

Puf3p, a Pumilio family RNA binding protein, localizes to mitochondria and regulates mitochondrial biogenesis and motility in budding yeast

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Puf3p binds preferentially to messenger RNAs (mRNAs) for nuclear-encoded mitochondrial proteins. We find that Puf3p localizes to the cytosolic face of the mitochondrial outer membrane. Overexpression of *PUF3* results in reduced mitochondrial respiratory activity and reduced levels of Pet1 23p, a protein encoded by a Puf3p-binding mRNA. Puf3p levels are reduced during the diauxic shift and growth on a nonfermentable carbon source, conditions that stimulate mitochondrial biogenesis. These findings support a role for Puf3p in mitochondrial biogenesis through effects on mRNA interactions. In addition,

Puf3p links the mitochore, a complex required for mitochondrial–cytoskeletal interactions, to the Arp2/3 complex, the force generator for actin-dependent, bud-directed mitochondrial movement. Puf3p, the mitochore, and the Arp2/3 complex coimmunoprecipitate and have two-hybrid interactions. Moreover, deletion of *PUF3* results in reduced interaction between the mitochore and the Arp2/3 complex and defects in mitochondrial morphology and motility similar to those observed in Arp2/3 complex mutants. Thus, Puf3p is a mitochondrial protein that contributes to the biogenesis and motility of the organelle.

Introduction

The Pumilio-Fem-3 binding factor (PUF) proteins are defined by the presence of a Pumilio (PUM-HD) domain. This domain is crucial for PUF protein function and has the capacity to bind to the 3' untranslated region (UTR) of mRNAs and to regulate transcript localization, translation, and/or decay (Zamore et al., 1997; Wickens et al., 2002). PUF proteins are found in eukaryotic cells, from yeast to humans. In *Drosophila melanogaster*, the PUMILIO protein binds to *hunchback* mRNA, to repress its translation at the posterior pole during early embryogenesis (Wreden et al., 1997). In *Caenorhabditis elegans*, PUF proteins regulate the switch from spermatogenesis to oogenesis through effects on *fem-3* translation (Zhang et al., 1997), and germ line stem cell propagation through effects on *gld-1* expression (Crittenden et al., 2002). Recent studies indicate that the PUF proteins of *Saccharomyces cerevisiae* are components of “posttranscriptional operons,” punctate, cytoplasmic structures that interact with RNAs encoding proteins that localize to the same subcellular location, are part of the same

protein complex, or act in the same cellular pathway (Gerber et al., 2004).

Our recent studies support an unexpected role for Jsn1p/Puf1p in mitochondrial motility and inheritance in budding yeast. During cell division, equal segregation of mitochondria between the mother cell and bud occurs by regulated, region-specific mobilization and immobilization of the organelle. That is, mitochondria are actively transported to the opposite poles of the yeast cell, i.e., the bud tip and mother cell tip. During poleward movement, mitochondria exhibit linear movement either in the anterograde direction, toward the bud tip, or in the retrograde direction, toward the mother cell tip. Thereafter, they are retained at the poles until the end of the cell division cycle (for review see Boldogh et al., 2005). The poleward movement of mitochondria during inheritance occurs using actin cables, bundles of F-actin that align along the mother-bud axis and serve as tracks for movement (Simon et al., 1997; Fehrenbacher et al., 2004; Pruyne et al., 2004). Binding of mitochondria to F-actin in vitro and association of mitochondria with actin cables during poleward movement and inheritance in vivo require the mitochore, an integral mitochondrial membrane protein complex consisting of the proteins Mmm1p,

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Abbreviations used in this paper: mtDNA, mitochondrial DNA; PUF, Pumilio-Fem-3 binding factor; UTR, untranslated region.

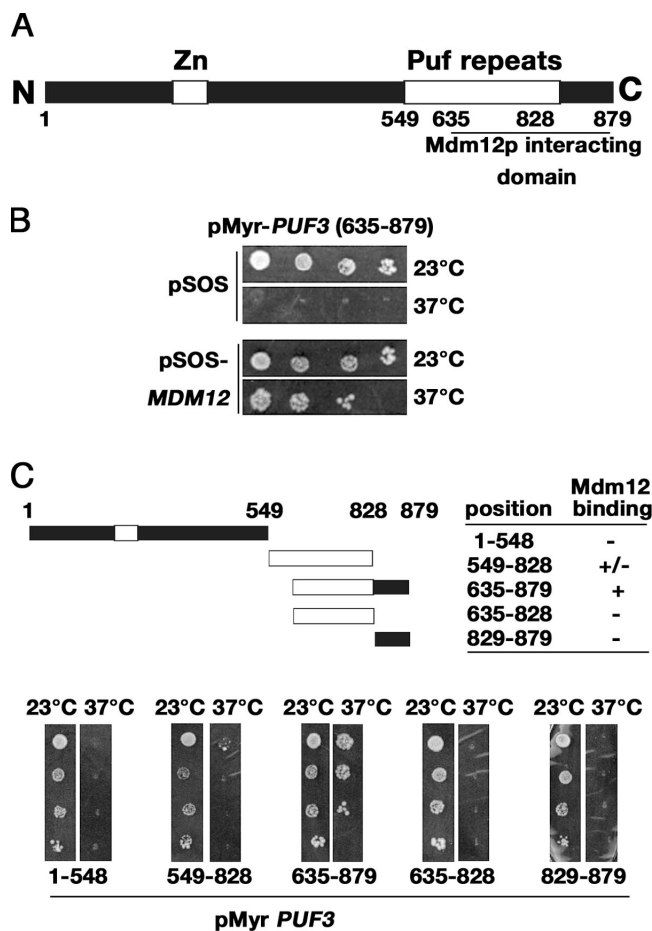


Figure 1. Puf3p interacts with Mdm12p. (A) Sequence elements of Puf3p. The Puf repeats region is represented as a white rectangle (aa 549–828). (B) The yeast *cdc25H* was transformed with the pMyr-PUF3 (635–879) plasmid in combination with either the pSOS-MDM12 or the pSOS plasmid. Serial dilutions of transformants were plated onto SCGal (-Leu-Ura) plates. Cells were grown at 23 or 37°C for 7 d. (C) The yeast *cdc25H* was transformed with the pMyr-PUF3 plasmids, which encode various truncated forms of PUF3 and pSOS-MDM12. Serial dilutions of transformants were plated onto SCGal plates. Cells were grown at 23 or 37°C for 7 d.

Mdm10p, and Mdm12p (Boldogh et al., 1998, 2003; Fehrenbacher et al., 2004).

Our studies support a role for Jsn1p/Puf1p in recruiting the Arp2/3 complex to mitochondria, for force generation during anterograde movement along actin cables. In budding yeast, the Arp2/3 complex localizes to endosomes (Moreau et al., 1996; Huckaba et al., 2004) and mitochondria (Boldogh et al., 2001, 2005), where it stimulates actin nucleation and generates force for intracellular movement. Mitochondria-associated Arp2/3 complex is required for normal mitochondrial morphology and for anterograde, but not retrograde, movement of the organelle during inheritance (Boldogh et al., 2001; Fehrenbacher et al., 2004). Jsn1p/Puf1p localizes to the cytoplasmic face of the mitochondrial outer membrane and interacts with mitochondria-associated Arp2/3 complex. Moreover, deletion of *JSN1* results in a decreased association of Arp2/3 complex with mitochondria, defects in mitochondrial morphology, and reduced levels of anterograde, but not retrograde, mitochondrial movement (Fehrenbacher et al., 2005). Together, these studies support

the model that Jsn1p contributes to recruiting the Arp2/3 complex to mitochondria in budding yeast. This, in turn, allows for Arp2/3 complex-mediated actin polymerization and force production for anterograde movement of mitochondria, using actin cables as tracks, from mother cells to buds during yeast cell division.

In the current work, we found that another PUF protein, Puf3p, interacts with the machinery for mitochondrial motility and inheritance. Previous studies indicate that Puf3p binds preferentially to mRNAs for nuclear-encoded mitochondrial proteins. Specifically, 87% of the 154 transcripts that bind to Puf3p encode proteins that localize to mitochondria and contribute to mitochondrial protein synthesis, respiration, organization, and/or biogenesis. Moreover, Pum-HD of Puf3p binds to a consensus motif in the 3' UTR of mRNAs that is found in the 3' UTR of many nuclear-encoded mitochondrial proteins (Gerber et al., 2004). Other lines of evidence indicate that Puf3p can promote the deadenylation (polyA-tail shortening) and decay of *COX17* mRNA in vitro and that deletion of *PUF3* results in a decrease in mRNA deadenylation and a twofold increase in the half-life of *COX17* mRNA in vivo (Olivas and Parker, 2000; Foat et al., 2005). These findings support the model that Puf3p affects mitochondrial biogenesis through effects on the stability and/or targeting of mRNAs for nuclear-encoded mitochondrial proteins. Here, we report that Puf3p localizes to mitochondria, where it regulates not only mitochondrial biogenesis but also mitochondrial motility during inheritance.

Results

Identification of Puf3p as an Mdm12p-interacting protein

Conventional two-hybrid screens have had limited success identifying proteins that interact with integral membrane proteins. Because mitochondria subunits are integral membrane proteins, we used an unconventional two-hybrid screen that tests for protein-protein interactions at the plasma membrane (Aronheim et al., 1994; Aronheim, 1997). The system takes advantage of the fact that Ras must be activated at the plasma membrane to stimulate cell proliferation by using a strain, *cdc25H*, that carries a temperature-sensitive mutation in *CDC25*, the guanyl nucleotide exchange factor for Ras. Incubation of *cdc25H* at 37°C results in a loss of Cdc25p function and traps Ras in its inactive GDP-bound form, producing growth arrest. Expression and targeting of hSos, the human guanyl nucleotide exchange factor for Ras, to the plasma membrane rescues the growth defect of the *cdc25H* strain at 37°C.

We fused the bait—full-length Mmm1p, Mdm10p, or Mdm12p—to hSos, and the target, a yeast cDNA library, to the plasma membrane-targeting myristylation signal of Src (see Materials and methods). If fusion proteins containing the bait and target interact when coexpressed in the *cdc25H* strain, hSos is recruited to the plasma membrane, where it activates the Ras pathway and supports growth at 37°C. We did not detect any reproducible two-hybrid interactions with Mmm1p or Mdm10p as bait. However, expression of *MDM12*-hSos resulted in growth

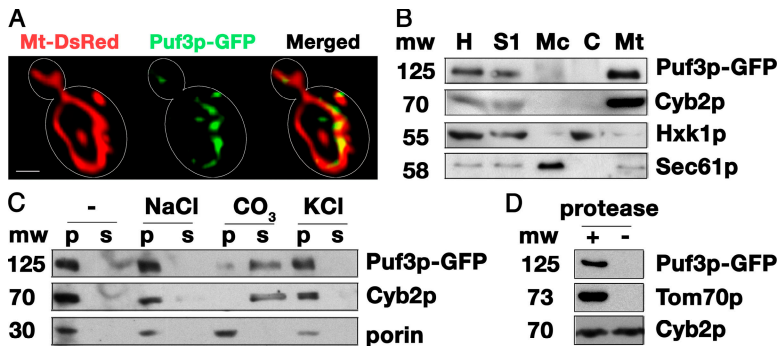


Figure 2. Puf3p is a peripheral mitochondrial outer membrane protein. (A) Puf3p-GFP colocalizes with mitochondrial tubules. Yeast (LGY005) expressing Puf3p that was tagged at its chromosomal locus with GFP and plasmid-borne (pTDT104GAL1+PreF₀ATPase-[subunit9]-DsRed) mitochondria-targeted DsRed were grown to midlog phase in glucose-based selective media. Z sections through the cell were collected using an epifluorescence microscope. The images were deconvolved and reconstructed into a 3D volume. The images shown are 2D projections of reconstructed 3D volumes. Mitochondria-targeted (mt) DsRed (A) is shown in red. Puf3p-GFP is shown in green. The merged image shows mitochondria (red) and Puf3p-GFP (green). Bar, 1 μ m. (B) Puf3p-GFP cofractionates with a mitochondrial marker protein upon subcellular fractionation. Puf3p-GFP cells (LGY001) were grown to midlog phase in SC medium and subjected to subcellular fractionation as described in Materials and methods. Equal amounts of protein from whole cell extracts (H), S1 (the supernatant recovered after centrifugation of homogenized spheroplasts at 2,000 g), microsomes (Mc), cytosol (C), and Nycodenz-purified mitochondria fractions (Mt) were analyzed using Western blots and antibodies that recognize the mitochondrial marker cytochrome *b*₂ (Cyb2p), the cytosolic marker hexokinase (Hxk1p), and an ER marker (Sec61p). Anti-GFP monoclonal antibody was used to detect Puf3p-GFP. mw, molecular weight of proteins in kilodaltons. (C) Nycodenz-purified mitochondria prepared from yeast expressing Puf3p-GFP (LGY001) were treated with 0.1 M NaCl, 0.1 M Na₂CO₃, or 1 M KCl as described in Materials and methods. Carbonate or NaCl extractable and inextractable material was analyzed using Western blots and antibodies raised against Puf3p-GFP, porin (Por1p, an integral mitochondrial membrane protein), and cytochrome *b*₂ (Cyb2p, a peripheral mitochondrial inner membrane protein). P, pellet; S, supernatant. (D) Nycodenz-purified mitochondria were washed with protease inhibitor-free breaking buffer and incubated with a protease inhibitor cocktail or trypsin and chymotrypsin (150 μ g/ml) for 30 min at 23°C. After addition of protease inhibitors to the protease-treated sample, mitochondria were separated from the reaction mixture. Proteins in the mitochondrial pellets were analyzed using Western blots and antibodies raised against Puf3p-GFP, Tom70p (a mitochondrial outer membrane protein), and cytochrome *b*₂ (Cyb2p).

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at 37°C in nine clones from a pool of 42,000 transformants. One of them, pMyr-*PUF3*, contained amino acids 635–879 of Puf3p, which encodes the C terminus and part of the PUM-HD RNA binding domain of the protein (Fig. 1 A). pMyr-*PUF3* (635–879) rescued the temperature-sensitive growth defect of *cdc25H* cells when coexpressed with pSos-*MDM12* but not with the pSos alone (Fig. 1 B). Thus, amino acids 635–879 of Puf3p interact with Mdm12p in the two-hybrid assay. Finally, we found that Mdm12p can bind to the full-length PUF repeat region on Puf3p (aa 549–828), and to aa 635–879, a region encompassing the C-terminal 2/3s of the PUF region and the C terminus of Puf3p (Fig. 1 C).

Puf3p localizes to the cytoplasmic face of mitochondrial outer membranes

The localization of Puf3p was determined by optical imaging and subcellular fractionation. For imaging studies, cells expressing Puf3p tagged at its chromosomal locus with GFP and mitochondria-targeted Ds-Red were examined by deconvolution microscopy. The morphology of mitochondria using targeted Ds-Red—long, tubular structures that aligned along the mother-bud axis and accumulated in the bud tip and mother cell tip—was similar to that observed in other wild-type cells. Thus, the Puf3p-GFP appears to be fully functional.

Previous studies indicate that PUF proteins localize predominantly to the cytoplasm in higher eukaryotes (Lehmann and Nusslein-Volhard, 1991; Zhang et al., 1997). In budding yeast, Puf3p localizes to punctate structures in yeast (Huh et al., 2003; Gerber et al., 2004). However, it was not clear whether Puf3p-containing structures colocalized with mitochondria. With the improved spatial resolution of deconvolution microscopy combined with visualization of Puf3p and mitochondria in the same cells, we found that Puf3p-GFP localizes to punctate and tubular structures that colocalized with mitochondria (Fig. 2 A). Although single projections of the 3D volumes are

shown, colocalization of Puf3p with mitochondria was confirmed by examining the projections at multiple angles. Given the localization of Puf3p to punctate structures in these and previous studies, it is likely that the tubular Puf3p-containing structures consist of multiple Puf3p puncta that are closely spaced on mitochondria.

For subcellular fractionation studies, whole cell extracts from cells expressing Puf3p-GFP were fractionated into mitochondria, microsomes, and cytosol by differential and Nycodenz gradient centrifugation (Fig. 2 B). Puf3p-GFP cofractionated with the mitochondrial marker protein cytochrome *b*₂ (Cyb2p); that is, Puf3p was enriched in the mitochondrial fraction and depleted in the fractions containing microsomes and cytosol to the same extent as Cyb2p upon subcellular fractionation. Carbonate extraction and protease-sensitivity studies were performed to determine the disposition of Puf3p on mitochondrial membranes. Puf3p could be extracted from mitochondrial membranes with Na₂CO₃ but remained associated with mitochondria after washes with KCl (Fig. 2 C). Moreover, Puf3p was degraded upon treatment of Nycodenz-purified mitochondria with trypsin and chymotrypsin (Fig. 2 D). This protease treatment degraded a mitochondrial surface protein (Tom70p) without affecting the integrity of the organelle, as assessed by the stability of Cyb2p, an intermembrane space protein. Together, these results indicate that Puf3p is a peripheral mitochondrial membrane protein that is associated with the cytosolic face of the mitochondrial outer membrane.

Puf3p regulates mitochondrial biogenesis

Previous findings (Gerber et al., 2004) indicated that deletion of *PUF3* had no effect on growth rates on fermentable carbon sources and produces a subtle decrease in growth rates on nonfermentable carbon sources. We find that overexpression of *PUF3* resulted in cell growth defects on glycerol at 37°C (Fig. 3 A). Using the DNA binding dye DAPI, we detected

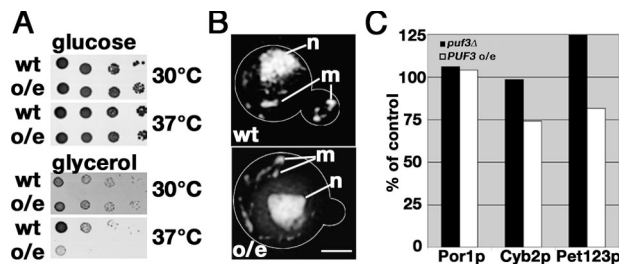


Figure 3. Overexpression of *PUF3* leads to respiratory deficiency at elevated temperature. (A) Growth of wild-type yeast (wt) that carry empty vector (pRS423; LGY008) and yeast that overexpress (o/e) *PUF3* from the pRS423-*PUF3* plasmid (LGY009) on selective, glucose- or glycerol-based solid medium, and cells were grown at 30°C for 3 d or at 37°C for 7 d. (B) Overexpression of *PUF3* has no effect on maintenance of mtDNA. Wild-type (LGY008) and *PUF3* overexpressing (LGY009) cells were grown in selective glucose-based liquid medium at 37°C for 48 h, stained with DAPI, and visualized by epifluorescence microscopy. The images shown are 2D projections of reconstructed 3D volumes that were obtained as for Fig. 1. m, mitochondrial DNA; n, nuclear DNA. Bar, 1 μ m. (C) Effect of deletion or overexpression of *PUF3* on the steady-state levels of three nuclear-encoded mitochondrial proteins. Mitochondria were isolated from midlog phase wild-type (LGY008), *puf3* Δ (OB1501), and *PUF3* overexpressing (LGY009) cells and analyzed by Western blots with antibodies that recognize porin (Por1p), cytochrome *b*₂ (Cyb2p), and Pet123p. The steady-state level of each protein was determined by densitometric analysis of Western blots and is expressed as a percentage of the levels detected in wild-type cells.

mitochondrial DNA (mtDNA) in 90 and 85% of wild-type and *PUF3* overexpressing cells examined, respectively (Fig. 3 B). Thus, the observed defect in respiration-driven growth at elevated temperatures in yeast overexpressing *PUF3* is not due to a loss of mtDNA.

Previous studies revealed that Puf3p binds to and affects the stability and/or targeting of mRNAs for nuclear-encoded mitochondrial proteins. Here, we studied the effects of deletion or overexpression of *PUF3* on the steady-state protein levels of Pet123p, porin (Por1p), and Cyb2p. Pet123p is a subunit of the mitochondrial ribosome that is encoded by a transcript that binds to Puf3p (Gerber et al., 2004). Por1p and Cyb2p localize to mitochondria but are encoded by mRNAs that do not exhibit high-affinity binding to Puf3p (Gerber et al., 2004; Fig. 3 C). The steady-state level of mitochondria-associated Pet123p was elevated in *puf3* Δ cells and reduced in cells overexpressing *PUF3*. Overexpression of a Puf3p mutant that bears a deletion in the PUF repeat region has no effect on Pet123p levels (unpublished data). In contrast, Por1p levels were not affected by deletion or overexpression of *PUF3*. These findings are consistent with the proposed function of Puf3p in binding to and stimulating the decay of specific mRNAs for nuclear-encoded mitochondrial proteins.

Although deletion of *PUF3* also had no effect on the steady-state levels of Cyb2p, *PUF3* overexpression resulted in a reduction in the amount of Cyb2p. Overexpression of a Puf3p mutant that bears a deletion in the PUF repeat region has no effect on Cyb2p levels (unpublished data). Because Puf3p does not exhibit high-affinity binding to *CYB2* mRNA, it is possible that Puf3p binds to nonrelevant targets when overexpressed. Alternatively, because Puf3p binds to transcripts that encode

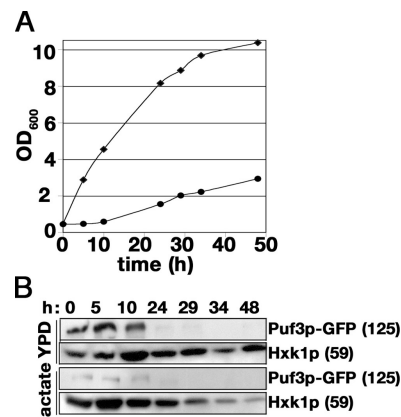


Figure 4. Puf3p is down-regulated during the diauxic shift and growth in nonfermentable carbon sources. (A) A yeast strain expressing Puf3p-GFP (LGY001) was inoculated in glucose- (circles) or lactate-based (squares) liquid media and incubated with aeration at 30°C overnight. At each time point indicated, cell growth was determined by optical density measurements at 600 nm. (B) Steady-state levels of Puf3p-GFP and a cytosolic marker protein, hexokinase (Hxk1p), as a function of cell growth on fermentable or nonfermentable carbon sources. At each of the time points indicated, equal amounts of total cellular protein were analyzed using Western blots and antibodies that recognize GFP or hexokinase. Numbers in parentheses are molecular weights of proteins in kilodaltons.

mitochondrial protein import proteins (e.g., Tim9p, Tim44p, Tim17p, Tom22p, Tom6p, Ssc1p, and Hsp60p), *PUF3* overexpression may affect Cyb2p levels through indirect effects on transcripts for proteins that affect Cyb2p import and/or stability.

Puf3p levels decline when mitochondrial biogenesis is up-regulated

Here, we tested whether Puf3p protein levels are altered in two situations in which mitochondrial biogenesis is up-regulated: the diauxic shift and the shift in growth from a fermentable to a nonfermentable carbon source. Upon growth of budding yeast on glucose as the sole carbon source, budding yeast undergo two sequential exponential growth phases (Johnston and Carlson, 1992). The first growth phase is largely a fermentative phase. The second phase is mostly driven by the aerobic metabolism of ethanol produced during fermentative growth. The transition between fermentation- and respiration-driven growth phases is the diauxic shift. During this transition, there is an up-regulation in mRNAs for enzymes involved in gluconeogenesis, the glyoxylate cycle, the tricarboxylate cycle, and respiration (DeRisi et al., 1997), and a large (up to 10-fold) increase in mitochondrial abundance (Johnston and Carlson, 1992). A similar induction is observed when yeast cells are shifted from growth on a fermentable to a nonfermentable carbon source.

Because respiration-driven growth is slower than fermentation-driven growth, the diauxic shift is detectable by monitoring growth rates as a function of time in glucose-based liquid medium (YPD). Under our growth conditions, this occurs after 10 h of growth (Fig. 4 A). We found that the steady-state level of Puf3p-GFP declines during the diauxic shift (Fig. 4 B) and that there is little detectable Puf3p-GFP in cells that are adapted

to growth on lactate, a nonfermentable carbon (Fig. 4 B). Together, these findings provide additional support for a role of Puf3p in the down-regulation of mitochondrial biogenesis. Given the localization of Puf3p described here, it appears that this regulation occurs on the surface of mitochondria in budding yeast.

Finally, we studied the level of Cyb2p and Pet123p in cells grown in different carbon sources. First, we confirmed a previous study that Cyb2p levels are increased by 15-fold in cells grown on nonfermentable carbon source compared with that observed in glucose-repressed cells (Lodi and Guiard, 1991). In addition, we found that Pet123p levels are elevated by 30% in wild-type and *puf3Δ* cells grown on a nonfermentable carbon source compared with wild-type, glucose-grown cells (unpublished data). These results are consistent with the established up-regulation of mitochondrial biogenesis upon release from glucose repression and provide correlative evidence for a role of Puf3p in this process.

Puf3p contributes to recruitment of the Arp2/3 complex to the mitochore

We identified two links between PUF proteins and the mitochondrial motility machinery. First, Jsn1p/Puf1p interacts with mitochondria-associated Arp2/3 complex and contributes to anterograde mitochondrial movement through effects on recruiting the Arp2/3 complex to the organelle (Fehrenbacher et al., 2005). Second, Puf3p interacts with the mitochore, the protein complex that is required to link mitochondria to actin cables for anterograde and retrograde movement during cell division. Here, we studied these protein–protein interactions and their role in mitochondrial motility and morphology.

To determine whether Puf3p is an Mdm12p binding partner, we tested whether Mdm12p coimmunoprecipitates and colocalizes with Puf3p. Both studies were performed using a yeast strain in which the chromosomal genes *PUF3* and *MDM12* were tagged with GFP and multiple copies of the Myc epitope, respectively. Because cells expressing Puf3p-GFP and Mdm12p-Myc exhibited normal growth rates on fermentable and nonfermentable carbon sources and normal mitochondrial morphology (unpublished data), both fusion proteins appear to be fully functional.

Puf3p-GFP was recovered in the pellet obtained upon immunoprecipitation of Mdm12p-Myc using a monoclonal anti-Myc antibody (Fig. 5 A). Puf3p-GFP was not recovered in the immunoprecipitated pellet from yeast expressing untagged Mdm12p or in antibody-free immunoprecipitation controls (Fig. 5 A). Moreover, OM45p, an abundant, unrelated integral mitochondrial outer membrane protein, did not coimmunoprecipitate with Mdm12p-Myc or Puf3p-GFP (unpublished data). Thus, Puf3p-GFP coimmunoprecipitates with Mdm12p-Myc. Consistent with this, punctate structures stained with Puf3p-GFP frequently colocalized with Mdm12p-Myc. Colocalization was greatest in punctate structures that exhibited high levels of Mdm12p-Myc and Puf3p-GFP (Fig. 5 B) and was detected when the imaging threshold was adjusted to eliminate structures with low GFP or Myc signal. These results indicate that the association between Puf3p and Mdm12p detected in our

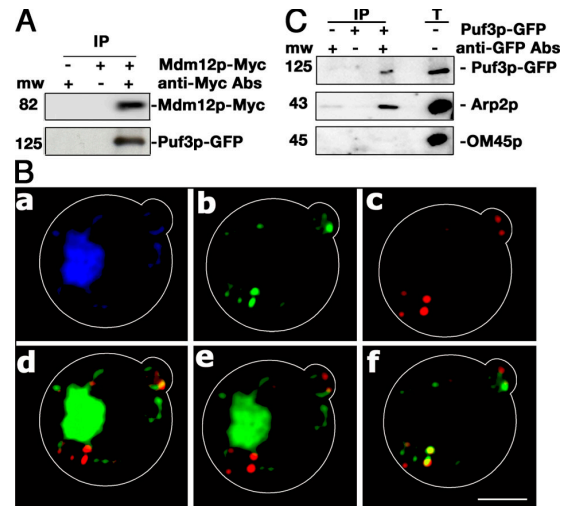


Figure 5. Puf3p has physical and functional interactions with mitochondria-associated Arp2/3 complex. (A) Coimmunoprecipitation of Puf3p with Mdm12p. Mitochondria were purified from strains expressing Puf3p-GFP (LGY001) or Puf3p-GFP and Mdm12p-Myc (LGY002) and solubilized with buffer containing 0.5% digitonin. Monoclonal anti-Myc antibody was added to immunoprecipitate Mdm12p-Myc (see Materials and methods). Immunoprecipitated proteins were probed using antibodies that recognize Myc or GFP. mw, molecular weight of proteins in kilodaltons. (B) Puf3p colocalizes with some Mdm12p-containing puncta and some mtDNA nucleoids. Yeast expressing Puf3p-GFP and Mdm12p-Myc (LGY002) were grown to midlog phase, fixed, converted to spheroplasts, and stained for Mdm12p using a monoclonal antibody against the Myc epitope and for DNA using DAPI (see Materials and methods). The images shown are 2D projections of reconstructed 3D volumes that were obtained as for Fig. 1. Puf3p-GFP was detected as punctate structures that partially colocalize with DAPI and punctate Mdm12p mitochondrial structures. (a–c) Localization of DAPI-stained nuclear and mtDNA (blue), Puf3p-GFP (green), and Mdm12p-Myc (red). (d) Merged image of DAPI-stained material (green) and Puf3p-GFP (red). (e) Merged image of DAPI-stained material (green) and Mdm12p-Myc (red). (f) Merged image of Puf3p-GFP (green) with Mdm12p-Myc (red). Bar, 1 μ m. (C) Coimmunoprecipitation of Arp2p with Puf3p. Mitochondria purified from wild-type strains expressing GFP-tagged (LGY001) or untagged Puf3p (BY4741) were solubilized with buffer containing 0.5% digitonin. Monoclonal anti-GFP antibody was added to the lysates, as indicated, to immunoprecipitate Puf3p-GFP. Immunoprecipitated proteins (IP) were analyzed using Western blot and antibodies that recognize GFP, Arp2p, or OM45p. T, 100 μ g of mitochondrial lysate from a wild-type strain expressing Puf3p-GFP (LGY001); mw, molecular weight of proteins in kilodaltons.

unconventional two-hybrid screen is both specific and physiologically relevant.

Genome-wide affinity purification screens revealed that Puf3p can bind to several subunits of the Arp2/3 complex (Ho et al., 2002). We found that Arp2p coimmunoprecipitated with Puf3p-GFP upon immunoprecipitation of mitochondrial extracts from Puf3p-GFP-expressing cells but not from cells expressing untagged Puf3p or from antibody-free control samples (Fig. 5 C). Under these conditions, OM45p, an abundant mitochondrial outer membrane protein, was not immunoprecipitated. Thus, Puf3p interacts with mitochondria-associated Arp2p.

Finally, we tested whether the mitochore coimmunoprecipitates with the Arp2/3 complex in a wild-type cell and a *puf3Δ* mutant. Mdm12p-Myc coimmunoprecipitated with Arp2p from mitochondrial extracts in wild-type cells (Fig. 6 A). Deletion of *PUF3* results in a 60% reduction in the amount of Arp2p that is recovered with Mdm12p-Myc after coimmunoprecipitation.

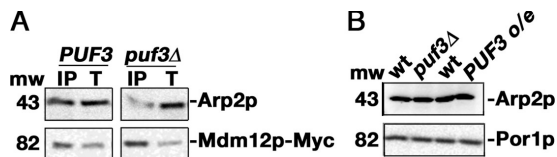


Figure 6. Deletion of *PUF3* results in a reduction in interactions between Mdm12p and mitochondria-associated Arp2p without affecting recruitment of Arp2p to the organelle. (A) Coimmunoprecipitation of Mdm12p and Arp2p in wild-type cells and *puf3Δ* mutants. Mitochondria, purified from wild-type (LGY002) and *puf3Δ* (LGY012) strains expressing Myc-tagged Mdm12p, were solubilized with buffer containing 0.5% digitonin and subjected to immunoprecipitation using a monoclonal anti-Myc antibody. Immunoprecipitated proteins (IP) were analyzed using Western blot and antibodies that recognize Myc or Arp2p. T, 100 μg of the mitochondrial lysate used for immunoprecipitation; mw, molecular weight of proteins in kilodaltons. (B) Effect of deletion or overexpression of *PUF3* on association of Arp2p with mitochondria. Mitochondria were purified from wild-type (wt) cells (BY4741), *puf3Δ* cells (OB1501), or yeast that overexpress plasmid-borne *PUF3* (LGY009) and analyzed by Western blot analysis using antibodies that recognize Arp2p or porin (Por1p, a mitochondrial marker protein).

Thus, we obtained evidence that mitochondria-associated Arp2/3 complex interacts with the mitochore and that Puf3p contributes to this interaction.

Because Jsn1p/Puf1p contributes to recruiting the Arp2/3 complex to mitochondria in budding yeast, it is possible that the reduced interactions between the mitochore and the Arp2/3 complex detected in *puf3Δ* cells are due to a decreased association between the Arp2/3 complex and mitochondria. To address this issue, we compared the amount of Arp2p that is recovered with mitochondria during subcellular fractionation in wild-type cells, *PUF3* deletion mutants, and cells that overexpress *PUF3*. Deletion or overexpression of *PUF3* had no effect on the recovery of Arp2p with mitochondria (Fig. 6 B), indicating that Puf3p and Jsn1p are not functionally redundant. In contrast, our results indicate that Puf3p contributes to recruiting the Arp2/3 complex to the mitochore.

Puf3p is required for normal mitochondrial motility and morphology

Previous studies indicate that mutations in the mitochore and Arp2/3 complex/Jsn1p/Puf1p have different effects on mitochondrial morphology and motility. Mutations of mitochore subunits result in an accumulation of large, spherical mitochondria that fail to move in either the anterograde or retrograde directions (Boldogh et al., 2003; Fehrenbacher et al., 2005). In contrast, mitochondria from yeast carrying mutations in *JSN1* or Arp2/3 complex subunits are largely tubular, not spherical. However, they are fragmented and aggregated and exhibit defects in anterograde, but not retrograde, mitochondrial movement. If Puf3p contributes to recruiting the mitochore to the Arp2/3 complex, then mitochondrial motility and morphology in *PUF3* deletion mutants should resemble those observed in the Arp2/3 complex or Jsn1p mutants.

Analysis of mitochondrial morphology in deconvolved 3D projections revealed that 14% of wild-type cells exhibited aggregation or fragmentation of mitochondria ($n = 55$). In contrast, 44% of cells in a *PUF3*-null population showed

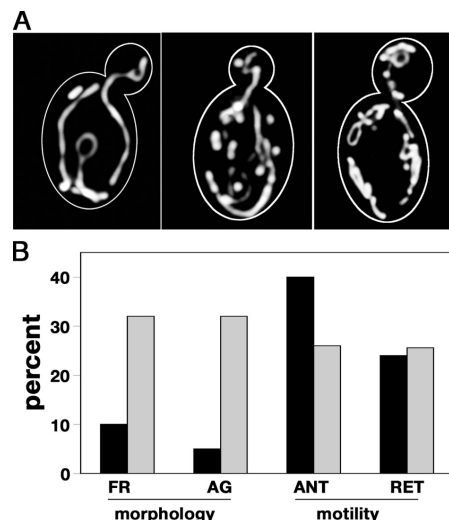


Figure 7. Mitochondrial morphology and motility are abnormal in cells lacking Puf3p. (A) Mitochondrial morphologies are shown for diploid wild-type cells (LGY013; left) and yeast bearing deletions in both copies of the *PUF3* gene (LGY014; middle and right). Cells expressing mitochondria-targeted GFP (pCS1-GFP) were grown to midlog phase in selective media and analyzed by fluorescence microscopy. The images shown are 2D projections of reconstructed 3D volumes that were obtained as for Fig. 2. Deletion of *PUF3* resulted in an increase of cells showing aggregated and fragmented mitochondria. Examples of mitochondria in *puf3Δ/puf3Δ* cells with fragmented (middle) or aggregated (right) mitochondria are shown. (B) Quantitation of defects in mitochondrial morphology and motility in diploid wild-type cells and yeast bearing deletions in *PUF3* (*puf3Δ/puf3Δ*). FG, fragmented mitochondria; AG, aggregated mitochondria; ANT, anterograde mitochondrial movement; RET, retrograde mitochondrial movement.

abnormal mitochondrial phenotypes, with 32% of cells containing aggregated mitochondria and 32% fragmented mitochondria ($n = 63$; Fig. 7). The observed defects in mitochondrial morphology were not due to defects in actin organization or in the interaction between mitochondria and actin cables (unpublished data). Thus, mitochondrial morphology in a *puf3Δ* mutant is similar to that observed in *JSN1* and Arp2/3 complex mutants.

Consistent with this, *puf3Δ* cells show defects in mitochondrial motility that are similar to those observed in yeast bearing a deletion in *JSN1* or mutation of Arp2/3 complex subunits (Fig. 7 B). In wild-type yeast, 40% of mitochondria moved in the anterograde direction, and 24% move in the retrograde direction during 1 min of analysis ($n = 123$). Deletion of *PUF3* had no obvious effect on retrograde mitochondrial movement. In contrast, we observed a 35% decrease in the amount of anterograde mitochondrial movement in a *puf3Δ* population as compared with wild type ($n = 109$; Fig. 7 B). These findings support a role for Puf3p in recruiting the mitochore to the Arp2/3 complex to promote anterograde mitochondrial motility and normal mitochondrial morphology in budding yeast.

Because Puf3p has been implicated in mitochondrial biogenesis and mRNA stability, it is possible that the motility defects observed in *puf3Δ* cells are a consequence of Puf3p effects on mRNA turnover that are critical for mitochondrial morphology or biogenesis. To address this issue, we measured mitochondrial motility in yeast bearing mutations in *MAS37*

or *TOM7* (Fig. 8). Mas37p is a subunit of the SAM/TOB complex, which mediates assembly of newly imported β -barrel proteins into the mitochondrial outer membrane and promotes the segregation of Mdm10p from the SAM/TOB complex (Wiedemann et al., 2003; Meisinger et al., 2006). Tom7p is a subunit of the protein-translocating mitochondrial outer membrane pore (Honlinger et al., 1996). Deletion of *TOM7* results in defects in mitochondrial protein import and produces defects in mitochondrial morphology that are similar to those observed in *mdm12* Δ cells (Meisinger et al., 2001, 2006). Deletion of *MAS37* or *TOM7* had no major effect on the level of anterograde and retrograde mitochondrial movement. Therefore, mutations that affect mitochondrial biogenesis or morphogenesis do not produce motility defects that resemble those observed in mitochore or mitochondria-associated PUF family proteins.

Finally, we studied poleward mitochondrial movement in wild-type and *puf3* Δ cells upon growth on media containing fermentable or nonfermentable carbon sources (Fig. 8). Because Puf3p is present in cells grown in glucose-based media and severely down-regulated in cells grown on glycerol-based media, mitochondria motility in yeast grown on nonfermentable carbon sources should be similar to that observed in *puf3* Δ cells. We did not detect any carbon source effects on the extent of retrograde mitochondrial movement. In contrast, we found that growth on glycerol results in a 50% decrease in anterograde mitochondrial motility compared with that observed in yeast grown on a fermentable carbon source. Thus, two conditions that result in severe down-regulation of Puf3p levels, i.e., deletion of the *PUF3* gene or growth on nonfermentable carbon source, produce a decrease in anterograde but not retrograde mitochondrial motility. Consistent with this, mitochondrial motility in wild-type cells that were grown in a glycerol-based medium was not appreciably different from that observed in *puf3* Δ deletion mutations that were grown in media containing either glucose or glycerol.

Discussion

Puf3p, one of the six PUF proteins found in *S. cerevisiae*, binds preferentially to cytoplasmic mRNAs for nuclear-encoded mitochondrial proteins and promotes the decay of bound mRNA by enhancing mRNA deadenylation (Olivas and Parker, 2000; Gerber et al., 2004). Another PUF protein of budding yeast, Jsn1p/Puf1p, localizes to mitochondria, where it contributes to the recruitment of the Arp2/3 complex to the organelle for anterograde movements that lead to inheritance (Fehrenbacher et al., 2005). Here, we report that Puf3p also localizes to mitochondria in budding yeast, where it contributes to both mitochondrial motility during inheritance and mitochondrial biogenesis.

Puf3p cofractionates with a mitochondrial marker protein during subcellular fractionation and localizes to punctate structures that colocalize with mitochondria in living yeast cells. Carbonate extraction, salt washes, and protease-sensitivity studies indicate that Puf3p is a peripheral membrane protein that is associated with the cytoplasmic face of the mitochondrial outer membrane. Thus, two of the six PUF proteins of budding yeast,



Figure 8. **Effect on mitochondrial motility of carbon source and mutation of genes implicated in mitochondrial biogenesis.** Mitochondrial motility was monitored in midlog phase wild-type (WT; LGY013) and mutant strains (LGY014, LGY016, and LGY017) grown in either glucose- or glycerol-based media. Quantitation of the percentage of mitochondria that were either nonmotile or exhibited anterograde or retrograde movement was performed as for Fig. 7.

Jsn1p/Puf1p and Puf3p, localize to mitochondria and to the same submitochondrial compartment.

Role for Puf3p in mitochondrial motility and inheritance

All known motor molecules contain two distinct activities: they link cargo to the cytoskeleton and generate forces for cargo movement along cytoskeletal tracks. Our previous studies indicate that these two activities occur during mitochondrial movement in budding yeast. However, the activities exist in two distinct protein complexes. The mitochore is required for the interaction of mitochondria with actin cables for poleward movement (Boldogh et al., 2003), whereas the Arp2/3 complex generates forces for movement of the organelle along its cytoskeletal tracks (Fehrenbacher et al., 2004). Here, we report that Puf3p contributes to mitochondrial movement during inheritance by linking the force generator for anterograde mitochondrial motility (the Arp2/3 complex) to the adaptor that links the organelle to the cytoskeleton (the mitochore).

Several lines of evidence support a link between Puf3p and the machinery for mitochondrial motility and inheritance. A genome-wide affinity precipitation screen revealed that Puf3p can bind to several subunits of the Arp2/3 complex (Ho et al., 2002). We found that that Puf3p binds to a mitochore subunit in a two-hybrid assay and coimmunoprecipitates with mitochore and Arp2/3 complex subunits. Thus, the interactions detected by two-hybrid and affinity-precipitation assays appear to be physiologically relevant.

Mutations of mitochore subunits result in the accumulation of large spherical, nonmotile mitochondria (Boldogh et al., 2005). In contrast, mitochondria in yeast *PUF3*, *JSN1*, or Arp2/3 complex mutants are fragmented and aggregated. Moreover, deletion of *PUF3*, *JSN1*, *ARC15*, or *ARP2* impairs anterograde, bud-directed mitochondrial movement, but has no obvious effect on retrograde, mother cell-directed movement of the organelle (Fehrenbacher et al., 2005). Thus, deletion of *PUF3* does not produce phenotypes that are similar to those observed in mitochore mutants. Rather, *puf3* Δ cells exhibit defects in mitochondrial morphology and anterograde motility that are similar to those observed in yeast bearing mutations in the Arp2/3 complex or in Jsn1p.

Although *puf3* Δ and *jsn1* Δ mutants exhibit similar defects in mitochondrial morphology and Jsn1p and Puf3p share structural similarity, these proteins do not appear to be functionally redundant. First, Puf3p is reduced 10-fold during the diauxic shift and in cells grown on a nonfermentable carbon source. In contrast, Jsn1p is readily detectable in cells grown on a nonfermentable carbon source (Fehrenbacher et al., 2005). Second, deletion of *JSN1* results in a 50% decrease in recruitment of Arp2/3 complex to mitochondria (Fehrenbacher et al., 2005). In contrast, deletion of *PUF3* has no effect on the association of the Arp2/3 complex with mitochondria. Finally, we find that mitochondrial motility in a *jsn1* Δ *puf3* Δ mutant does not resemble that observed in either *jsn1* Δ or *puf3* Δ single mutants (unpublished data). Thus, we obtained genetic evidence that these proteins have distinct roles in mitochondrial motility.

Rather, our findings support the model that Puf3p stimulates mitochondrial motility through effects on recruiting mitochondria-associated Arp2/3 complex to the mitochore complex. Arp2/3 complex subunits coimmunoprecipitate with and are therefore physically associated with mitochore subunits. Moreover, deletion of *PUF3* results in a large (>60%) decrease in the amount of Arp2p that coimmunoprecipitates with the mitochore subunit Mdm12p. Because deletion of *PUF3* does not completely abolish interactions of Arp2/3 complex with the mitochore, there may be other proteins that play a role in this process. Nonetheless, our data indicate that Puf3p serves as an adaptor to link Arp2/3 complex, the force generator for anterograde mitochondrial movement, to the mitochore, the protein complex that is required for reversible interaction of mitochondria with the cytoskeletal track for movement. Thus, Puf3p may stimulate anterograde mitochondrial movement by bringing the two fundamental functions of a motor molecule—present in two separate protein complexes—together into one physical and functionally efficient unit.

Role for Puf3p in mitochondrial biogenesis and respiratory activity

Previous findings support a role for Puf3p in regulating the stability of mRNAs for mitochondrial proteins (Gerber et al., 2004). Moreover, a recent study indicates that Puf3p destabilization of mitochondrial mRNAs is carbon source dependent. Specifically, transcripts for nuclear-encoded mitochondrial proteins, which undergo Puf3p-dependent degradation, are degraded in yeast grown on fermentable carbon sources but are stabilized in yeast grown on nonfermentable carbon sources (Foat et al., 2005).

Our findings provide additional support for a role of Puf3p in the regulation of mitochondrial biogenesis. The transcript for Pet123p, a subunit of the mitochondrial ribosome, binds to Puf3p with high affinity (Gerber et al., 2004). We find that deletion of *PUF3* results in an increase in the level of Pet123p but has no obvious effect on the levels of two mitochondrial proteins whose mRNAs do not bind to Puf3p. Conversely, overexpression of *PUF3* results in a decrease in Pet123p levels. Thus, Puf3p levels are inversely proportional to the levels of a protein whose transcript exhibits high-affinity binding to Puf3p. This finding provides additional support for a

role of Puf3p in regulation of mitochondrial biogenesis through effects on mRNAs for nuclear-encoded mitochondrial proteins. Moreover, because Pet123p is a subunit of the mitochondrial ribosome, our results also indicate that Puf3p can affect mitochondrial biogenesis through effects on synthesis of mtDNA-encoded mitochondrial proteins.

We also provide evidence that Puf3p contributes to the down-regulation of mitochondrial biogenesis during glucose repression. Growth of yeast on glucose represses mitochondrial biogenesis, up to 10-fold in some genetic backgrounds (Carlson, 1987). Previous microarray studies have revealed an increase in 893 mRNAs and a decrease in 1,233 mRNAs during the diauxic shift (DeRisi et al., 1997). Thus, regulation of transcription is a key contributor to glucose repression. However, it is possible that regulation of mRNA stability may also contribute to glucose repression. As described above, Puf3p promotes mRNA decay and regulates mRNA stability in a carbon source-dependent manner. We find that Puf3p is present in cells grown on glucose and is down-regulated during both the diauxic shift and adaptation to growth on nonfermentable carbon sources. Moreover, we find that Pet123p, a protein whose transcripts exhibits high-affinity binding to Puf3p, is reduced in cells grown on glucose compared with nonfermentable carbon sources. These findings support the model in which Puf3p regulation of mRNA stability contributes to down-regulation of mitochondrial biogenesis during glucose repression.

Finally, previous findings indicate that mRNAs for many nuclear-encoded mitochondrial proteins localize to mitochondria in yeast and human cells (Suissa and Schatz, 1982; Egea et al., 1997; Corral-Debrinski et al., 2000; Marc et al., 2002). In light of this and our findings regarding Puf3p localization and function in mitochondrial biogenesis, it is tempting to speculate that Puf3p could have one additional function. That is, it may serve, like Puf6p, to transport mRNAs to specific sites (Gu et al., 2004). In this scenario, Puf3p could bind to the 3' UTR of mitochondrial mRNAs and guide those transcripts to the surface of the organelle. In agreement with this hypothesis, it has been reported that the 3' UTR of mitochondrial mRNAs is required for their localization to the vicinity of the organelle, a function that is conserved from yeast to human cells (Sylvestre et al., 2003).

Is there a relationship between Puf3p functions in down-regulation of mitochondrial biogenesis and up-regulation of mitochondrial motility?

We find that Puf3p is a bifunctional protein that (1) links the adaptor for mitochondrial-cytoskeletal interactions to the force generator for cytoskeleton-dependent anterograde mitochondrial movement and (2) regulates mitochondrial biogenesis and respiratory competence through effects on the stability and/or localization of mRNAs for select nuclear-encoded mitochondrial proteins. Our previous studies revealed that the mitochore is functionally equivalent to the kinetochore. That is, it links the minimum heritable unit of mitochondria (mitochondrial membranes and mtDNA) to actin cables for segregation of the organelle during cell division. The finding that the mitochore is also

associated with Puf3p provides additional evidence for a role of the mitochore in mitochondrial motility and inheritance. Moreover, it raises the possibility that the machinery regulating mitochondrial transcript stability and/or trafficking may be part of the minimal heritable unit of the organelle.

What is the benefit of having a single protein with dual functions in mitochondrial biogenesis and bud-directed motility? Anterograde mitochondrial movement results in the transfer of the organelle from mother to daughter cell and is therefore a critical contributor to mitochondrial inheritance. Because mitochondrial inheritance is essential for cell viability, there is a greater burden on the cell division machinery to ensure that mitochondria are inherited when the organelle is present in low abundance. In light of this, Puf3p function in mitochondrial biogenesis during glucose repression and anterograde mitochondrial motility may promote cellular vitality because it down-regulates mitochondrial abundance in response to environmental conditions and ensures that the few mitochondria that are present during repression of mitochondrial biogenesis are transferred from mother to daughter cell during cell division. Thus, Puf3p serves both as a modulator of mitochondrial abundance and as an effector that allows the cell division machinery to compensate for changes in mitochondrial abundance.

Materials and methods

Yeast strains and growth conditions

Yeast strains used in this study are listed in Table I. Strains were derivatives of BY4741 or BY4743 (Open Biosystems). Yeast cells were cultivated and manipulated according to Sherman (2002).

Construction of plasmids

Standard molecular techniques for cloning procedures were used (Sambrook et al., 1989). In all cases, PCR was performed using *Pfu* Ultra HF DNA polymerase (Stratagene) according to the manufacturer's instructions.

Baits used for the two-hybrid screen were encoded on plasmids (pSos-*MMM1*, -*MDM10*, and -*MDM12*) that express fusion proteins of Mmm1p, Mdm10p, or Mdm12p with the hSos (Aronheim et al., 1994; Aronheim, 1997). Primers used for construction of these plasmids are listed in Table II. In all cases, the entire open reading frame of each gene of interest was amplified by PCR from *S. cerevisiae* genomic DNA. The primers used for these amplifications contained restriction sites (BamHI or MluI). To subclone amplified genes into the pSos plasmid, PCR products were cut with BamHI and MluI and inserted into the polylinker region of the pSos plasmid after linearization with BamHI and MluI. The nucleotide sequences of the cloning junctions were sequenced to verify that the bait proteins were expressed in frame with the hSos protein. DNA encoding the bait proteins was sequenced to verify the absence of mutations.

pRS423-*PUF3* is a multicopy plasmid containing the complete *PUF3* coding sequence flanked by 300 kb upstream of the ATG start codon and 300 kb downstream of the TGA stop codon. The *PUF3* open reading frame was amplified by PCR from *S. cerevisiae* genomic DNA. Primers used for this cloning were as follows (underlined sequences correspond to XhoI and BamHI restriction sites sequences): forward, 5'-TGCACTCGAGACCATTAGCACACTTGAGAATGTATATTGG-3', and reverse, 5'-TGCAGGATCCCTCTCTATCTGTTGCAGAAATAAGAAGAGCG-3'. The PCR product was cut with XhoI and BamHI and integrated into the polylinker of the pRS423 plasmid after linearization with XhoI and BamHI.

LG015 (pRS423-*PUF3*[1-548]-GFP) transformed cells overexpress a chimeric protein in which the PUM-HD domain of Puf3p has been replaced with GFP. For this construction, part of the open reading frame of *PUF3* (aa 1-548) was amplified by PCR from *S. cerevisiae* genomic DNA with the following primers: forward, 5'-TGCACTCGAG-ACCATTAGCACACTTGAGAATGTATATTGG-3', and reverse, ATGCGGATCCGATCTTTGCAAACTCTAAGG-3'. The underlined sequences in the forward and reverse primers correspond to XhoI and BamHI restriction sites, respectively. The residue shown in bold font is a G that was inserted into the reverse primer and allows GFP to be expressed in frame. GFP was amplified from the plasmid pFA6a-GFP (S65T)-*TRP1* (Longtine et al., 1998) with the following primers: forward, CAACATGGTCCGGATCCCCGGTTAATAA-3', and reverse, 5'-ATGCGAATTCGAGCTCGTTAAAC-3'. The underlined sequences in the forward and reverse primers correspond to BamHI and EcoRI restriction sites, respectively. PCR products were cut with XhoI and BamHI (for truncated *PUF3*) and BamHI and EcoRI (for GFP). Both fragments were ligated, and the resulting product was inserted into the polylinker of the pRS423 vector as an XhoI-EcoRI fragment. The nucleotide sequence of the cloning junction between *PUF3* and the GFP was sequenced to verify that the GFP was expressed in frame with the Puf3p.

Table I. Yeast strains used in this study

Strain	Genotype	Source or reference
<i>cdc25H</i>	<i>MATa, ade2-101, his3-200, leu2-2, lys2-801, trp1-901, ura3-52, cdc25-2 Gal</i>	Stratagene (Aronheim, 1997)
BY4741	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ</i>	Open Biosystems
LG001	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, PUF3::GFP-HIS3MX6</i>	This study
LG002	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, PUF3::GFP-HIS3MX6, MDM12::13Myc-KANMX6</i>	This study
LG003	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, PUF3::GFP-HIS3MX6, MDM10::13Myc-KANMX6</i>	This study
LG004	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, PUF3::GFP-HIS3MX6, MMM1::13Myc-KANX6</i>	This study
LG005	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, PUF3::GFP-HIS3MX6 [pRS426ADH+PreF_oATPase-DsRed]</i>	This study
LG006	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ [pCS1-GFP]</i>	This study
LG007	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, puf3Δ::KANMX6, [pCS1-GFP]</i>	This study
LG008	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ [pRS423]</i>	This study
LG009	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ [pRS423-PUF3]</i>	This study
LG010	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, puf3Δ::KANMX6, [pRS426ADH+PreF_oATPase-DsRed]</i>	This study
LG011	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, mdm12Δ::KANMX6, PUF3::GFP-HIS3</i>	This study
LG012	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, puf3Δ::KANMX6, MDM12::13Myc-KANMX6</i>	This study
LG013	<i>MATa/α, his3Δ, leu2Δ, met15Δ, ura3Δ [pCS1-GFP]</i>	This study
LG014	<i>MATa/α, his3Δ, leu2Δ, met15Δ, ura3Δ puf3Δ::KANMX4/puf3Δ::KANMX4 [pCS1-GFP]</i>	This study
LG015	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ [pRS423-PUF3(trunc)-GFP]</i>	This study
LG016	<i>MATa/α, his3Δ, leu2Δ, met15Δ, ura3Δ tom7Δ::KANMX4/tom7Δ::KANMX4 [pCS1-GFP]</i>	This study
LG017	<i>MATa/α, his3Δ, leu2Δ, met15Δ, ura3Δ mas37Δ::KANMX4/mas37Δ::KANMX4 [pCS1-GFP]</i>	This study
OB1501	<i>MATa, his3Δ, leu2Δ, met15Δ, ura3Δ, puf3Δ::KANMX6</i>	Open Biosystems

Table II. Primers used for preparation of Sos fusion genes

Plasmid	Primer direction	Primer
pSos-MDM12	Forward	5'-TGCAGGATCCCTTTTGATATTAATTGGAGTACATTGG-3'
pSos-MDM12	Reverse	5'-TGCAACGCGTTACTCATCACCATGTTGAAATCC-3'
pSos-MMM1	Forward	5'-TGCAGGATCCCTGATAGTGAGAATGAATCC-3'
pSos-MMM1	Reverse	5'-TGCAACGCGTTATAACTCTGTAGGCTTTTCTTCTCTCG-3'
pSos-MDM10	Forward	5'-TGCAGGATCCCTACCCTATATGGACCAAGTACTAAGGGC-3'
pSos-MDM10	Reverse	5'-TGCAACGCGTCATGTGGAGTACTGGAATTGTATGCC-3'

The sequences underlined in the primers correspond to BamHI and MluI restriction sites in forward and reverse primers, respectively.

Construction of yeast strains

The carboxy terminus of Puf3p was tagged GFP using PCR-based insertion into the chromosomal copy of the *PUF3* locus (Longtine et al., 1998; Nowakowski et al., 2001). The carboxy terminus of Mdm12p was tagged with 13 tandem copies of the Myc epitope (13Myc) using the same technique. Table III lists primers used to tag these genes. Yeast cells were first transformed with the PCR products using the lithium acetate method (Gietz et al., 1995). PCR was used to confirm the proper integration of tags into the target locus. Expression and localization of GFP- and 13Myc-tagged proteins were analyzed via Western blot. GFP and 13Myc localization were visualized in cells directly (GFP) or by immunofluorescence staining using a monoclonal anti-Myc antibody (13Myc).

Two-hybrid screen

The two-hybrid screen was performed essentially as described previously (Aronheim et al., 1994; Aronheim, 1997). After cotransformation of a *cdc25H* strain with the bait (pSos-MMM1, -MDM10, or -MDM12) and the Cytotrap XR *S. cerevisiae* yeast cDNA library (Stratagene), single colonies were allowed to grow on solid media consisting of synthetic complete, glucose-based medium -Leu and -Ura (SC glucose-Leu-Ura) for 3–4 d at 23°C. Colonies that grew at 23°C were replica-plated onto synthetic complete, galactose-based medium -Leu and -Ura (SC galactose-Leu-Ura). Cotransformants that grew on SC galactose-Leu-Ura plates at 37°C were retained for further characterization. Library plasmids were isolated from clones that showed consistent galactose-dependent growth at 37°C. Isolated plasmids were transformed into *cdc25H* cells in combination with the bait (pSos-MMM1, -MDM10, or -MDM12) or the pSos vector. Plasmids that supported growth of the *cdc25H* strain only in the presence of bait containing MMM1, MDM10, or MDM12 were sequenced.

Live-cell imaging of mitochondria

Mitochondria were visualized using two different fusion proteins. For analysis of mitochondrial morphology and motility, a centromeric plasmid containing the open reading frame of the citrate synthase 1 fused to GFP (pCS1-GFP; Okamoto et al., 2001) was used. For Puf3p-GFP and mitochondria colocalization studies, mitochondria were visualized using a fusion protein expressed from the plasmid pTDT104GAL1 + PreF₀ATPase-(subunit9)-DsRed (Boldogh et al., 2003). Both hybrid proteins were expressed from plasmids using their own promoters. Cells were grown to midlog phase (OD₆₀₀ 0.3–0.9) in SC-Ura media and placed on an SC-Ura-containing agarose pad on a microscope slide. A coverslip was applied to the slide, and the slide was sealed with Valap (1:1:1 Vaseline/lanolin/paraffin). Images were collected with a microscope (E600; Nikon) using a Plan-Apochromat 100×, 1.4 NA objective lens and a cooled charge-coupled device camera (Orca-ER; Hamamatsu). Illumination with a 100-W mercury arc lamp was controlled with a MAC5000 shutter controller and Ludl filter

wheel (Ludl Electronic Products Ltd.). Ludl filter wheels or a Dual View image splitter (Optical Insights) were used for two-color imaging. Hardware control and image enhancement were performed using Openlab software (Improvision, Inc.).

For 3D imaging, 25 z sections were obtained at 0.2-μm intervals through the entire cell using a piezo-electric focus motor mounted onto the objective lens of the microscope (Polytech PI). Out-of-focus light was removed by deconvolution of each image section, and each series of deconvolved images was projected and rendered using Volocity software (Improvision, Inc.).

Quantification of mitochondrial morphology and movement in vivo

Mitochondrial morphology was analyzed using deconvolved, rendered images viewed from multiple angles. During cell division in wild-type cells, mitochondria accumulate at the bud tip and the mother cell tip. Mitochondria can also accumulate at the mother-bud neck in wild-type cells, presumably because the mother-bud neck is a bottleneck for transport. Therefore, to evaluate abnormal mitochondrial aggregation or fragmentation, analysis was restricted to the mother cell at regions other than the mother-bud neck or the mother cell tip.

Mitochondria were defined as motile if they displayed linear movement for three consecutive still frames taken at 1-s intervals. In all cases, the only portion of the organelle that was evaluated for movement was the tip of the organelle in the mother cell in a single focal plane. Polarized movement was defined as that which achieved a net displacement toward or away from the bud and is expressed as the percentage of all observable mitochondrial tips over the time-lapse course (60 s).

Other methods

The bicinchoninic acid assay (Pierce Chemical Co.) was used for protein concentration determinations. Gel electrophoresis and Western blot analysis were performed as described previously (Boldogh et al., 1998). Yeast mitochondria were isolated as described previously (Lazzarino et al., 1994). Immunofluorescence and visualization of DNA using DAPI was performed as described previously (Fehrenbacher et al., 2005). Immunoprecipitation, carbonate extraction, and protease-sensitivity studies were performed as described previously (Fehrenbacher et al., 2005).

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Table III. Primers used to tag various genes at their chromosomal loci

Gene	Primer direction	Primer
<i>PUF3</i>	Forward	5'-TGTTGAGAAACTGCAGCATTGGTTGAAAATGCGGAGGTGCGGATCCCCGGGTTAATTA-3'
<i>PUF3</i>	Reverse	5'-ATAGTAAAAAGTGAAAGGAGAACGATGATAACACTAATCAGAATTCGAGCTCGTTTAAAC-3'
<i>MDM12</i>	Forward	5'-GCATGGCCAAAGTTGGATTAATCTGGATTCAACGATGCTGATGAGCGGATCCCCGGGTTAATTA-3'
<i>MDM12</i>	Reverse	5'-TTATGTAGACACTATTTCAAACATCTTTGTTAAATTAGAATTCGAGCTCGTTTAAAC-3'

The sequences underlined in the primers correspond to the tagging plasmid sequence.

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