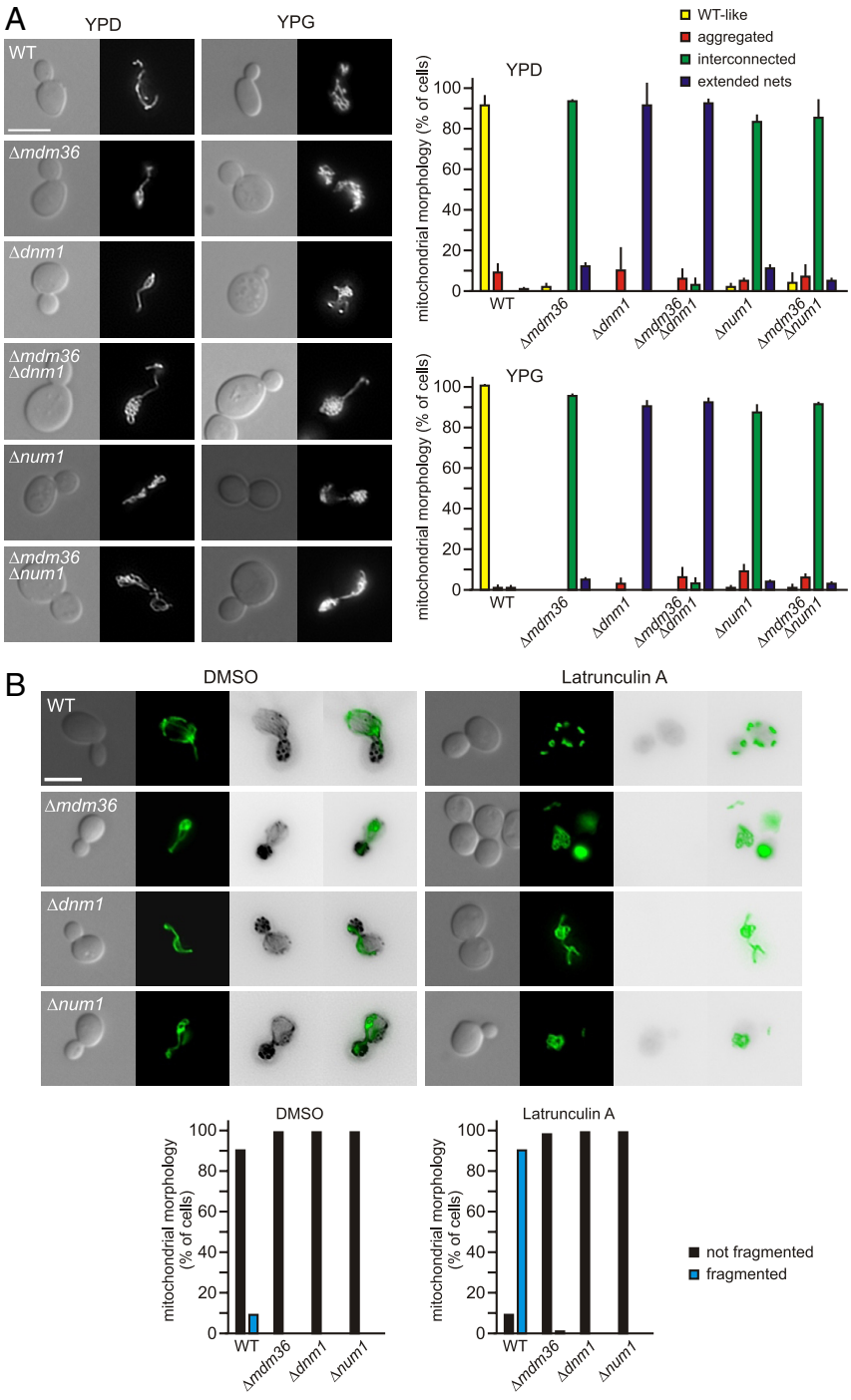

Figure 3. Mdm36 is required for mitochondrial division. (A) Yeast strains expressing mitochondria-targeted GFP were grown to logarithmic growth phase in glucose- (YPD) or glycerol (YPG)-containing media and analyzed by differential interference contrast (DIC) and fluorescence microscopy. Left, representative cells. Bar, 5 μm. Right, quantification of mitochondrial phenotypes (error bars represent SDs for three independent experiments with 100 cells per strain and growth condition). (B) Yeast cells were grown to logarithmic growth phase in glucose-containing medium (YPD) and either mock-treated with DMSO (left) or treated with 10 μM latrunculin A for 1 h at 30°C. Cells were analyzed by fluorescence microscopy after rhodamine-phalloidin staining. Top, representative cells. Images from left to right: DIC, green fluorescence (mitochondria-targeted GFP), red fluorescence (rhodamine-stained actin shown as a reversed black and white image to better visualize faint actin cables and patches), merged image of GFP and rhodamine staining. Bar, 5 μm. Bottom, mean values of two independent experiments with 100 cells per sample.

tions of Num1 and Dnm1 coassemble in punctate structures. These observations point to a role of Num1 in Dnm1-dependent mitochondrial division (Cerveny *et al*., 2007a). We observed that Δ*mdm36* and Δ*num1* single mutants and a Δ*mdm36* Δ*num1* double mutant show an indiscernible mitochondrial phenotype (Figure 3A), suggesting that Mdm36 and Num1 affect mitochondrial division in a similar manner.

To obtain more direct evidence for a role of Mdm36 in mitochondrial division, we assayed mitochondrial fragmentation upon depolymerization of the actin cytoskeleton. Treatment of yeast cells with the actin-depolymerizing drug latrunculin A induces rapid fragmentation of mitochondrial tubules in wild-type cells (Boldogh *et al*., 1998). This fragmentation depends on the mitochondrial division machinery, because it is blocked in Δ*dnm1* and Δ*mdv1* mutants (Jensen *et al*., 2000; Cerveny *et al*., 2001). Wild-type, Δ*mdm36*, Δ*dnm1*, and Δ*num1* cells expressing mitochondria-targeted GFP were grown to logarithmic growth phase and either mock-treated or treated with latrunculin A. Depolymerization of the actin cytoskeleton was controlled by staining with rhodamine phalloidin, and cells were analyzed by fluorescence microscopy (Figure 3B). We observed fragmentation of mitochondria in ~90% of wild-type cells. Fragmentation was efficiently blocked in Δ*dnm1* and Δ*mdm36* cells, suggest-

ing that Mdm36 is critical for mitochondrial division. Although Cerveny *et al*. (2007a) observed some fragmentation of mitochondria in latrunculin A-treated Δ*num1* cells and ascribed it to residual division activity in this strain, we observed a complete block of mitochondrial fragmentation in Δ*num1* (Figure 3B). We conclude that Mdm36 is critical for mitochondrial division in yeast, similar to Dnm1 and Num1.

Mdm36 Associates with Mitochondria

Because Mdm36 is involved in mitochondrial fission, we considered the possibility that it at least transiently associates with mitochondria. Because Mdm36 apparently is a low abundant protein it is not possible to detect GFP fusion proteins (Huh *et al*., 2003) or epitope-tagged proteins expressed from the endogenous promoter (our unpublished observations). To test whether the Mdm36 protein has the capacity to interact with mitochondria, we synthesized radiolabeled Mdm36 by in vitro translation and incubated it with isolated wild-type mitochondria. Because it was not possible to import the protein into a protease-protected location (our unpublished observations), we tested whether it peripherally associates with mitochondria. Isolated mitochondria were incubated with radiolabeled Mdm36 and floated in a sucrose gradient. A small but significant amount of Mdm36 floated together with the organelles to the top of the gradient indicating binding to mitochondria (Figure 4A). This association was dependent on mitochondrial proteins, because removal of surface-exposed proteins by pre-treatment of mitochondria with trypsin abolished binding of Mdm36 to mitochondria (Figure 4A). Mitochondrial association did not require preexisting Mdm36 or mitochondria-associated Dnm1 or Num1, because it was also observed with mitochondria isolated from the respective deletion mutants (Figure 4A). To obtain a second line of evidence for a mitochondrial association of Mdm36 we overexpressed an epitope-tagged variant (Mdm36-HA) from the *GAL1* promoter (Gelperin *et al*., 2005) and analyzed subcellular fractions by Western blotting. Mdm36-HA was detected with antibodies directed against the HA epitope in mitochondria isolated from Mdm36-HA–expressing cells (Figure 4B). A minor amount of Mdm36-HA was also found in the cytosol (Figure 4B). We conclude that Mdm36 has the capacity to interact with mitochondria in a receptor-dependent manner. The relatively low efficiency of this interaction in flotation experiments and the presence of some Mdm36-HA in cytosolic fractions suggest that mitochondrial binding of Mdm36 is rather weak or transient.

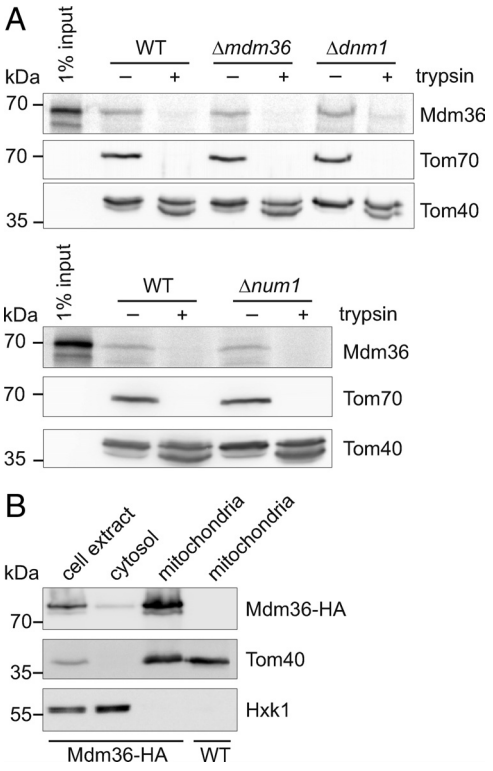

Figure 4. Mdm36 binds to mitochondria. (A) Isolated sucrose gradient-purified mitochondria were either pretreated with trypsin (+) or left untreated (−) and then incubated with in vitro-translated Mdm36 and floated by sucrose density gradient centrifugation. Mdm36 was detected by SDS-PAGE, blotting to nitrocellulose, and autoradiography. The mitochondrial outer membrane proteins Tom70 and Tom40 served as controls for efficient trypsin treatment and were detected by immunoblotting. Tom40 forms a characteristic fragment upon trypsin treatment. (B) Cells overexpressing Mdm36 fused to a His_6/HA/protein A tag (Mdm36-HA) under control of a *GAL1* promoter were grown in galactose-containing medium, and total cell extract, cytosol and mitochondria were prepared. Similar amounts of protein of each fraction were analyzed by immunoblotting using antibodies against the HA epitope (Mdm36-HA) Tom40 and the cytosolic protein hexokinase (Hxk1). Mitochondria isolated from a *MDM36* wild-type strain lacking the HA epitope were analyzed (WT) as a control for specificity of the HA antibody.

Cells Lacking Mdm36 Have a Reduced Number of Dnm1 Clusters at Mitochondrial Tips

Dnm1 assembles into dynamic punctate structures on the mitochondrial surface to mediate membrane fission (Otsuga *et al*., 1998; Bleazard *et al*., 1999; Sesaki and Jensen, 1999; Legesse-Miller *et al*., 2003). Previous studies have shown that assembly of Dnm1 on mitochondria is reduced—albeit not blocked completely—in mutants lacking Fis1, Mdv1, or Caf4 (Mozdy *et al*., 2000; Tieu and Nunnari, 2000; Schauss *et al*., 2006). To test whether assembly of Dnm1-containing division complexes on mitochondria is compromised in the absence of Mdm36 or Num1, we analyzed wild-type, Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* cells expressing Dnm1-GFP from its normal chromosomal locus. Mitochondria were stained with mitochondria-targeted RFP, strains were grown on glucose- and glycerol-containing media, and at least 50 cells per strain were analyzed by confocal microscopy. We observed efficient assembly of Dnm1-GFP on mitochondria in all strains tested (Figure 5A). After completion of division, Dnm1 complexes remain transiently associated with one tubular tip that has been generated as a product of the division reaction (Legesse-Miller *et al*., 2003). Careful inspection of the confocal image stacks revealed a reduced number of Dnm1 complexes at mitochondrial tips in Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* cells (Figure 5B and Supplemental Table S1). Because association of Dnm1 with a free mitochondrial tip in most cases probably is a product of a division event, these observations point to a reduced activity of the Dnm1-containing mitochondrial division machinery in cells lacking Mdm36 or Num1.

Attachment of Mitochondria to the Cell Cortex Is Impaired in Cells Lacking Mdm36

Num1 is a large 313-kDa protein that is anchored via its pleckstrin homology domain to the cell cortex (Kormanec *et al*., 1991; Farkasovsky and Küntzel, 1995). It has been suggested that Num1 might affect mitochondrial morphology by acting as a cortical anchor for mitochondria-associated Dnm1 (Cerveny *et al*., 2007a; Schauss and McBride, 2007).

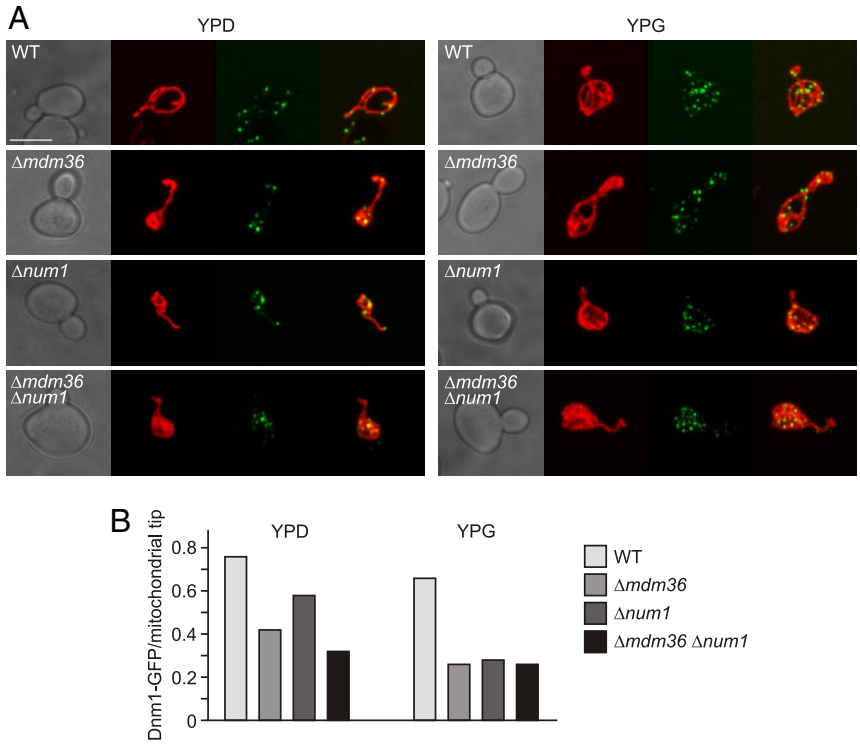

Figure 5. Association of Dnm1-GFP with free mitochondrial ends is reduced in the absence of Mdm36 and Num1. (A) Yeast strains coexpressing mitochondria-targeted RFP and Dnm1-GFP were grown to logarithmic growth phase in glucose- (YPD) or glycerol (YPG)-containing media. Then, cells were fixed and analyzed by confocal microscopy. Maximum intensity projections of confocal z-stacks of representative cells are shown. Images from left to right: bright field, red channel (mitochondria), green channel (Dnm1-GFP), merged red and green image. Bar, 5 μm. (B) At least 50 cells per strain were analyzed by inspection of single frames of confocal z-stacks for the presence of Dnm1-GFP spots located at free mitochondrial tips. The graph shows an excerpt of data contained in Supplemental Table S1.

Because the Δ*mdm36* and Δ*num1* mutants show very similar mitochondrial phenotypes, we asked whether attachment of mitochondria to the cell cortex might be compromised in the absence of Mdm36. First, we analyzed wild-type, Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* cells by 3D confocal time-lapse microscopy. We recorded z-stacks of cells expressing mitochondria-targeted GFP every ~7.2 s over a period of ~6 min. These data revealed that mitochondrial shape changes occurred more frequently and were more pronounced in Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* mutants than in wild-type cells (Supplemental Figure S3 and Supplemental Videos 1–8). Furthermore, two-dimensional traces of mitochondrial tips covered a larger area in the mutants than in wild type (Figure 6A) and revealed a higher mean velocity in Δ*mdm36* (40.5 ± 10.4 nm/s), Δ*num1* (44.4 ± 10.0 nm/s), and Δ*mdm36* Δ*num1* (39.1 ± 7.6 nm/s) compared with wild-type (26.6 ± 5.7 nm/s) cells. Intriguingly, impetuous mitochondrial movement could be efficiently blocked by treatment of cells with latrunculin A (Supplemental Videos 9–12), suggesting that it is dependent on the actin cytoskeleton. These observations reveal an increased actin-dependent motility of mitochondria in Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* strains.

Next, we analyzed the alignment of mitochondria with the cell cortex in Δ*mdm36*, Δ*num1*, Δ*mdm36* Δ*num1*, and wild type cells. We stained the cell wall of mitochondria-targeted RFP-expressing cells by biotin labeling and addition of Alexa Fluor 488-conjugated streptavidin or by calcofluor staining. Then, we recorded confocal z-stacks consisting of 30 planes per cell, and we used 14 planes covering the volume around the cell equator for generation of maximum intensity projections. These data show that a large part of the mitochondrial network is localized close to the cell periphery in wild-type cells, whereas mitochondria are predominantly seen in the cell center in Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* mutants (Figure 6B). These observations suggest that connections of mitochondria and the cell cortex have largely been lost in Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* cells. A weakened cell cortex attachment is consistent with the increased mitochondrial motility described above. The fact that the phenotype of the Δ*mdm36* Δ*num1* double deletion strain phenocopies that of the single deletions is compatible with a common function of Mdm36 and Num1 in the same cellular pathway.

It has been shown that a subfraction of Dnm1-GFP spots colocalizes with Num1-RFP and that this interaction may be important for attachment of mitochondria to the cell cortex (Cerveny *et al*., 2007a). We asked, whether Mdm36 promotes the association of Dnm1 and Num1. To test this, we analyzed Δ*mdm36* and wild-type cells coexpressing Dnm1-GFP and Num1-RFP by fluorescence microscopy. Consistent with the results reported by Cerveny *et al*. (2007a), we observed a significant colocalization of Dnm1-GFP and Num1-RFP in the presence of Mdm36. In contrast, Dnm1-GFP spots could only rarely be found in the vicinity of Num1-RFP in Δ*mdm36* cells (Figure 6C and Supplemental Figure S4). Together, these data suggest that Mdm36 plays an important role for the attachment of mitochondria to the cell cortex via Dnm1 and Num1.

DISCUSSION

Several lines of evidence suggest that Mdm36 is a novel component required for mitochondrial fission. First, highly interconnected net-like mitochondria characteristic for fission mutants are found in Δ*mdm36* cells. Second, Mdm36 acts antagonistically to components of the fusion machinery, because mitochondrial fragmentation in Δ*fzo1* mutants is alleviated by deletion of the *MDM36* gene, and mitochondrial content mixing is largely restored in Δ*mdm30* Δ*mdm36* double mutants. Third, mitochondrial fragmentation induced by depolymerization of the actin cytoskeleton is blocked in Δ*mdm36* mutants. And fourth, the number of Dnm1-containing division complexes at mitochondrial tips is reduced. Together, these data provide strong evidence for

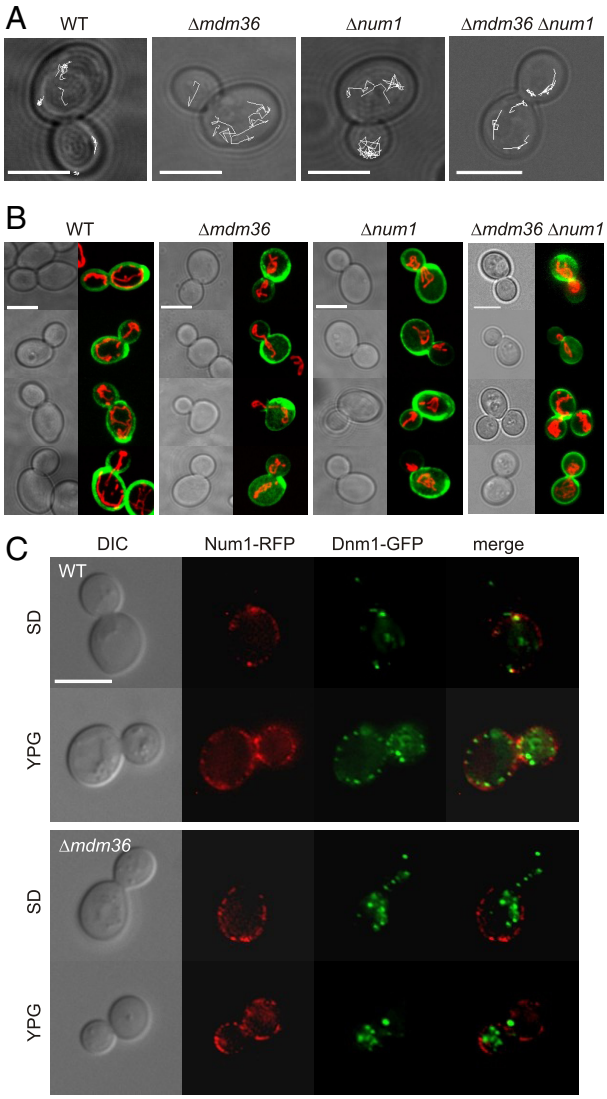

Figure 6. Mitochondrial motility, attachment to the cell cortex and Dnm1/Num1 colocalization are altered in the absence of Mdm36. (A) Yeast cells expressing mitochondria-targeted GFP were grown to logarithmic growth phase in YPD and analyzed by confocal microscopy. For each cell, 50 confocal z-stacks consisting of 10 confocal planes were recorded over a period of 355 s (i.e., one z-stack was taken every ~7.2 s). Movements of free mitochondrial tips were traced in maximum intensity projections using ImageJ 1.41 software (Abramoff *et al*., 2004) and highlighted in bright field images. The traces represent five mitochondrial tips in the wild-type, Δ*num1*, and Δ*mdm36* Δ*num1* cell, and six tips in Δ*mdm36*. Bars, 5 μm. Maximal intensity projections used to generate these data are shown in Supplemental Figure S3 and Supplemental Videos 1, 3, 5, and 7. Supplemental Videos 2, 4, 6, and 8 show additional representative cells. (B) Yeast cells expressing mitochondria-targeted RFP were grown to logarithmic growth phase in YPD. The cell wall was labeled by covalent attachment of biotin and addition of Alexa Fluor 488-conjugated streptavidin or calcofluor staining. Then, cells were fixed and analyzed by confocal microscopy. For each cell, confocal z-stacks consisting of 30 planes were recorded (covering 3.83–8.26 μm in *z*-axis, depending on the size of the cell). Maximal intensity projections were generated using 14 planes located around the cell equator (covering 1.79–3.86 μm in *z*-axis). Left, bright field image; right, merged image of mitochondria (red) and cell wall (green) fluorescence. Bars, 5 μm. (C) Yeast cells expressing Dnm1-GFP and Num1-RFP were grown to logarithmic growth phase in glucose- (SD) or glycerol (YPG)-containing media and analyzed by differential interference contrast and epifluorescence microscopy. Bar, 5 μm. Additional representative cells are shown in Supplemental Figure S4.

a role of Mdm36 in mitochondrial fission. However, several observations indicate that Dnm1 retains some residual activity in the absence of Mdm36. Extended mitochondrial networks are less frequently found in Δ*mdm36* cells than in Δ*dnm1*; mitochondrial fragmentation is only partially alleviated in Δ*fzo1* Δ*mdm36* double mutants; and Dnm1-GFP assembles efficiently on Δ*mdm36* mitochondria. Thus, Mdm36 apparently is an accessory component rather than an essential part of the mitochondrial division machinery.

Although clearly distinguishable from Δ*dnm1*, the mitochondrial phenotype of Δ*mdm36* strikingly resembles that of Δ*num1*, suggesting that Mdm36 and Num1 might act in the same cellular pathway. Num1 was first described as a cell cortex-associated protein that affects nuclear migration and orientation of the mitotic spindle (Kormanec *et al*., 1991; Farkasovsky and Küntzel, 1995). First evidence for a role in mitochondrial dynamics came from the discovery that Δ*num1* cells contain abnormal mitochondria (Dimmer *et al*., 2002) and that the Num1 protein can be found in the mitochondrial proteome (Sickmann *et al*., 2003). Cerveny *et al*. (2007a) showed that *NUM1* is a high copy suppressor of certain *dnm1* alleles and that Num1 physically interacts with Dnm1, establishing a role of Num1 in mitochondrial fission. Furthermore, the accumulation of mitochondria in buds of Δ*dnm1* Δ*num1* double mutant cells points to a role of a Num1/Dnm1 complex as an anchor for mitochondria at the cell cortex that ensures retention in the mother cell during cell division (Cerveny *et al*., 2007a). Consistent with such a role, a large number of Dnm1 clusters on mitochondria are oriented toward the cell cortex (Schauss *et al*., 2006).

Three observations point to a role of Mdm36 in the formation of mitochondrial cell cortex attachment points. First, mitochondria are more motile in Δ*mdm36*, Δ*num1*, and Δ*mdm36* Δ*num1* cells; second, the localization of mitochondria near the cell cortex is lost in the absence of Mdm36 and/or Num1; and third, the colocalization of Dnm1 and Num1 is largely abolished in the absence of Mdm36. Together, these data argue that the joint activity of Mdm36, Num1, and Dnm1 anchors mitochondria at the cell cortex and that this activity constrains free mitochondrial movement and migration toward the cell center.

Although a cell cortex anchor conceivably plays an important role for equal partitioning of mitochondria during cell division (Cerveny *et al*., 2007a; Schauss and McBride, 2007), its function in mitochondrial fission is less obvious. Interestingly, generation of tension on membranes is critical for membrane fission by dynamin (Roux *et al*., 2006). Thus, it has been suggested that the Num1/Dnm1 cell cortex anchor together with actin dynamics generates tension on mitochondria that is used by the Dnm1/Fis1/Mdv1 machinery for membrane fission (Schauss and McBride, 2007; Lackner and Nunnari, 2009). Our results support this model and add Mdm36 as a new player to this process. In this scenario, a complex containing Dnm1 and Num1 attaches mitochondrial tubules to the cell cortex. It remains unknown whether Mdm36 is an integral part of this complex and whether it directly interacts with cell cortex components, because GFP fusions cannot be detected in vivo (Huh *et al*., 2003) and numerous attempts to generate specific antibodies failed (our unpublished observations). However, the lack of colocalization of Num1 and Dnm1 in Δ*mdm36* indicates that Mdm36 is required for formation of this complex. The observation that increased mitochondrial motility in cells lacking Mdm36 or Num1 is actin dependent is consistent with the view that cell cortex attachment points provide a counterforce for cytoskeleton-dependent processes pulling on mitochondria, such as mitochondria-associated myosin mo-

tors (Altmann *et al*., 2008) and/or retrograde flow of actin cables (Fehrenbacher *et al*., 2004). Tension generated by this mechanism together with constriction of mitochondrial tubules by Dnm1/Fis1/Mdv1 then contributes to the severing of mitochondrial membranes. According to this model, the absence of Mdm36 leads to the loss of mitochondrial cell cortex attachment points. As a consequence, mitochondria move more freely within the cell, have an elevated velocity and a higher tendency for a localization away from the cell cortex. Furthermore, the efficiency of the Dnm1/Fis1/Mdv1 fission machinery is probably attenuated in Δ*mdm36* cells because of reduced tension on mitochondria. Therefore, the number of Dnm1-GFP clusters at mitochondrial tips (i.e., products of successful division events) is reduced in the absence of Mdm36 and Δ*mdm36* cells show phenotypes characteristic of fission mutants. In sum, our studies establish an important role for Mdm36 in mitochondrial distribution and morphology. We propose that the activity of Mdm36 contributes to a functional link of mitochondrial motility and division. It will be interesting to see whether similar mechanisms exist in division of mitochondria in mammalian cells.

ACKNOWLEDGMENTS

We thank Hiromi Sesaki (Johns Hopkins University School of Medicine), Stefan Jakobs (Max-Planck-Institut für Biophysikalische Chemie), Kai Stefan Dimmer (Universität Tübingen), and Mark Dürr (Universität Bayreuth) for yeast strains and plasmids; Stefan Geimer and Rita Grotjahn for help with electron microscopy; Annette Suske for technical support; Stefan Jakobs and Johannes Förtsch for help with life cell microscopy; and the students Yvonne Baberowski, Jan Fiedler, Patricia Händle, Marisa Schäffner, Bastian Seubert, and Torsten Winkler for contributions during the initial phase of this work. We thank the Deutsche Forschungsgemeinschaft for financial support through grants 2174/4-1 and 4-2.

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