Bot1p Is Required for Mitochondrial Translation, Respiratory Function, and Normal Cell Morphology in the Fission Yeast Schizosaccharomyces pombe

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Mitochondria are energy-generating organelles that participate in multiple cell signaling cascades, which allow continuous communication with the rest of the cell. Recent studies underline the close relationship between mitochondria and cell morphology control, indicating that while mitochondrial movement, morphology, and function are regulated by the cytoskeleton in mostly uncharacterized ways, mitochondrial function is essential for both maintenance of normal morphology, particularly in neuronal cells, and for normal cell cycle progression (1, 29).

The fission yeast Schizosaccharomyces pombe has been extensively used as a powerful genetic model organism for studying the molecular mechanisms of morphology and cell cycle control. S. pombe is a rod-shaped cell that grows in a polarized fashion at its extremities and divides by medial fission. Polarized growth is cell cycle regulated (30). In early G2 phase, cells switch to bipolar growth (new-end takeoff) (30). When cells reach a critical size, growth ceases and cells enter mitosis (reviewed in references 25 and 45). The regular rod shape of S. pombe cells allows ready identification of morphological defects and has been used to isolate morphological and/or cell polarity mutants mainly by visual screening (38, 41). These mutants led to the identification of numerous cell functions involved in the regulation of cell morphology in fission yeast (5, 25).

Mitochondria host many intermediary metabolism reactions as well as the electron transport chain and oxidative phosphorylation system pathways required for the aerobic synthesis of ATP. Mitochondrial biogenesis requires the contribution of two genomes and of two compartmentalized protein synthesis systems (nuclear and mitochondrial). Only a small portion of the structural subunits (seven in the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe) that form the respiratory complexes are encoded on the mitochondrial DNA (mtDNA). The mtDNA also encodes two tRNAs, for a set of 25 tRNAs and for some mitochondrial ribosome proteins, including Rps3 in the case of S. pombe (4). Mitochondria are semiautonomous organelles containing their own independent translational machinery. A large number of nuclear genes code for components of the mitochondrial protein synthetic system, including ribosomal proteins, aminoacyl-tRNA synthetases, and initiation, elongation, and termination factors (33, 40). Nuclear genes also code for proteins that function in biogenesis of the translational machinery rather than in translation itself (2, 9, 37). In S. pombe mitochondrial protein translation is regulated by the guanine nucleotide exchange factor EF-Ts, conserved in higher eukaryotes, which controls the translation elongation factor EF-Tu (7). S. pombe is a petite-negative yeast and, similarly to mammalian cells, cannot survive the loss of mtDNA and mitochondrial protein synthesis (36).

Mitochondria are not isolated organelles and form a dy-
namic reticulum that changes structure and localization in response to the metabolic state of the cell. In fission yeast mitochondrial movement and distribution are mediated by the microtubule cytoskeleton, as is also observed in mammalian cells (16, 43, 46, 47). Recent evidence has shown that mitochondria are an integral part of several signal transduction cascades involved in metabolism, cell cycle control, and differentiation (reviewed in references 6 and 29). In this respect, studies with S. cerevisiae and human cells have suggested that some of the mitochondrial ribosomal proteins may have additional functions in the control of cell metabolism and growth (13, 29).

In this paper we present the characterization of a novel protein that we named Bot1p. bot1 is a gene that is essential for cell viability. bot1A mutant cells expressing lower levels of Bot1p display an altered cell morphology and cell size and an alteration of the actin cytoskeleton. We demonstrate that Bot1p localizes to the mitochondrial ribosome and that decreased levels of Bot1p lead to mitochondrial network fragmentation, decreased cell respiration, and decreased mitochondrial protein translation, indicating that Bot1p function is essential for mitochondrial protein synthesis.

**MATERIALS AND METHODS**

**Yeast strains and cell culture.** The genotypes and sources of the Schizosaccharomyces pombe strains used in this study are listed in Table 1. All fission yeast strains used were isogenic to 972. Cells were cultured in yeast extract rich medium or MIN medium (31, 32) with the appropriate supplements at the indicated temperatures. Genetic manipulations and analysis were performed by standard procedures (31, 32).

**Construction of a strain carrying a null allele of bot1.** The Bot1p protein was originally identified in a two-hybrid screen for proteins that interact with the morphology control kinase Orb8p (42). The screen was performed as previously described (8, 21, 22, 44). The functional role of this interaction, if any, is still unclear. Deletion of bot1+ was performed by replacing the whole bot1 open reading frame (ORF) (residues 1 to 316) with the ura4+ sequence. The 5' and 3' flanking sequences were obtained by PCR from the bot1-containing c41CS (Sanger Center, United Kingdom) cosmids. After transformation, nine ura4+ diploids were analyzed for the presence of the bot1 deletion by Southern blotting. Eight were found to contain the deletion. Two diploids were chosen, sporulated, and analyzed by tetrad analysis. Ten complete tetrads showed 2:2 segregation of the deletion phenotype, which was lethal. The viable spores produced ura4+ colonies and wild-type-looking cells. The deleted spores produced nonviable microcolonies with morphologically aberrant cells (Fig. 1, a). To test the effects of lowered levels of Bot1p, a bot1+::ura4+ bot1+ ade6-M210/ade6-M216 ura4-D18/urad4-D18 ade6::ura4+ ura4-D18 leu1-32::leu1-32 h+ h+ diploid was transformed with the integrative plasmid pJK148 containing the gene bot1+ under the control of the repressible nmt1 (no message in yeast) promoter; the diploid was then sporulated, and haploid ura4+ leu1+ colonies were selected. In medium lacking thiamine, expression of bot1+ from the full-strength nmt1 promoter or bot1-GFP from the attenuated nmt81 promoter fully rescues the lethality and phenotype of the bot1+ deletion. Repression of the nmt1 and nmt81 promoter activity by addition of 15 µM thiamine (5 µg/ml) resulted in misshapen, bottle-shaped cells.

For visualization of Bot1p in vivo and in yeast extracts, we integrated C-terminally green fluorescent protein (GFP)- and Myc-tagged versions of bot1+ into the chromosomal DNA at the bot1 locus. This was done by transforming the plasmid pBK(sup3-5)bot1-GFP or pBK(sup3-5)bot1-13Myc in the strain PN567. The resulting strains (FV806 and FV811) carry one copy of the tagged bot1+ gene under the control of the endogenous promoter and one wild-type copy of bot1+ with no promoter.

**Immunofluorescence microscopy.** Cells in liquid cultures were grown exponentially for at least eight generations, at densities below 10^7 cells/ml, before the start of the experiment. Immunofluorescence was performed as described previously (31). For actin and microtubule staining, we used AlexaFluor 488 phalloidin (Molecular Probes), a monoclonal antiantibody (Amersham/GE Healthcare, Piscataway, NJ), and a monoclonal antibotulin antibody (TATI; a kind gift of K. Gull from the Sir William Dunn School of Pathology at the University of Oxford, Oxford, United Kingdom). We used a CY3-conjugated anti-mouse antibody as a secondary antibody (Sigma, St. Louis, MO). Cells were immobilized on coverslips using phosphate-buffered saline containing antifade (Molecular Probes, Eugene, OR) as mounting medium and photographed using a Zeiss Axioshot microscope equipped with OpenLab (Improvision, Lexington, MA) software. To visualize mitochondria, 10 µl of 5 µM MitoTracker Red CMXRos (Molecular Probes) was added to 1 ml of fission yeast cell culture. The cell culture was then incubated at 32°C for 20 to 25 min while shaking. Cells were then visualized as described above.

**Cell wall digestion.** Cell wall resistance to the β-glucanase Zymolyase was determined as follows. Cells were grown to mid-logarithmic phase in minimal medium at 30°C. At that point cells were harvested, washed in Tris-EDTA buffer, and resuspended at an optical density of 600 nm (OD600) of 1.0 (2 × 10^7 cells) in the same buffer containing 100 µg/ml β-glucanase (Zymolyase 20T; ICN). Cell suspensions were incubated at 30°C while shaking, and cell lysis was monitored by OD492 measurement (4).

**Mitochondrial localization of Bot1p.** Mitochondria with intact outer membranes were prepared from cells carrying a copy of bot1-13Myc under the control of the endogenous promoter integrated at the bot1 locus (FV806), following the

![FIG. 1. Phenotype of a bot1 null mutant strain. A diploid strain (FV805) carrying a null mutation of bot1+ was exposed to sporulation conditions. Tetrads were dissected on yeast extract plates, and spores were allowed to germinate at 32°C. Cells micromanipulated from a bot1+ colony (a) and whole bot1Δ mutant cell colonies (b and c) at the indicated times are shown.](https://example.com/fig1.png)
method of Glick and Pon (15) except that Zymolyase 20T (ICN Biochemicals Inc., Aurora, OH) instead of Glusulase was used for the conversion of cells to spheroplasts. When required, mitochondria suspended at a protein concentration of 8 ng/ml in 0.6 M sorbitol–20 mM HEPES (pH 7.5) were disrupted by sonic irradiation using a VirSonic 100 sonicator at intensity 4 for 5 seconds. Proteins were precipitated by addition of an equal volume of 50% (v/v) trichloroacetic acid (TCA) diluted in phosphate-buffered saline, stained with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI), and visualized under a fluorescence microscope after excitation with UV light. To detect loss of mtDNA, total DNA was purified from wild-type and botΔ cells and digested overnight with the restriction enzyme HindIII. The DNA was separated on an agarose gel and transferred to nitrocellulose, and probed with antibodies against Myc (Abcam), subunit β of the mitochondrial ATP synthase (F1), and Tom70 (a kind gift of Mike Yaffe, UCSD, San Diego).

Mitochondrial ribosome preparation. Mitochondria with intact outer membranes were prepared by the method of Glick and Pon (15) from strain FV806. Samples of 4 mg of mitochondria were extracted in 400 μl of extraction buffer (20 mM HEPES, 0.5 mM PMSF, 500 mM KCl, 10 mM MgCl2, 2% digitonin) for 30 min in ice. Following a clarifying spin at 21,000 rpm (Beckman TL110 rotor) for 15 min at 4°C, the supernatant was loaded onto a linear 20 to 40% sucrose gradient containing 50 mM HEPES, 500 mM KCl, 0.5 mM MgCl2, and 0.2% digitonin. The gradient was centrifuged at 28,000 rpm (Beckman SW55 Ti rotor) for 8 h at 4°C, and 14 fractions were collected from the bottom. The ribosome profile was obtained by measuring absorbance at 260 nm, and Bot1p-Myc was detected by Western blot analysis using an anti-Myc antibody (Abcam).

Oxygen consumption measurements in whole cells. Endogenous cell respiration rates were assayed in whole cells in the presence of either 2% glucose or 2% galactose and 0.1% glucose using a Clark-type polarographic oxygen electrode from Hansatech Instruments (Norfolk, United Kingdom) at 25°C as described previously (3). Cell respiration was inhibited with 700 μM potassium cyanide (KCN), a specific inhibitor of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain. The specific activities reported were corrected for KCN-insensitive respiration.

Mitochondrial protein synthesis in vivo. Mitochondrial protein synthesis was followed in vivo using a method previously established for radiolabeling of mitochondrial products in S. cerevisiae (3), with a few modifications. Briefly, cells were grown to an absorbance of 0.5 at 595 nm (10^7 cells/ml) in 50 ml of medium containing yeast nitrogen base without amino acids, 2% galactose, and 0.1% glucose. Cells (3 × 10^7) were harvested, pelleted by centrifugation, washed with 1 ml of 40 mM potassium phosphate (pH 6.0) containing 2% galactose and 0.1% glucose, and resuspended in 500 ml of the same buffer supplemented with 10 mg/ml cycloheximide to inhibit cytoplasmic protein synthesis. The cells were incubated with 6 μl of [35S]methionine at 30°C for 15 min (10 ml/cm^2); Amares/Sh GE Healthcare, Piscataway, NJ). After pulses ranging from 15 min to 2 h, the reactions were terminated by addition of 75 μl of a solution containing 1.8 M NaOH, 1 M β-mercaptoethanol, and 0.01 M PMSF and diluted with 500 μl of H2O. Proteins were precipitated by addition of equal volume of 50% trichloroacetic acid. The mixture was centrifuged, and the pellet was washed once with 0.5 M Tris base and once with water and resuspended in 30 μl of sample buffer (26). The radiolabeled proteins were separated on a 17.5% polyacrylamide gel, transferred to nitrocellulose, and visualized by overnight exposure on Kodak X-OMat film.

mtDNA visualization. Cells were fixed with ice-cold methanol, washed and diluted in phosphate-buffered saline, stained with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI), and visualized under a fluorescence microscope after excitation with UV light. To detect loss of mtDNA, total DNA was purified from wild-type and botΔ cells and digested overnight with the restriction enzyme HindIII. The DNA was separated on an agarose gel and transferred to a nitrocellulose membrane. The membrane was hybridized sequentially with two different probes, which span the N-terminal 1,000-bp fragment of the mitochondrial cox1 gene (forward primer, ATGAAGCTTTGTTGGGACTTATGGTTAATAAGA; reverse primer, ATTTTTTGTAGTAAAGGGAAATGCTCAAA) and the complete ORF of the nuclear rps4 gene (forward primer, AAT GTGCGACATGAAATTGGGCTGACACT; reverse primer, TAAACCAGG GATCCGGGCAAAAGCCATGACGTACCA). The membrane was hybridized sequentially with two different probes, which span the N-terminal 1,000-bp fragment of the mitochondrial cox1 gene (forward primer, ATGAAGCTTTGTTGGGACTTATGGTTAATAAGA; reverse primer, ATTTTTTGTAGTAAAGGGAAATGCTCAAA) and the complete ORF of the nuclear rps4 gene (forward primer, AAT GTGCGACATGAAATTGGGCTGACACT; reverse primer, TAAACCAGG GATCCGGGCAAAAGCCATGACGTACCA). The membrane was hybridized sequentially with two different probes, which span the N-terminal 1,000-bp fragment of the mitochondrial cox1 gene (forward primer, ATGAAGCTTTGTTGGGACTTATGGTTAATAAGA; reverse primer, ATTTTTTGTAGTAAAGGGAAATGCTCAAA) and the complete ORF of the nuclear rps4 gene (forward primer, AAT GTGCGACATGAAATTGGGCTGACACT; reverse primer, TAAACCAGG GATCCGGGCAAAAGCCATGACGTACCA). The membrane was hybridized sequentially with two different probes, which span the N-terminal 1,000-bp fragment of the mitochondrial cox1 gene (forward primer, ATGAAGCTTTGTTGGGACTTATGGTTAATAAGA; reverse primer, ATTTTTTGTAGTAAAGGGAAATGCTCAAA) and the complete ORF of the nuclear rps4 gene (forward primer, AAT GTGCGACATGAAATTGGGCTGACACT; reverse primer, TAAACCAGG GATCCGGGCAAAAGCCATGACGTACCA). The membrane was hybridized sequentially with two different probes, which span the N-terminal 1,000-bp fragment of the mitochondrial cox1 gene (forward primer, ATGAAGCTTTGTTGGGACTTATGGTTAATAAGA; reverse primer, ATTTTTTGTAGTAAAGGGAAATGCTCAAA) and the complete ORF of the nuclear rps4 gene (forward primer, AAT GTGCGACATGAAATTGGGCTGACACT; reverse primer, TAAACCAGG GATCCGGGCAAAAGCCATGACGTACCA).
After 44 h of incubation in medium containing thiamine, the actin dots in most cells appear to be redistributed throughout the cell cortex, although they are still localized to the cell tips in a few cells (see cell at the top of the micrograph) (Fig. 2A, panel p). We found that 71% of the cells display actin patches redistributed throughout the cell cortex, while 29% still show a distribution of the actin patches close to the cell tips (n = 33). In contrast, the distribution of microtubules does not appear to be altered by the decrease of Bot1p levels (Fig. 2A, panels i to l).

Finally, since loss of Bot1p leads to an abnormal cell shape and an alteration of the actin cytoskeleton, we asked whether other morphological parameters are altered in bot1Δ mutants. We found that bot1Δ mutant cells expressing low levels of Bot1p (44 h after thiamine addition) are substantially more resistant to digestion by Zymolyase, a 1,3-β-D-glucan hydrolase, than wild-type cells or bot1Δ cells expressing Bot1p in the absence of thiamine (Fig. 2B). Resistance to Zymolyase digestion suggests an alteration of the β-glucan composition in the walls of cells expressing low levels of Bot1p protein.
Bot1 protein localizes to the mitochondria. The bot1 gene (GenBank accession number AF352796; locus SPBC14C8.16c) encodes a novel 315-amino-acid protein. A BLAST search (NCBI) identified homologues in other systems but restricted to fungi (Fig. 3). Among them were one in *Saccharomyces cerevisiae* (ORF YGR165w; 23% identity and 43% similarity over 270 amino acids of alignment) and one in *Candida albicans* (Gen Bank accession number XP_714982.1, locus CaO19.11043 joined to CaP19.11044; 24% identity and 43% similarity over 201 amino acids). The *C. albicans* and *S. cerevisiae* homologues are closer in similarity to each other (39% identity and 54% similarity over the whole length) than to *S. pombe* Bot1p (Fig. 3A). Homologues of Bot1p are also found in other fungi, such as *Yarrowia lipolytica*, *Kluyveromyces lactis*, *Candida glabrata*, *Ustilago maidis*, and *Neurospora crassa*. A phylogenetic tree is presented in Fig. 3B. Although the function(s) of these genes is unknown at present, the *S. cerevisiae* putative homologue, YGR165w, has been identified as a putative component of the small mitochondrial ribosomal subunit by Tag-based affinity purification (TAP)-mediated isolation of yeast mitochondrial ribosomes followed by mass spectrometric analysis and has been named MrpS35p (14).

Thus, to investigate the subcellular localization of Bot1p, we used a strain carrying the endogenous *bot1* gene tagged with a sequence encoding GFP. We found that the Bot1p-GFP protein (Fig. 4A, panel a) colocalizes with mitochondria visualized with Mitotracker Red (Fig. 4A, panel b). Cellular subfractionation also allowed us to localize a Myc-tagged version of Bot1 in the mitochondrial fraction but not in the postmitochondrial supernatant, which contains soluble cytoplasmic proteins (Fig. 4C; comparison between mitochondrial fraction and postmitochondrial supernatant). Since the homologue of fission yeast *bot1* in *S. cerevisiae*, YGR165w, encodes a putative component of the small mitochondrial ribosomal subunit, we performed two different assays to determine the intramitochondrial localization of Bot1p. First, we determined the intramitochondrial Bot1p localization by performing a proteinase K protection assay on mitochondria purified from cells expressing the *bot1* gene tagged with a sequence encoding the Myc epitope under the control of the endogenous promoter (strain FV806; Fig. 4B). Consistent with an intramitochondrial localization of Bot1p, we found that Bot1p-Myc is protected from proteinase K digestion in intact mitochondria (Fig. 4B). Next, we tested the association of Bot1p with the mitochondrial ribosome (Fig. 4C). We solubilized highly purified mitochondria with digitonin as described in Materials and Methods and separated the soluble contents on a 20 to 40% sucrose gradient (Fig. 4C). After the gradient was fractionated, the ribosome profile was obtained by measuring absorbance at 260 nm (bottom) and Bot1p-Myc was detected by Western blot analysis of the different fractions using an anti-Myc antibody (top). Our results
indicate that fission yeast Bot1p-Myc cosediments with the small mitochondrial ribosomal subunit.

To determine to what extent the Myc- and GFP-tagged Bot1 fusion proteins complement the bot1 phenotype, we analyzed the cell shape, growth rate, and cell respiration rate in wild-type, FV806, and FV811 strains. We determined that cells expressing Bot1p-Myc or Bot1p-GFP display cell shapes, cell growth rates, and cell respiration rates similar to those of wild-type cells (not shown).

The association of Bot1p with the mitochondrial ribosomes suggested that this protein could be involved in mitochondrial protein synthesis. Thus, to test mitochondrial protein translation rates, we followed the incorporation of radioactively labeled [35S]methionine into newly synthesized mitochondrial proteins in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis, in cells grown in the presence of 2% galactose and 0.1% glucose. The assay, commonly used in studies on S. cerevisiae (2), has been specifically adapted to S. pombe cells and is described in Materials and Methods. We found that cells expressing Bot1p-Myc incorporate labeled methionine into mitochondrial proteins to 70% of the levels found in wild-type cells, while cells expressing Bot1p-GFP incorporate methionine to 50% of the levels in wild-type cells (Fig. 4D). Our findings indicate that the GFP and Myc fusion proteins are able to fully complement the loss of morphology and viability observed in cells lacking Bot1p. Conversely, we found that mutants exclusively expressing GFP- or Myc-tagged versions of the Bot1 protein display, to different degrees, a reduced rate of mitochondrial translation. These observations and the fact that Bot1p localizes to the small subunit of the mitochondrial ribosome prompted us to test the effect of Bot1p loss on mitochondrial morphology and mitochondrial protein translation (Fig. 5).

Loss of Bot1p leads to mitochondrial fragmentation, decreased mitochondrial protein translation, and decreased respiration. To test for the effects of Bot1p loss on mitochondrial function, we visualized mitochondria in a bot1Δ strain stably expressing GFP-tagged Bot1p under the control of the nmt1(81) promoter using the cell-permeant dye Mitotracker Red, which specifically accumulates into mitochondria in a membrane potential-dependent manner. In the absence of thiamine, cells and mitochondria have a phenotypically normal appearance (Fig. 5A, panels a and d), with 79% of the cells displaying a continuous, unfragmented mitochondrial network, similarly to wild-type cells (n = 50). After 26 h of exponential growth in medium containing thiamine, we found that the mitochondrial network becomes less continuous and more fragmented in a large percentage (50%) of cells (Fig. 5A, panel b). After 48 h the mitochondrial network appears completely fragmented in 100% of cells (n = 50) (Fig. 5A, panel c) and cells display a characteristic loss of normal morphology (Fig. 5A, panel f). Furthermore, we observed that Mitotracker Red incorporation was diminished in the 48-h sample (Fig. 5A, panel c). Since incorporation of Mitotracker Red is dependent on mitochondrial membrane potential, these observations suggest a decline in membrane potential in the 48-h sample.

Abnormal mitochondrial morphology/distribution and membrane potential decline are usually accompanied by lesions in the mitochondrial respiratory chain, affecting the ability of the cells to respire. We analyzed the oxygen consumption capacity of bot1Δ cells (Fig. 5B). Cells were grown exponentially in two different growth media, one containing 2% galactose and 0.1% glucose and one containing 2% glucose (Fig. 5B). We found that even in the absence of thiamine, cell respiration is substantially decreased (approximately to 25% of wild-type values) in bot1Δ mutants expressing Bot1p-GFP, again suggesting that the addition of the large GFP tag to Bot1p significantly impairs its role in regulating mitochondrial respiration. GFP-tagged Bot1p is stable and readily accumulates in the absence of thiamine (Fig. 5B, inset, lanes 2 and 6).

Although we did not obtain data concerning the turnover of Bot1p-GFP, our results showed that after 24 h of thiamine supplementation to the medium, the detected residual levels of the protein are below 10% of the levels at time zero (Fig. 5B, inset, lanes 3 and 7), and further decrease to virtually undetectable levels after 48 h of incubation in the presence of thiamine (Fig. 5B, inset, lanes 4 and 8). Following thiamine addition, cell respiration further diminishes, particularly in the 2% glucose culture (Fig. 5B, 26- and 48-h samples).

To establish whether Bot1 plays a role in mitochondrial protein synthesis, we followed the incorporation of radioactively labeled [35S]methionine into newly synthesized mitochondrial proteins in vivo in bot1Δ pJK nmt81 bot1GFP cells grown in the presence of 2% galactose and 0.1% glucose (Fig. 5C). We found that already in the absence of thiamine, which allows expression of Bot1p-GFP from the attenuated nmt81
promoter, the \( \text{bot}1 \Delta \) strain shows low levels of incorporation of \([\text{\textsuperscript{35}}S]\)methionine into mitochondrial gene products (one-seventh of wild-type levels for Cox1/Cytb and one-ninth for Atp8/9), demonstrating that mitochondrial protein translation is substantially less efficient in this strain than in the wild-type control. Following thiamine addition, mitochondrial protein translation progressively decreases further, as shown in the 26-h and 48-h samples (Fig. 5C). We found that in \( \text{bot}1 \Delta \) cells expressing Bot1p-GFP under the control of the \( nmt81 \) promoter, Cox2/Cytb protein levels further decrease 3.5-fold and Atp8/9 protein levels further decrease 2.8-fold, compared to initial levels, at 48 h after thiamine addition. These observations indicate that decreasing Bot1p levels negatively impact mitochondrial protein translation.

Mitochondrial translational mutants of \( S. \text{cerevisiae} \) (2) have a tendency to lose their mtDNA and survive by fermentation, but this not the case for \( S. \text{pombe} \), which does not tolerate the absence either of mtDNA or of respiratory function. Furthermore, for the yeast \( S. \text{cerevisiae} \) it has been recently shown that some mitochondrial proteins are essential for mtDNA stability.

FIG. 5. Decreased levels of Bot1p disrupt mitochondrial structure and function. (A) Mitochondrial morphology and distribution. A \( \text{bot}1 \Delta \) strain expressing Bot1p-GFP under the control of the \( nmt1 \) promoter (FV809) was grown in the absence of thiamine (a and d) or in the presence of thiamine for 26 h (b and e) or 48 h (c and f) at 32°C. Mitochondria were visualized using Mitotracker Red. The cell wall was visualized with Calcofluor. Bar, 5 \( \mu \text{m} \). (B) Endogenous cell respiration. Oxygen consumption was measured in wild-type (WT) and \( \text{bot}1 \Delta \) mutant cells grown in the absence of thiamine or treated with thiamine for 24 and 48 h using a Clark-type polarographic oxygen electrode from Hansatech at 25°C as described previously (2). Cells were grown in minimal medium containing either 2% galactose and 0.1% glucose or 2% glucose, and the respiratory assays were performed in the same media. The specific activities reported were corrected for KCN-insensitive respiration. The bars indicate the means ± standard deviations from at least three independent sets of measurements. In the inset is shown a Western blot analysis of the Bot1p-GFP levels in the corresponding strains. Lanes 1 and 5, wild-type 972; lanes 2 and 6, \( \text{bot}1 \Delta \) pJK \( \text{bot}1 \)-GFP (without thiamine); lanes 3 and 7, \( \text{bot}1 \Delta \) pJK bot1-GFP (with thiamine, 24 h); lanes 4 and 8, \( \text{bot}1 \Delta \) pJK bot1-GFP (with thiamine, 48 h). Lanes 1, 2, 3, and 4, minimal medium with 2% galactose and 0.1% glucose; lanes 5, 6, 7, and 8, minimal medium with 2% glucose. (C) In vivo labeling of mitochondrial gene products. Wild-type and \( \text{bot}1 \Delta \) mutant cells grown in the absence of thiamine or treated with thiamine for 26 and 48 h were labeled with \([\text{\textsuperscript{35}}S]\)methionine at 30°C for the indicated amount of time in the presence of cycloheximide as described in Materials and Methods. The \( S. \text{cerevisiae} \) W303-1A wild-type strain was labeled and run in parallel to facilitate the identification of the translation products. The mitochondrial translation products are identified in the margin. The \( S. \text{cerevisiae} \) Var1p ribosomal protein and the \( S. \text{pombe} \) Rps3p ribosomal protein have different sizes and consequently exhibit different electrophoretic mobilities. (D) mtDNA stability. (a) \( \text{bot}1 \Delta \) cells grown in the absence of thiamine, expressing Bot1p-GFP under the control of the \( nmt81 \) promoter (FV809). (b) FV809 bot1Δ cells grown in the presence of thiamine for 48 h. To detect mtDNA in vivo, cells were labeled with DAPI as explained in Materials and Methods and observed under a fluorescence microscope. (c) Southern blot detecting the mitochondrial gene \( \text{cox}1 \) and the nuclear gene \( \text{rga}4 \). (d) Quantification of band intensity, expressed as signal ratio of \( \text{cox}1 \) to \( \text{rga}4 \), in the Southern blot shown in panel c.
by interacting with mitochondrial nucleoids (11, 20). Reduced mtDNA levels in bot1Δ mutants could also explain the defective mitochondrial translation phenotype observed in this strain. To explore the mtDNA content in bot1Δ mutants, we stained wild-type and mutant cells with DAPI and visualized the mitochondrial nucleoids under a fluorescence microscope. After observing more than 300 cells, we concluded that the nucleoids are still present in bot1Δ cells grown in the presence of thiamine for 48 h (Fig. 5D). We confirmed these findings by observing that the ratio of the mitochondrial genome to the nuclear genome does not decrease in bot1Δ cells during culture in the presence of thiamine, as shown by detecting the mitochondrial gene coxl and the nuclear gene rga4 by Southern blotting.

Thus, our findings collectively showed that loss of Bot1p leads to mitochondrial fragmentation, decreased mitochondrial protein synthesis, and decreased cell respiration and demonstrated that Bot1p plays a direct role in the assembly and/or function of the mitochondrial translational apparatus.

**Overexpression of Bot1p leads to increased cell size and increased cell respiration.** Since loss of Bot1p leads to changes in cell length and cell size (Fig. 2A, panel d), to mitochondrial fragmentation, and to decreased cell respiration, we asked whether Bot1p overexpression would also affect these parameters. Indeed, we found that bot1+ cells containing a multicopy plasmid expressing Bot1p from the full-strength nmt1 promoter are substantially longer (Fig. 6A, panel c) than control cells carrying an empty plasmid (Fig. 6A, panel a). Cells overexpressing Bot1p are on average 160% longer than control cells (n = 100), and cell elongation is consistent with a delay in G2, as observed by fluorescence-activated cell sorter analysis (not shown). In cells overexpressing Bot1p, the appearance of the mitochondrial network is not significantly altered (Fig. 6A, panel d) compared to control cells (Fig. 6A, panel b) suggesting that Bot1p overexpression does not negatively affect mitochondrial structure. The actin cytoskeleton structure also is unaffected (not shown). Cell respiration is slightly increased in cells overexpressing Bot1p, by a small (~8 to 10%) but significant amount in cells grown in the presence of 2% glucose (P < 0.05) (Fig. 6B). The efficiency of mitochondrial protein synthesis and the pattern of the mitochondrial translation products are mostly unaffected by Bot1p overexpression (Fig. 6C). The only exception is the appearance of an extra band corresponding to a protein of electrophoretic mobility between those of Atp6 and Rps3, which does not correspond to an identified mitochondrial encoded protein sequence (Fig. 6C). To analyze Bot1 localization when overexpressed, we expressed Bot1-GFP from the same plasmid. In these conditions, Bot1-GFP is expressed at a fourfold-higher level than in a strain expressing Bot1-GFP under the control of the endogenous promoter (strain FV811) (not shown). We found that overexpressed Bot1-GFP is still localized to the mitochondria (Fig. 6D, panel b). Thus, our findings indicate that Bot1p overexpression leads to an increase in cell size.

**DISCUSSION**

In this paper, we present the identification of a novel *Schizosaccharomyces pombe* protein, Bot1p, that is conserved among fungi and plays a fundamental role in mitochondrial protein synthesis by associating with mitochondrial ribosomes. Consistent with this function, it also plays a role in the regulation of mitochondrial integrity and cell respiration. Bot1p is a protein conserved among other fungi and yeasts, and it is related to the *S. cerevisiae* protein MrpS35p. Interestingly, MrpS35p has been identified in a tag-mediated isolation of proteins associated with the small subunit of the yeast mitochondrial ribosome (14). Neither the Bot1p nor the MrpS35p protein has sequence similarity to any previously known ribosomal proteins (14). It should also be noted that neither Bot1p nor MrpS35p is related to human MRPS35 (23). *S. cerevisiae* MRPS35 deletion mutants fail to grow on a nonfermentable carbon source (14); however, no further detailed characterization has been yet performed on any protein of this family.

We show that *S. pombe* Bot1p is localized exclusively to mitochondria as shown both by in vivo localization of a GFP-tagged version of the protein under a fluorescence microscope and by Western blot analysis of a Myc-tagged version of the
protein, both expressed at the endogenous level. Furthermore, we found that it associates with the small subunit of the mitochondrial ribosome and it is required for mitochondrial protein synthesis. Consistent with a role in mitochondria protein translation, decreased levels of Bot1p lead to loss of mitochondrial network continuity and integrity. This is not an effect of a disrupted microtubular network, which has a role in the localization of mitochondria, as previously described (1, 46), since the microtubule pattern in bot1Δ mutants appears to be normal. Furthermore, we show that decreased levels of Bot1p lead to decreased respiration and decreased uptake of Mitotracker Red in the mitochondria, suggesting a reduced mitochondrial membrane potential. The cell respiration defect results from a defect in the mitochondrial ability to translate proteins as demonstrated by our in vivo protein synthesis experiments. Our results represent the first characterization of the mitochondrial protein translation function of Bot1p/MrpS35.

At this point, we have not established whether Bot1p is a structural component of the ribosomal small subunit or a ribosome assembly factor. In the yeast Saccharomyces cerevisiae, at least 34 small-subunit and 49 large-subunit mitochondrial ribosomal proteins (Mrps) have been described (14). While not all have been fully characterized, some also have other cellular functions: for example, in yeast, MrpL31 and Ygl068 may be involved in cell cycle control (39). Additionally, factors have been found which do not associate tightly with the ribosomes but are involved in ribosome biogenesis (2). Others proteins are also involved in the complex processing steps that modify rRNAs and that are required for the assembly of a functional ribosome (9, 37). Thus, future experiments will address the role of Bot1p in ribosomal structure, biogenesis, and translation control.

We found that strains carrying null alleles of bot1 show progressive alterations in cell shape. Forty-eight hours following spore germination, cells are longer, enlarged, and assume a characteristic bottle-shape form. This phenotype was recapitulated in mutants expressing reduced levels of Bot1p, where changes in cell shape correlate with an alteration of the actin cytoskeleton and increased resistance to glucanase digestion, indicating an alteration of cell wall structure (10, 17). Thus, our observations are consistent with the notion that loss of Bot1p and mitochondrial function, by inhibiting cell respiration, affects cellular processes that require a high energetic level, such as cytoskeleton dynamics and cell wall formation. The particular shape of these mutant cells likely reflects cellular processes that are very sensitive to energy deprivation, particularly in an organism like Schizosaccharomyces pombe, which is compulsorily aerobic. It is also possible that loss of respiration may not be the only cause of shape alteration. Indeed, pharmacological inhibition of the mitochondrial respiratory chain with the cytochrome c oxidase inhibitor KCN did not induce a similar alteration of the cell shape and instead produced uniformly small cells (F. Verde, unpublished data). Respiratory mutants were not previously reported as presenting a cell morphology alteration (7, 13). In mutants of mspl, which encode a dynamin-related protein involved in mitochondrial maintenance, an alteration of cell morphology is clearly not observed (18). Thus, it is possible that the morphological phenotype of bot1Δ cells reflects the disruption of other mitochondrial functions, for example, their important role in the biosynthesis of fatty acids (29).

An interesting observation is the fact that bot1Δ mutant cells also appear to be not only wider but also longer, suggesting a delay in cell cycle progression. Indeed, we found that such an increase in cell volume did not occur in a mutant with a mutation of the cell cycle regulatory kinase Wee1p (see Fig. S1 in the supplemental material). Thus, these findings suggest that in fission yeast low energy levels trigger a cell cycle arrest that is regulated by cell cycle control factors. It is interesting to note that a p53-dependent cell cycle checkpoint monitoring the metabolic activity of mitochondria has been described in mammalian cells and Drosophila, where it is thought to promote cell survival by blocking the commitment to another round of cell division (reviewed in reference 29). It will be interesting to establish in the future which mechanism is involved in such a cell cycle delay in Schizosaccharomyces pombe.

Interestingly, Bot1p overexpression also leads to cell elongation, slower growth, and a cell cycle delay in G2. This effect is likely not due to an effect either on the microtubular and actin network or on mitochondrial function, since the mitochondrial web appears to be intact and mitochondrial protein synthesis is not affected. Actually, in Bot1p-overexpressing cells, cell respiration is increased by a small but significant amount in cells grown in the presence of 2% glucose, which are aerobically repressed by 50%. This increase is interesting since it suggests that Bot1p, which is necessary for mitochondrial protein translation, may also positively regulate cell respiration when its levels are increased or, alternatively, decrease cell sensitivity to glucose-dependent repression of respiration. Also, it is possible that the effects of overexpression may reflect a function of Bot1p in mitochondrial signaling. Recent evidence suggests that mitochondria interact in several way with other cellular functions and that they are part of multiple cell signaling cascades (reviewed in reference 29). Furthermore, several mitochondrial factors, generally when overexpressed, have been found to interact with cell cycle, cell growth, or checkpoint control functions (6, 24, 34, 35, 39, 48). For example, in mammalian cells, the mitochondrial ribosomal protein MrpL41 has a role in stabilizing p53, p27KIP1, and p21WAF1/CIP1 (24, 48), and mitochondrial ribosomal protein MRPS36 overexpression delays cell cycle progression by inducing p21WAF1/CIP1 expression and regulating the expression and phosphorylation of p53 (6). Future experiments, addressing which cell size/cell cycle control pathway(s) is affected by Bot1 under- and overexpression, may shed light on the signaling mechanisms that govern cell growth in response to metabolic changes in fission yeast.

In conclusion, we presented the characterization of a novel factor, Bot1, which associates with mitochondrial ribosomes and is essential for mitochondrial protein translation in fission yeast and whose loss affects mitochondrial integrity, cell respiration, and cell morphology. Future analysis of the function of Bot1 and other proteins involved in mitochondrial protein translation will help decipher the interdependence between mitochondrial protein synthesis, cell respiration, and the overall control of cell growth.

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