Instability of the Mitofusin Fzo1 Regulates Mitochondrial Morphology during the Mating Response of the Yeast Saccharomyces cerevisiae*

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Mitochondria form a highly dynamic network that is shaped by continuous fission and fusion of these organelles. In the yeast Saccharomyces cerevisiae two machineries are involved in this process, one of which includes the mitochondrial fusion promoting GTPase Fzo1. Although a role for the F-box protein Mdm30 in regulating the stability of Fzo1 has been proposed, the molecular basis for the regulation of the fission to fusion ratio of mitochondria remains unknown. To discern the mechanism of the regulation of mitochondrial morphology, we arrested cells at different stages of the cell cycle and examined mitochondrial morphology as well as the stability of mitochondrial fission and fusion proteins. In response to a G1 arrest evoked by the mating pheromone α factor the mitochondrial network fragmented into small pieces, which was accompanied by dramatic down-regulation of Fzo1. Mating pheromone also triggered the degradation of Fzo1 produced under the control of a constitutive promoter, and Fzo1 was stabilized upon proteasome inhibition, indicating a role for the proteasome system in the degradation of Fzo1. However, deletion of MDM30 did not stabilize Fzo1 after mating pheromone treatment, showing a different mechanism from the previously reported process of steady state Fzo1 regulation. We show an example for a regulated change of the mitochondrial fission to fusion ratio during the life cycle of budding yeast. Proteasomal degradation of Fzo1 in response to the mating pheromone is proposed to mediate the remodeling of the mitochondrial network during the process of mating.

In the yeast Saccharomyces cerevisiae, mitochondria consist of long tubules that are located in the cell periphery. Because of constant fission and fusion of individual mitochondria, they form a dynamic mitochondrial network. Although many proteins have an impact on mitochondrial morphology (1), six proteins seem to build up the core mitochondrial fission and fusion machinery (2). Mitochondrial fusion is governed by the large outer mitochondrial membrane GTPase, Fzo1 (3, 4), the outer mitochondrial membrane protein, Ugo1 (5), and the dynamin-related GTPase, Mgm1, which is located in the intermembrane space (6). Deletion of either of these genes results in the loss of fusion-competent mitochondria and, because of ongoing mitochondrial fission, the mitochondria fragment into small pieces that eventually form clumped mitochondrial masses (7). Mitochondrial fission is mediated by the large dynamin-related GTPase Dnm1 (8), the adaptor protein Mdv1, and the outer mitochondrial membrane protein Fis1. Mutation of either of these fission proteins leads to highly interconnected, often net-like mitochondria. Interestingly, mutation of the fusion machinery together with the fission machinery restores the wild type mitochondrial morphology. However, this reflects a static morphology rather than the dynamic equilibrium of ongoing mitochondrial fission and fusion in wild type budding yeast.

Mitochondrial morphology in budding yeast is able to adapt to nutrition status or growth phase (9). Growth on respiratory substrates, such as glycerol, leads to a more elaborate mitochondrial network, probably because of an increased need for oxidative phosphorylation. Reaching the stationary growth phase causes fragmentation of mitochondria. However, the regulation of the activity of the mitochondrial fission and fusion machinery that governs these physiological morphological changes remains largely unknown.

The proteins of the fission and fusion machinery are widely conserved from yeast to man. Drosophila melanogaster possesses two orthologs of the yeast mitofusin Fzo1, one of which is the founding member of the family of mitofusins, Fuzzy onions (fzo), that is involved in the generation of a specialized mitochondrion during sperm development. The other, Drosophila dmfn, seems to be a more general mitofusin and is expressed in many cell types (10). Mammals have the two mitofusins Mfn1 and Mfn2 (11), both of which are involved in maintaining mitochondrial morphology (12). Opa1 (Mgm1 in yeast), another dynamin homologue, also participates in mitochondrial fusion. To date, the mitochondrial fission machinery in mammals consists of the Dnm1 ortholog Drp1 and the Fis1 ortholog hFis1 (13). Drp1 and Fis1 are involved not only in the fission of mitochondria but also in apoptosis (14–17). Although the importance of a proper balance between mitochondrial fission and fusion is underlined by diseases such as dominant optic nerve atrophy, caused by mutations in Opa1 (18), and Charcot-Marie-Tooth neuropathy, resulting from mutations in Mfn2 (19), it remains speculative how constant mitochondrial fission and fusion facilitate eukaryotic cell viability. Revealing the regulatory mechanisms of mitochondrial morphology in the budding yeast may help us to understand the molecular basis of these diseases.

To investigate a possible involvement of the E31 ubiquitin

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ligase SCF and proteasome-dependent protein degradation in the regulation of fission and fusion of mitochondria, we arrested cells at different points of the cell cycle and analyzed the stability of the reported SCF substrate Fzo1 (20) and other proteins involved in mitochondrial fission and fusion. Unexpectedly, we found that treatment with the yeast mating factor induces the degradation of Fzo1 and the fragmentation of mitochondria independently of SCF^Mat30^ (23). The stability of other components of the mitochondrial fission and fusion machinery is unaffected after activation of the pheromone signaling pathway, suggesting a new regulatory mechanism for a physiologically induced mitochondrial morphology rearrangement.

MATERIALS AND METHODS

**Yeast Procedures**—Standard procedures were used for mating, transformation, sporulation, and tetrad dissection of yeast cells. All strains used were W303 (MATa ade1-2 can1-100 his3-11 leu2-3 trp1-1 ura3-52). Cells were grown at 26 °C in complex XY medium (20 g/liter bacto peptone, 10 g/liter yeast extract, 10 mM KH2PO4, 0.2 g/liter tryptophan, and 0.1 g/liter adenine) supplemented with 2% glucose. Cells containing plasmids were grown in synthetic complete medium with glucose lacking the appropriate nutrient. Cells were treated with 50 ng/ml α factor (Sigma), 50 μM MG132 in 1% MeSO (Sigma), or 15 μg/ml nocodazole in 1% MeSO (Sigma).

**DNA Constructs and Genetic Manipulations—**3xHA- or 13xMyc-tagged versions of Fzo1, Mdv1, Dnm1, and Fis1 were generated using the pFA-3xHA-KANmx or pFA-13xMyc-KANmx plasmids (21) and the following synthetic oligonucleotides: 5’-TATTATGTGTTGAAAGACCGTCATATTTAACAAATGATTTAATTTGATTTTACACGGGTTAATTAA-3’ and 5’-TCTAGCTCGAGCTGCTTTTACCAAA-3’ and 5’-TCCCTCGAGGCTTTACCAAA-3’ and 5’-TATTATGTTGAAAGACCGTCATATTTAACAAATGATTTAATTTGATTTTACACGGGTTAATTAA-3’. The pAN113 was cut with EcoRI/HindIII and transformed into yeast to generate Δmdm30 strains. To generate TEF2MyC/FZO1, FZO1 was amplified using 5’-CTAGCTCGAGCTGCTTTTACCAAA-3’ and 5’-CTAGCTCGAGCTGCTTTTACCAAA-3’ and cloned SpeI/KpnI into pBluescript. A NdeI/BamHI fragment was replaced with the 3xHA-tag, and the CYC1 terminator. The FZO1 sequence was verified by sequencing.

**Protein Analysis**—For preparation of protein lysates, cells were harvested by centrifugation and washed once with ice-cold water, resuspended in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris/HCl, pH 7.5, 50 mM NaF, 5 mM EDTA, 0.1% IGEPAL CA-630, and 1% Triton X-100), supplemented with protease inhibitor mix (Sigma), and mixed with acid-washed glass beads. The cells were broken by shaking in a mixer mill (Retsch) for 5 min. Cell debris was removed by a 3-min centrifugation at 4 °C. Protein lysates were heated to 98 °C with 2× SDS loading buffer for 5 min. Protein lysates were analyzed by Western blot using mouse monoclonal antibodies 12CA5 (Roche Applied Science) and 9E10 (Roche Applied Science), anti-porin antibody (Molecular Probes), and horseradish peroxidase-coupled anti-mouse antibodies (Amer sham Biosciences).

**Microscopy**—Cells for confocal microscopy were fixed with 3.7% formaldehyde (Sigma) for 10 min, washed in phosphate-buffered saline, and embedded in 0.2% agarose into German borosilicate chamber slides (Nunc). Pictures were taken using an LSM510 Meta (Zeiss) equipped with a 100× oil immersion objective. Confocal pictures were projected into one plane using LSM 5 image browser (Zeiss) and overlaid using Photoshop (Adobe).

**RESULTS**

**Fzo1 Is Unstable in a Factor-arrested but Not in Nocodazole-treated Cells**—It was previously shown that Fzo1 stability can be regulated by the F-box protein Mdm30 (20). F-box proteins are substrate recruiting factors for the E3 ubiquitin ligase complex SCF (24), an important factor in controlling major cell cycle transitions. Cell cycle-dependent phosphorylation of SCF substrates often targets them for degradation. Because mitochondria in mammalian cells had been reported to fragment during cell division through an unknown mechanism, we examined the stability of a functional 3xHA-tagged version of Fzo1 in different cell cycle stages. The mating pheromone α factor was used to arrest cells in the G1 phase of the cell cycle. As shown in Fig. 1A, 5 h after treatment with a factor Fzo1 was no longer detectable, whereas Fzo1 was stable in untreated control cells.

To examine the stability of Fzo1 in M phase, we arrested cells at the metaphase to anaphase transition by evoking the spindle checkpoint with the spindle poison nocodazole and compared them with cells treated with a factor. As shown in Fig. 1B, untreated cells showed stable Fzo1 levels, whereas nocodazole-treated cells had no Fzo1 detectable.
mito-GFP) were treated with highly fragmented mitochondria (Fig. 2A, lower panel). As expected from the stability of Fzo1 during spindle checkpoint activation, mitochondria in nocodazole-treated cells remained tubular and interconnected (Fig. 2B), showing that a cell cycle arrest per se does not lead to mitochondrial fragmentation. Deletion of DNMI leads to the formation of interconnected, fission-incompetent mitochondria (25). To examine the role of the mitochondrial fission machinery in the mating pheromone-evoked mitochondrial fragmentation, Δdnm1 cells were treated with α factor, and mitochondrial morphology was analyzed by confocal microscopy (Fig. 2C). 99% of untreated control cells showed long and tubular mitochondria, often with regions of condensed mitochondrial networks. During α factor treatment most of the cells (from 95% at 2 h to 85% at 6 h) still displayed this mitochondrial phenotype. Mitochondria were never fragmented into small round units as in wild type cells but were sometimes completely condensed without any tubules (from 5% at 2 h to 15% at 6 h). Thus, the observed mitochondrial fragmentation after pheromone treatment is mediated by the Dnm1-dependent mitochondrial fission machinery.

Mating pheromone evokes a G1 cell cycle arrest by activating the cyclin-dependent kinase inhibitor Far1 (26). Far1 then inactivates G1 cyclin-dependent kinase activity by binding to Cdc28-cyclin complexes. To further characterize the observed mitochondrial fragmentation in G1 arrested cells, we used a strain carrying the temperature-sensitive cdc28-4 allele and analyzed the mitochondrial morphology using mito-GFP and confocal microscopy. The cdc28-4 allele allows growth at room temperature but leads to a G1 cell cycle arrest at 37 °C because of insufficient cyclin-dependent kinase activity. Mitochondrial morphology in 87% of cdc28-4 cells and in 93% of wild type control cells at room temperature was tubular (Fig. 2D). After a shift to 37 °C, 86% of the cdc28-4 cells displayed a fragmented and often clumped mitochondrial morphology, whereas only 20% of the wild type control cells showed an altered mitochondrial morphology.

Mating pheromone-induced cell cycle arrest, as well as the cdc28-4-mediated cell cycle block, is characterized by low cyclin-dependent kinase activity. Nocodazole treatment on the other hand leads to a cell cycle arrest with high cyclin-dependent kinase activity. Therefore, mitochondrial fragmentation is induced selectively by two independent processes that decrease the cyclin-dependent kinase activity. Because mitochondrial morphology depends on ongoing fission and fusion, mitochondrial fragmentation shows that fission activity prevails over fusion activity. This physiological alteration of mitochondria morphology by α factor that occurs during the yeast mating process correlates with the disappearance of Fzo1.

In higher eukaryotes, mitochondrial fragmentation is part of the cell death program (27, 28), and treatment with mating pheromone is a stimulus for programmed cell death in yeast (29). A recent study showed that mitochondrial fragmentation in yeast occurs after treatment with acetic acid and that inhibition of mitochondrial fission with a dominant negative inhibitor of Drp1 inhibits the cell death (17). Thus, the observed mitochondrial fragmentation after treatment with α factor may reflect the initiation of a programmed cell death pathway due to unsuccessful mating. Therefore, we treated cells of a wild type strain, a Δdnm1 strain, and a Δfis1 strain with high concentrations of α factor (100 μg/ml) for 7 h and compared the

**FIG. 2. Mitochondria fragment after treatment with α factor.** A, exponentially growing cells of strain yAN101 (mito-GFP Δbar1) were treated with α factor for 2, 4, and 6 h or left untreated (column 0) and processed for microscopy. To compare mitochondria after treatment with α factor with mitochondria in Δfzo1 cells, exponentially growing cells of strain yAN150 (Δfzo1 mito GFP Δbar1) were fixed for microscopy. Pictures were taken and processed with LSM 5 Image Browser (Zeiss). Mitochondrial morphology in cells (n > 200) was observed by microscopy and categorized as long mitochondria or fragmented and clumped mitochondria. B, cells of strain yAN101 (mito-GFP Δbar1) in early logarithmic phase were treated with nocodazole and processed for microscopy as described in A. C, cells of strain yAN124 (Δdnm1 Δbar1 mito-GFP) were treated with α factor and analyzed by confocal microscopy as described in A. D, logarithmic phase cells of strain yAN021 (mito-GFP Δbar1) and yAN039 (mito-GFP cdc28-4) were grown in XYD medium at 26 °C before shift to 37 °C. Cells were then processed for microscopy as described in A. wt, wild type.

1B, levels of Fzo1 in G1 cells were greatly diminished compared with the levels in asynchronously grown cultures. In contrast, Fzo1 levels were not influenced by nocodazole treatment. Even after a prolonged cell cycle arrest due to spindle checkpoint activation, Fzo1 levels were as high as in untreated control cells. These data indicate a mating pheromone-dependent regulation of the mitochondrial fusion machinery. The stability of Fzo1 after a long treatment with nocodazole shows that Fzo1 is not per se unstable in a prolonged cell cycle arrest.

**α Factor Treatment Influences Mitochondrial Morphology—** Because Fzo1 disappeared after treatment with α factor we examined the mitochondrial morphology in a factor-treated cells. The mitochondria of these cells were visualized using mitochondria-targeted GFP (kindly provided by B. Wester mann). Untreated control cells showed several elongated, sometimes branched, mitochondria (Fig. 2A, 0 h α factor, and B, 0 h nocodazole), often located on the periphery of the cell. In contrast, 38% of the cells treated with α factor for 2 h showed highly fragmented mitochondria (Fig. 2A). Longer incubation
survival of these cells by spotting serial dilutions onto agar plates (data not shown). We found no difference in the survival of these three strains. Because mitochondria do not fragment in pheromone-treated △△△△ cells (Fig. 2C), this result indicates that the observed mitochondrial fragmentation after α factor treatment plays no cell death promoting role.

**Fzo1 Levels Are Down-regulated upon α Factor Treatment in a Proteasome-dependent Manner**—To analyze the mechanism of Fzo1 down-regulation in response to α factor, Mdv1-Fzo1 was expressed under the control of the constitutive TEF2 promoter (30). Exponentially growing TEF2-Mdv1-Fzo1 cells were treated with α factor or left untreated. Mdv1-Fzo1 levels were analyzed by Western blotting. Fig. 3A shows that constitutively produced Mdv1-Fzo1 is unstable after treatment with α factor, whereas Mdv1-Fzo1 is stable in untreated control cells. This shows that Fzo1 levels after treatment with α factor are regulated in a posttranslational fashion. The mating pheromone-induced degradation of Fzo1 outweighs the ongoing constitutive expression from the unregulated TEF2 promoter.

A role for the F-box protein Mdm30 was proposed in the regulation of Fzo1 stability (20). To address whether a factor triggers Mdm30-mediated degradation of Fzo1, we constructed a ΔΔΔΔ strain containing a 3xHA-tagged version of Fzo1. Either ΔΔΔΔ or wild type cells were treated for 6 h with α factor. Fzo1 levels were analyzed using Western blot. As shown in Fig. 3B, the deletion of MDM30 does not prevent the α factor-triggered decrease of Fzo1. Fzo1 protein is down-regulated in ΔΔΔΔ cells with similar kinetics compared with wild type cells. To investigate a possible role of ubiquitin-mediated proteasome-independent degradation of Fzo1 after α factor treatment, we used the specific proteasome inhibitor MG132 (31). Treatment with MG132 greatly stabilized Fzo1 upon a factor treatment compared with control cells treated only with α factor and the solvent MeSO (Fig. 3C). This suggests that α factor activates a proteasome-dependent degradation of Fzo1. However, degradation of Fzo1 after treatment with α factor is not prevented by the deletion of MDM30. This indicates that two mechanisms involving proteasome-dependent degradation exist for the regulation of Fzo1 stability, one functioning during steady state regulation of mitochondrial morphology and a distinct pathway activated by α factor.

**Impact of α Factor Treatment on Other Components of the Mitochondrial Fission and Fusion Machinery**—Because treatment with α factor leads to a dramatic alteration in the balance between mitochondrial fission and fusion and greatly influences the levels of the mitochondrial proteins Fzo1, we determined whether this is reflected in the level of other mitochondrial fission or fusion proteins. To this end, we used strains producing 3xHA-epitope-tagged versions of Dnm1, Ugo1, and Mgm1 (kindly provided by J. Shaw) and 13xMyc epitope-tagged versions of Fis1 and Mdv1. Cells in early logarithmic phase were treated with α factor for 6 h. At different time points cells were harvested, and protein lysates were prepared.

Levels of Ugo1, Mgm1, Dnm1, and Fis1 were analyzed by Western blot using anti-HA and anti-Myc antibodies. The detection of the outer mitochondrial membrane protein porin, using anti-porin antibodies, served as a loading control. We found that the levels of the fission protein Fis1 are unaffected by treatment with α factor (Fig. 4). The levels of Ugo1, Dnm1, and Mgm1 show only a minor decrease after treatment with mating pheromone. Mdv1-Myc13 showed reproducibly high unspecific degradation; therefore we conclude that Mdv1 is most likely not influenced by treatment with α factor.

These results show that the mitochondrial fission machinery is intact even after a prolonged treatment with mating pheromone and that mitochondrial fission can occur under these conditions. However, the fusion machinery consisting of Fzo1, Ugo1, and Mgm1 is impaired by the degradation of Fzo1. Consequently, ongoing mitochondrial fission and insufficient fusion activity results in mitochondrial fragmentation during the yeast mating response.

**Fzo1 Degradation Depends on an Intact Mating Signaling Pathway**—To further characterize the α factor-induced degradation of Fzo1, we used mutants of the mating response signaling pathway. Therefore, we deleted the genes for the MAP kinases Fus3 and Kss1 as well as the gene for the transcription factor Ste12 (32) in a strain carrying a 3xHA-epitope-tagged version of Fzo1. Fus3 and to a lesser extent Kss1 are activated after the binding of the mating factor to the pheromone receptor. These kinases in turn activate the transcription factor Ste12. Ste12 then initiates the transcriptional program leading to mating. Deletion of either the MAP kinase Fus3 or the transcription factor Ste12 inhibited the degradation of Fzo1.
after treatment with a factor in contrast to wild type cells (Fig. 5). However, the deletion of Kss1 did not significantly stabilize Fzo1 upon treatment with a factor. These results show that the degradation of Fzo1 depends on an intact mating pheromone signaling. Complete inactivation of the mating response by STE12 deletion blocks the degradation of Fzo1. Also a strong impairment of mating signaling by FUS3 deletion inhibits Fzo1 down-regulation, although a slight disturbance of the mating response by the deletion of KSS1 still allows Fzo1 degradation after treatment with a factor. Activation of Ste12 by the MAP kinases Fus3 and Kss1 leads to the transcriptional regulation of over 400 genes (33). These may include a factor involved in regulation of Fzo1 stability.

**DISCUSSION**

The family of mitofusins plays an important role in the maintenance and shaping of the mitochondrial network from yeast to man. Although several activities of the protein machinery for mitochondrial fission and fusion are known, such as membrane localization domains, mitochondrial tethering domains, and GTPase activities, the regulation of these proteins and, thereby, the regulation of mitochondrial morphology are completely unknown. By analyzing mitochondrial morphology in different stages of the cell cycle, we were able to identify a dramatic change in mitochondrial morphology associated with the process of mating. The identification of a complete fragmentation of mitochondria upon treatment with a factor gave us the opportunity to explore the mechanism of regulation of mitochondrial fusion during this transition in the life cycle of the budding yeast. This deficit in mitochondrial fusion competence was accompanied by a loss of Fzo1 protein. This down-regulation of Fzo1 induced by a factor involved proteasome-mediated protein degradation rather than regulation on a transcriptional level. Because none of the other mitochondrial fusion proteins was unstable under these conditions and Dnm1 activity is necessary for the a factor-mediated change in mitochondrial morphology, degradation of Fzo1 seems to be the sole cause for the observed mitochondrial fragmentation. Stabilization of Fzo1 by inhibiting proteasome activity is indicative of a role for ubiquitin-mediated proteasome-dependent degradation. Two scenarios are imaginable. Activation of an E3 ubiquitin ligase for Fzo1 in response to a factor or degradation of a Fzo1-stabilizing factor that would then allow Fzo1 to be degraded. Although the exact mechanism of Fzo1 degradation after treatment with a factor remains to be elucidated, the previously described pathway for instability of Fzo1 involving the E3 ligase SCF together with the F-box protein Mdm30 is not involved. Therefore, we propose two independent pathways for the regulation of Fzo1 by proteasome-mediated protein instability, one pathway involving Mdm30 during steady state mitochondrial morphogenesis and another pathway not utilizing Mdm30 that is activated for more dramatic down-regulation of Fzo1.

The physiological role of mitochondrial fragmentation in response to a factor can be explained as follows. The observed mitochondrial fragmentation is not simply caused by the fact that the cells are in the G1 phase after treatment with a factor because G1 cells during an undisturbed cell cycle display tubular mitochondria. The altered mitochondrial morphology in cdc28-4 cells with a low cyclin-dependent kinase activity at the restrictive temperature indicates that the mitochondrial fragmentation after treatment with a factor is a direct cellular response to the drop in cyclin-dependent kinase activity upon activation of pheromone signaling. Mitochondria may fragment during mating in preparation for the fusion with mitochonaria from the mating partner after zygote formation. After mating, mitochondria of the two mating partners fuse very rapidly, which results in a complete blending of the mitochondrial contents. Mitochondrial fragmentation before zygote formation would support a fast and thorough mitochondrial mixing after cell fusion following stabilization of Fzo1 expression. After cell fusion mitochondria could fuse very efficiently and mix their genetic material. In fact, recombination of mitochondrial DNA takes place as soon as the zygote is formed (9). Fast and thorough mitochondrial mixing may pose an advantage for newly formed diploid cells by providing a uniform population of mitochondria and allowing repair of damaged mitochondrial DNA. Another major transition in the life cycle of budding yeast is also accompanied by changes in the ratio between mitochondrial fission and fusion. During the process of sporulation, a specific temporal pattern of fragmentation and fusion of mitochondria can be observed, and disturbance of mitochondrial morphology due to mutations of the fission or fusion machinery interferes with the generation of respiratio proficient spores (34, 35).

Taken together, our results are the first example of a regulatory mechanism for the control of mitochondrial morphology. We show with the example of the mating response that receptor-mediated G protein-coupled signal transduction can regulate mitochondrial morphology. Proteasomal degradation of Fzo1 likely mediates mitochondrial fragmentation by modulating the fission to fusion ratio of mitochondria, allowing the rapid generation of a uniform population of mitochondria in the newly formed zygote. Further evaluation of the impact of such signaling modules on the stability of mitofusins in higher eukaryotes may give insights into the regulatory processes governing the equilibrium between fission and fusion that controls mitochondrial morphology.

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