Proteasomes Associated with the Blm10 Activator Protein Antagonize Mitochondrial Fission through Degradation of the Fission Protein Dnm1*

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Background: Blm10 binds to the proteasome core particle and stimulates its proteolytic activity.
Results: Loss of BLM10 results in impaired respiration, elevated oxidative stress sensitivity, increased mitochondrial fission, and stabilization of the fission protein Dnm1.
Conclusion: Blm10 proteasome-mediated Dnm1 degradation is a regulatory mechanism to maintain correct mitochondrial function.
Significance: Blm10 is involved in mitochondrial quality control under oxidative stress.

The conserved Blm10/PA200 activators bind to the proteasome core particle gate and facilitate turnover of peptides and unfolded proteins in vitro. We report here that Blm10 is required for the maintenance of functional mitochondria. BLM10 expression is induced 25-fold upon a switch from fermentation to oxidative metabolism. In the absence of BLM10, Saccharomyces cerevisiae cells exhibit a temperature-sensitive growth defect under oxidative growth conditions and produce colonies with dysfunctional mitochondria at high frequency. Loss of BLM10 leads to reduced respiratory capacity, increased mitochondrial oxidative damage, and reduced viability in the presence of oxidative stress or death stimuli. In the absence of BLM10, increased fragmentation of the mitochondrial network under oxidative stress is observed indicative of elevated activity of the mitochondrial fission machinery. The degradation of Dnm1, the main factor mediating mitochondrial fission, is impaired in the absence of BLM10 in vitro and in vivo. These data suggest that the mitochondrial functional and morphological changes observed are related to elevated Dnm1 levels. This hypothesis is supported by the finding that cells that constitutively overexpress DNM1 display the same mitochondrial defects as blm10Δ cells. The data are consistent with a model in which Blm10 proteasome-mediated turnover of Dnm1 is required for the maintenance of mitochondrial function and provides cytoprotection under conditions that induce increased mitochondrial damage and programmed cell death.

The proteasome is the major proteolytic system in the cytoplasm and nuclei of eukaryotic cells (1). It is required for the maintenance of protein homeostasis through its ability to eliminate damaged and misfolded proteins. Furthermore, its activity is vital for numerous cellular processes that are regulated by the temporally specific degradation of pathway components (1). Because the proteasome provides building blocks for protein synthesis, it also has a metabolic function under nutrient-limiting conditions (2).

Proteasomal activity is subject to tight regulation. The central regulatory mechanism is defined by the topology of its proteolytic core complex, the core particle (CP1 or 20S proteasome). The barrel-shaped structure of the CP is formed by four stacked rings, which sequester the proteolytic sites within the interior chamber (3). Access to the degradation chamber is regulated by adjustable gates, located at both ends of the cylinder. To promote substrate degradation, the CP must interact with proteasome activators, which open the gate, thus allowing substrate entry (5). To date, three types of proteasome activators are known as follows: the conserved regulatory particle (RP195/PA700) (6, 7); activators of the PA28 protein family present in higher eukaryotes (8), and the conserved Bln10/PA200 activator family (9, 10). The RP is required for the ATP-dependent unfolding and degradation of ubiquitinated proteasome substrates. PA28 activators mediate ATP- and ubiquitin-independent turnover of specific proteasome substrates (11, 12). As RP and PA28 activators, Bln10/PA200 proteins enhance the proteasomal peptidase activity (9, 10, 13, 14) and accelerate the degradation of substrates with intrinsically disordered domains such as Tau (14) or acetylated histones (15) in the absence of ATP or ubiquitin, similar to the activity of PA28...
Regulated Turnover of Dnm1 by Blm10 Proteasomes

proteins (11, 12). Blm10 has also been associated with proteasome holocomplex formation (16, 17).

In yeast and in mammalian cells, Blm10/PA200 proteasomes form a substantial proteasome subpopulation, where the CP/20 S cylinder is flanked on one side with Blm10/PA200 and with RP/19S on the opposite side (10, 18). Blm10 proteasome-mediated turnover is involved in the transcriptional down-regulation of ribosome biosynthesis in response to nutrient depletion through degradation of the ribosome-related transcription factor Sfp1 (19). Loss of PA200 in mice results in defective spermatogenesis (15, 20), and the protein is recruited to chromatin upon γ-irradiation-induced DNA damage (15, 18). PA200 also plays a role in the maintenance of glutamine/glutamate homeostasis in mammalian cells (21). In addition, overexpression of BLM10 in yeast causes growth defects on nonfermentable carbon sources at elevated temperatures (14); recently, increased frequency of cells with dysfunctional mitochondria in cells lacking BLM10 was reported (22). These observations indicate that the cellular functions of Blm10/PA200 proteasomes might be related to the regulation of metabolic adaptation and stress response.

The ubiquitin proteasome system (UPS) has been implicated in the maintenance of mitochondrial structural dynamics and homeostasis. The E3 ubiquitin ligase Rsp5 is required for correct mitochondrial inheritance during vegetative growth (23), and proteasomal inhibition in vivo results in the ubiquitination and accumulation of a model substrate located in the mitochondrial intermembrane space (24). Additional reports demonstrate proteasome-mediated degradation of proteins, which associate with the outer mitochondrial membrane (25). These findings support a model in which the proteasome provides a protein quality control function for mitochondrial proteins. A particular mitochondrial-related function mediated by the UPS is the regulation of mitochondrial dynamics. In most eukaryotic cells, mitochondria form a dynamic network and are subject to continuous fission and fusion. Unopposed fission or fusion in response to deletion of the specific factors involved results in a reduction of mitochondrial functionality (26). The fusion of mitochondria promotes repair and complementation processes to optimize the respiratory capacity of the organelle (27), whereas damaged mitochondria are segregated from the network by fission, promoting mitophagy (28). Thus, mitochondrial fission and fusion are thought to provide an organelar quality control mechanism.

A screen for mitochondrial morphological defects upon down-regulation of essential genes in yeast revealed that loss of individual proteasome subunits leads to altered mitochondrial morphology (29). Altered mitochondrial morphology was also observed for mutants of proteasome RP subunits, surprisingly with the following opposite outcome: mutations in RPN11 result in fragmented mitochondria (30, 31), whereas an RPN3 mutant exhibits fused mitochondria (31). It is assumed that the underlying mechanism for altered mitochondrial morphology in proteasome mutants is related to proteasome-mediated turnover of Fzo1/mitofusin, a mitochondrial fusion protein (32–34).

In this study, we provide evidence for an additional regulatory function of the proteasome in mitochondrial homeostasis, mediated by the proteasome activator Blm10. In the absence of BLM10, a high frequency of colonies with dysfunctional mitochondria is observed, and the cells display a temperature-dependent growth defect under conditions that require functional mitochondria. Mitochondria isolated from blm10Δ cells exhibit reduced respiratory activity and increased oxidative damage. Consistent with these findings, we observed decreased viability in the absence of BLM10 after exposure to oxidizing reagents such as hydrogen peroxide and menadione. In the presence of oxidative stress, cells lacking BLM10 exhibit increased mitochondrial fragmentation. The primary protein, which drives mitochondrial fragmentation, is Dnm1. Loss of BLM10 leads to Dnm1 stabilization, and overexpressing BLM10 phenocopied the mitochondrial functional and structural changes of bnm1Δ cells. These data argue for a model in which the regulated turnover of Dnm1 mediated by Blm10 proteasomes is required for normal mitochondrial function and under oxidative stress. Mitochondrial fragmentation is also an integral mechanism of programmed cell death/apoptosis, and changes in Dnm1 activity affect the cellular response to death stimuli (28, 35). In agreement with Blm10 proteasome-mediated regulation of Dnm1 abundance, we find that loss of BLM10 confers hypersensitivity to hydrogen peroxide or to high doses of acetic acid. Finally, we demonstrate that the impact of Blm10 on mitochondrial dynamics requires proteasome interaction, because a BLM10 mutation that abrogates CP binding recapitulates the effects of BLM10 deletion. The impact of Blm10 on Dnm1 abundance is also evident from in vitro studies, which demonstrate that Blm10 mediates proteasomal turnover of Dnm1. Our data indicate that Blm10 proteasome-mediated degradation of Dnm1 is a regulatory mechanism to counteract mitochondrial fission and provides a cytoprotective function under conditions that induce mitochondrial stress.

EXPERIMENTAL PROCEDURES

Strains, Media, Growth Conditions, and Chemicals—All strains and plasmids used in this work are listed in Tables 1 and 2, respectively. They were obtained using standard genetic techniques. All strains are isogenic to BY4741 or BY4742 (36) and are S288C-derived. Complete gene deletion, promoter exchange, or tag integration was performed at the genomic locus by homologous recombination using standard techniques (37). Primer sequences are available upon request. Unless otherwise noted, strains were grown at 30 °C in yeast peptone/dextrose (YPD) and were harvested at OD660 nm ~1 for log phase cells, at OD660 nm ~12 for PDS phase cells, and after 5 days for stationary phase cells. Cycloheximide (CHX), hydrogen peroxide, menadione, and acetic acid were purchased from Sigma.

Phenotypic Analysis—To quantitate the frequency of petite colonies, strains were grown in YPD overnight, harvested, diluted to ~100 cells per plate and spread onto YPD plates. Petite frequency was determined after 48 h by visual inspection. To confirm that petite colony formation originates from mitochondrial dysfunction, normal and petite colonies were grown on YP/glycerol plates containing 3% glycerol. For phenotypic analysis, strains were grown overnight in YPD and diluted in 96-well plates to a density of 6 × 10⁶ cells per well followed by...
5-fold serial dilutions. They were spotted onto solid media, and colony growth was monitored. Carbon sources in the complete media used were as follows: YPD, 2% glucose; YP/acetate, 0.1 M acetate; and YP/glycerol, 3% glycerol. To analyze survival after oxidative stress, logistically growing cells were treated with 10 mM H₂O₂ or 300 μM menadione for 3.5 or 1.5 h, respectively, prior to spotting onto solid YPD medium.

Quantitative Real Time PCR—Total RNA was isolated from WT cells grown in YP/glycerol for the time indicated by acidic precipitation using the High Capacity cDNA kit (Applied Biosystems). Primers were designed using the software Primer3Plus. The sequences are available upon request. cDNA from 1 μg of RNA was subjected to quantitative RT-PCR using the SYBR Green PCR Master Mix (Applied Biosystems) in an IQ5 Bio-Rad real time PCR detection system. The reactions were performed in 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 40 s after an initial activation at 95°C for 2 min. Negative controls were run simultaneously for each reaction. Data were analyzed using the IQ5 Optical System software. To compare the relative mRNA expression between the individual genes and the endogenous reference gene ACT1, the comparative threshold cycle (Cₜ) method was used. The amount of target, relative to the reference gene as described in the figure legends, is given by 2⁻ΔΔCₜ. All reactions were performed in triplicate. Error bars indicate the mean ± S.E. of three independent experiments.

Respiration Assay—The oxygen consumption of WT or btm10Δ cells was measured at 30°C using an Oroboros instrument (Oroboros, Graz, Austria). Respiratory rates (JO) were determined from the slope of a plot of O₂ concentration versus time. For all assays, 2 ml of growing cell suspension were used. Respiration assays of growing cells were performed in the growth medium plus 2% lactate when triethylthiophosphate (TET) (Sigma) was added at 0.2 mM and carbonyl cyanide m-chlorophenyldihydrazone (CCCP) (Sigma) at 10 μM. Respiratory state value (RSV) is defined as (JObasal − JO2CCCP)/(JO02CCCP − JO2TET). RSV represents the percentage of state 4 oxidative phosphorylation compared with the basal respiration capacity (38).

Isolation of Yeast Mitochondria—Yeast cells were cultured overnight at 30°C in YP/lactate medium) and harvested by centrifugation at 4,000 × g for 10 min at 4°C, washed with 50 ml of ice-cold water, and resuspended in 0.6 M sorbitol, 50 mM Tris-HCl, pH 7.5, supplemented with complete protease inhibitor mixture (Sigma). 4 ml of 0.45-mm diameter sterile glass beads were added to each tube, and the cells were lysed by vortexing six times for 30 s each, at 2-min intervals, on ice. All subsequent steps were carried out at 4°C. Glass beads and unbroken cells were removed by centrifugation at 4,000 × g for 10 min; supernatants were centrifuged (14,000 × g, 10 min, 4°C), and the resulting pellets containing mitochondria were resuspended in 0.6 M sorbitol, 50 mM Tris-HCl, pH 7.5.

Aconitase Activity Assay—Mitochondria were diluted to 0.05 mg/ml in 25 mM KH₂PO₄, pH 7.25, containing 0.05% Triton X-100. Aconitase activity was assayed as the rate of NADP reduction (340 nm, ε = 6200 M⁻¹ cm⁻¹) by isocitrate dehydrogenase upon addition of 1.0 mM sodium citrate, 0.6 mM MnCl₂ (a cofactor of isocitrate dehydrogenase), 0.2 mM NADP, and 1.0 unit/ml isocitrate dehydrogenase to solubilized mitochondria (0.05 mg/ml mitochondrial protein). Isocitrate dehydrogenase (Sigma) was exchanged into a buffer composed of 25 mM KH₂PO₄, pH 7.25, by gel filtration (PD-10 column, GE Healthcare) prior to incubations. Use of oxalomalate (2.0 mM), a competitive inhibitor of aconitase, ensured the specificity of the assay.

Live Cell Fluorescence Microscopy—To visualize mitochondria, mitochondrially targeted pCoX4-GFP (pOK29) (39) was introduced into the strains indicated via transformation. Btm10
Regulated Turnover of Dnm1 by Blm10 Proteasomes

Localization during the fermentative phase of yeast growth (log) or after the switch to oxidative metabolism (PDS) was determined using a plasmid with an N-terminal BLM10 GFP fusion (yCPLac111-GFP-BLM10) under the endogenous promoter in btm10Δ cells. Live cell fluorescence was monitored using a fluorescence microscope (Olympus BX61) at the Albert Einstein Imaging Facility with a ×60 or ×100 NA 1.4 objective (PlanApo). Fluorescence or differential interference contrast images were captured with a cooled CCD camera (Sensicam QE) using IPLab 4.0 software. Images were identically processed using Image software 1.42q. For excitation of GFP, a 450–490-nm bandpass filter was used. Emitted light was detected with a 520-nm long pass filter (filter set Olympus).

Detection of Steady State Protein Levels—Cells from WT and mutant strains were harvested in the respective growth phases and stored at −80 °C. Cells were disrupted by alkaline lysis, and the resulting powder was thawed, supplied with additional lysis buffer at 0.4 M NaCl, and cleared via centrifugation. The supernatant was incubated with nickel-nitritotriacetic acid resin (Qiagen) at 4 °C for 1 h. Bound proteins were washed with 10 mM imidazole and eluted with 250 mM imidazole. The eluted samples were dialyzed against 50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM DTT, and 10% glycerol, aliquoted, and frozen at −80 °C.

Dnm1 turnover was tested at 30 °C in 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 2.5 mM MgCl2, 0.5 mM EDTA, 0.6% Nonidet P-40 supplemented with 350 mM His6·Dnm1 in the absence of proteasomes or in the presence of 10 mM CP or 10 mM Blm10–CP. At the times indicated, aliquots were supplemented with SDS sample buffer and boiled for 3 min to stop the reaction. Dnm1 levels were visualized via SDS-PAGE and immunoblotting with anti poly-histidine antibody (Sigma). Anti-CP antibodies were from Biomol. The ECL-based chemiluminescence signals were recorded using ImageQuant LAS 4000 mini instrument and quantified with the ImageQuant TL software.

Detection of Programmed Cell Death—Survival after treatment with cell death stimuli was tested as described previously (41). Briefly, WT, btm10Δ, btm10ΔC3, DNM1-OE, and fis1Δ cells were grown overnight in YPD medium at 30 °C. Overnight cultures were diluted to OD660 = 0.2 in YPD and incubated at 30 °C until mid-log phase was reached. Cultures were supplemented with 250 mM acetic acid and incubated for 4 h at 30 °C. Subsequently, 5-fold serial dilutions of treated and untreated cells were spotted on solid YPD medium to test for survival.

RESULTS

Loss of BLM10 Results in Reduced Fitness during Respiratory Growth and under Oxidative Stress—We reported previously that BLM10 overexpression decreases the viability of yeast cells under conditions that induce oxidative metabolism at elevated temperatures, such as growth on nonfermentable carbon sources (14). We also demonstrated that BLM10 expression is strongly induced in response to the metabolic switch from fermentation to oxidative metabolism in yeast (19). Furthermore, increased frequency of petite colonies, indicative of impaired mitochondrial function (42) upon loss of BLM10 in different strain backgrounds was observed (Fig. 1, A–C) (22). Because of their ability to ferment carbon sources, yeast cells can dispense with functional mitochondria, and thus petite strains are viable, yet growth is abolished if nonfermentable carbon sources are
Regulated Turnover of Dnm1 by Blm10 Proteasomes

scripts during growth in medium with glycerol as a carbon source as tested via quantitative RT-PCR (Fig. 1E, dark gray bars). This finding is consistent with the increased expression of BLM10 observed upon entry into oxidative metabolism or upon treating cells during logarithmic growth with the TORC1 inhibitor rapamycin (19). Genes for CP and RP subunits (PRE1 and RPN11) were not up-regulated during growth on glycerol (Fig. 1E).

Loss of BLM10 Leads to Reduced Respiratory Capacity and Increased Oxidative Damage of Mitochondria—We tested the respiratory capacity of blm10Δ and WT cells by high resolution respirometry using an Oroboros Oxygraph-02K, which records mitochondrial oxygen consumption. We measured respiration of WT cells and cells deleted for BLM10 at basal level and in the presence of CCCP and TET. Addition of CCCP causes dissipates of the proton gradient across the mitochondrial membrane. The proton gradient across the mitochondrial membrane has a crucial regulatory function on the capacity of the respiratory chain complexes. Removing the membrane potential by addition of CCCP allows for the recording of maximum respiration. blm10Δ cells exhibited an ~40% increase in maximum respiration as compared with WT mitochondria (Fig. 2A, left panel). Subsequently, respiration in the presence of the ATP synthase inhibitor TET was determined, which allows for determining nonphosphorylating respiration caused by proton leakage across the inner mitochondrial membrane (LEAK flux). TET addition led to ~20% higher respiration rate in cells without Blm10 as compared with WT. From these data we calculated an in vivo RSV (38) and found that cells lacking Blm10 exhibit an ~60% reduction in respiratory chain activity (Fig. 2A, right panel). Finally, we measured the activity of aconitase in isolated mitochondria. The activity of mitochondrial aconitase is exquisitely sensitive to oxidative stress (43). We observed severely impaired mitochondrial aconitase activity in the absence of BLM10 (Fig. 2B), indicative of elevated oxidative stress within the mitochondria upon loss of BLM10. We therefore reasoned that Blm10 mutants might exhibit increased sensitivity during oxidative stress. When we treated cells with hydrogen peroxide or menadione, we found this hypothesis confirmed as in the absence of BLM10 or in the presence of a CP binding-incompetent Blm10 mutant reduced viability after oxidative stress induced by either hydrogen peroxide (H₂O₂) or menadione was observed (Fig. 2C, right panels).

Loss of BLM10 Affects the Morphological Integrity of Mitochondria during Oxidative Stress—Next, we investigated the structural integrity of the mitochondrial network in the absence or presence of oxidative stress. To that end, we visualized mitochondria utilizing GFP fused to cytochrome c oxidase subunit 4 as a reporter (39) in blm10Δ and WT cells in the absence or presence of H₂O₂. The mitochondrial morphology in untreated cells was largely comparable (Fig. 3, right panels). During the time course of the experiment, we also did not observe major morphological changes in WT cells after H₂O₂ treatment (Figs. 3, left panels, and 4, A and B). In Blm10 mutants however, we observed a significant increase in mitochondrial fragmentation after incubation with H₂O₂ (Figs. 3, left panels, and 4, A and B). Approximately 50% of blm10Δ and

used. The petite colonies in BLM10-deleted yeast strains are unable to grow on the nonfermentable carbon source glycerol (Fig. 1B) confirming that they do not contain functional mitochondria. In addition, growth of blm10Δ cells is impaired on the nonfermentable acetate at higher temperature (Fig. 1C). These observations point to a previously unidentified function of Blm10 proteasomes in the maintenance of mitochondrial function. We recently demonstrated that deletion of the last three residues of Blm10 containing the HbYX motif at the C terminus (blm10ΔC3) prevents CP binding and causes a loss-of-function phenotype (14, 19). Similar to complete BLM10 deletion, cells expressing the CP binding-incompetent Blm10 mutant exhibit elevated levels of colonies with dysfunctional mitochondria and a temperature-sensitive growth defect on nonfermentable carbon sources (Fig. 1, A–C). Thus, the role of Blm10 in the maintenance of correct mitochondrial function requires its association with and activation of the proteasome.

We hypothesized that if BLM10 function is specifically required during respiratory growth, we might observe an increase in BLM10 expression if cells are grown in the presence of nonfermentable carbon sources. In agreement with this hypothesis, we observed an ~10-fold induction of BLM10 tran-
Regulated Turnover of Dnm1 by Blm10 Proteasomes

FIGURE 2. Loss of BLM10 results in respiratory defects and increased mitochondrial oxidative stress. A, respiratory capacity of WT or blm10Δ cells grown in lactate was determined using an Oroboros high resolution respirometer. Left, basal respiration was measured in the absence of drugs. To determine maximal respiration, the uncoupling reagent CCCP was added, which dissipates the proton gradient across the mitochondrial membrane. Leak flux was analyzed in the presence of TET, an inhibitor of ATP synthase. Right, RSV is defined as \((\text{U}_{\text{respiration}} - \text{U}_{\text{TET}})/\text{U}_{\text{CCCP}} - \text{U}_{\text{TET}}\) and represents the percentage of stimulation of oxidative phosphorylation compared with the basal respiration capacity. In vitro, aconitase activity was determined in mitochondria isolated from WT, blm10Δ, DNM1-OE, and DNM1-OE blm10Δ cells grown in lactate. B, WT, blm10ΔC3, or blm10Δ cells were treated with 10 mM H2O2 or with 300 μM menadione for 3.5 and for 1.5 h, respectively. Serial dilutions of treated and untreated cells were spotted on YPD and grown at 30 °C. Error bars, S.E.

Blm10ΔC3 cells exhibited a fragmented phenotype as compared with ~18% in the WT.

Increased mitochondrial fragmentation could be caused by increased activity of the mitochondrial fission machinery. A crucial protein, which drives the mitochondrial fission process, is the dynamin-like GTPase Dnm1 (44). We hypothesized that Blm10 proteasomes might be involved in the turnover of Dnm1 and that the increased mitochondrial fragmentation observed in Blm10 mutants might originate from impaired Dnm1 degradation. To investigate whether elevated Dnm1 levels are sufficient to increase mitochondrial fission under oxidative stress, we treated cells overexpressing DNM1 (DNM1-OE) with H2O2 and observed a similar increase in mitochondrial fragmentation as observed for Blm10 mutants (Figs. 3, left panels, and 4, A and B). To explore whether Dnm1 is a key factor, which determines increased mitochondrial fission in the presence of Blm10 mutants, we analyzed the mitochondrial morphology after H2O2 treatment in cells deleted for DNM1 in the presence or absence of BLM10. In dnm1Δ cells, mitochondrial fission is blocked resulting in a fused mitochondrial network (Figs. 3, right panels, and 4A, lower panel) (44). Furthermore, in the absence of DNM1 H2O2 does not trigger mitochondrial frag-

mentation, and this phenotype was not dependent on the presence of BLM10 (Figs. 3, left panels, and 4, A and B). These observations suggest that the increased mitochondrial fission observed in Blm10 mutants during oxidative stress might be caused by impaired Dnm1 turnover. This hypothesis predicts that Dnm1 overexpression should exacerbate the temperature-sensitive growth defect we observed in Blm10 mutants. To test this, we created a strain deleted for BLM10 and overexpressing DNM1 (DNM1-OE blm10Δ). In cells overexpressing DNM1, deletion of BLM10 results in a growth arrest under the same experimental conditions (Fig. 4C). These data corroborate the hypothesis that the temperature-sensitive growth defect of blm10Δ cells is related to impaired Dnm1 turnover.

As described above, we detected increased oxidative damage in mitochondria isolated from blm10Δ cells (Fig. 2B). To test whether increased Dnm1 levels could produce a similar phenotype, we measured aconitase activity in mitochondria isolated from cells overexpressing DNM1 and observed a significant reduction. Co-deletion of BLM10 in that strain led to additive effects (Fig. 2B). Finally, we tested whether overexpression of Dnm1 leads to impaired growth on nonfermentable carbon sources as observed for Blm10 mutants. A growth defect on acetate at 37 °C was evident in cells overexpressing DNM1, and co-deleting BLM10 in cells overexpressing DNM1 led to a growth arrest (Fig. 4C). In summary, the phenotypic character-
istics as well as the mitochondrial structural and functional changes observed in\textit{Blm10} mutants are phenocopied by \textit{Dnm1} overexpression.

Abundance of Dnm1 Is Regulated by Blm10 Proteasomes—Our results suggest that Dnm1 might be a physiological target for Blm10 proteasomes. To investigate Dnm1 abundance in \textit{blm10}/H9004 cells and in cells harboring a CP binding-incompetent \textit{Blm10} mutant, we studied the steady state levels of Dnm1 in the different growth phases of yeast. We found Dnm1 levels elevated in \textit{blm10}/H9004 cells in stationary phase (Fig. 5A), where yeast mitochondria are known to fragment (45). Next, we tested the rate of Dnm1 turnover \textit{in vivo} via a CHX chase assay. The addition of lethal doses of the translation inhibitor CHX blocks new synthesis. Thus, the degradation of a protein \textit{in vivo} can be monitored (19). We examined Dnm1 levels in WT, in \textit{Blm10} mutants, and in cells with reduced proteasome abundance through deletion of the proteasome-related transcription factor \textit{Rpn4} (1, 46). We observed impaired Dnm1 turnover in \textit{blm10Δ}, \textit{blm10ΔC3}, and in \textit{rpm4Δ} cells (Fig. 5, B and C), suggesting that Dnm1 stability is regulated by the proteasome and involves the proteasome activator Blm10. To unambiguously determine the impact of Blm10 on proteasome-mediated Dnm1 degradation, we purified Dnm1 and endogenous Blm10 proteasome core particle complexes (Blm10-CP) from yeast and tested Dnm1 turnover in a purified system (Fig. 6). In the absence of CP or Blm10-CP, the level of Dnm1 remains largely unchanged during the time course of the experiment. Addition of CP in the absence of Blm10 led to a slight reduction in Dnm1. Blm10-CP complexes, however, rapidly degraded Dnm1. ATP or ubiquitination of Dnm1 was not required for turnover of the protein. These data demonstrate that Blm10 induces proteasome-mediated Dnm1 turnover.

\textit{Blm10} Activates Dnm1 Degradation, but Does Not Affect the Abundance of Other Fission Components nor Fzo1—To investigate whether other components of the fission machinery are regulated by proteasomal degradation, we tested the turnover of Fis1 and Mdv1 \textit{in vivo}. Within the time frame of our experi-
Regulated Turnover of Dnm1 by Blm10 Proteasomes

FIGURE 6. Dnm1 is degraded by Blm10 proteasomes in vitro. A, Dnm1 degradation was investigated in a purified system in the presence of 10 nM Blm10-CP (left lanes), CP (middle lanes), or in the absence of proteasomes (right lanes). Dnm1 detection was achieved via an N-terminal His6 tag using an anti-polyhistidine antibody. The same membranes were tested for CP levels using an anti-CP antibody (lower signals). B, left panel, 0.7 μg of CP or Blm10-CP used in A were separated via SDS-PAGE and stained with Coomassie Blue. *, Blm10 degradation products. Blm10-CP used in C, in vivo turnover of the fission proteins Fis1 and Mdv1 was determined as in Fig. 5A with Fis1- and Mdv1-specific antibodies after blocking new synthesis with lethal doses of the ribosome inhibitor CHX. Right panel, chemiluminescence intensities of the bands shown in the blots presented in A were recorded in an ImageQuant LAS 4000 mini system and quantified using the ImageQuant TL software.

FIGURE 7. Decreased viability of Blm10 mutants in the presence of high doses of acetic acid. A, after treatment of WT, blm10Δ, blm10ΔC3, DNM1-OE, and fis1Δ with 250 mM acetic acid for 4 h, treated and untreated cells were serially diluted and spotted on YPD to test for survival. B, to monitor Blm10 localization, a plasmid containing the BLM10 gene under its endogenous promoter was N-terminally tagged with GFP and transformed in a BLM10 deletion strain. GFP fluorescence was analyzed in living cells in logarithmically growing cells (left panel) and after the diauxic shift (right panel).

ments, the level of both proteins remained unchanged (Fig. 6C); thus, proteasomal degradation of mitochondrial fission proteins appears to be restricted to Dnm1. Rather than from elevated mitochondrial fission, increased fragmentation might also originate from reduced activity of the fusion machinery. The yeast mitofusin is regulated through proteasomal degradation and abrogated function of the UPS leads to stabilization of mitofusins and reduced fragmentation of the mitochondrial network (47). If Blm10 would affect the fusion machinery, we would expect decreased abundance of Fzo1. In contrast, for rpnΔ4 cells, however, which exhibit elevated Fzo1 levels, Fzo1 abundance is unchanged in Blm10 mutants (Fig. 6D). These data suggest that the impact of Blm10 on mitochondrial dynamics is specific to Dnm1.

Loss of BLM10 Confers Hypersensitivity to Death Stimuli—A conserved function for Dnm1/Drp1 proteins is their involvement in programmed cell death (28, 48). In response to a death stimulus such as high concentrations of acetic acid, the yeast mitochondrial fragment and this process are mediated by Dnm1 (48). Overexpression of Dnm1 reduces the viability after a death stimulus (Fig. 7A, right panel) (48). We therefore hypothesized that blm10Δ cells might exhibit reduced viability after acetic acid treatment. When cells lacking BLM10 were treated with 250 mM acetic acid, they inhibit reduced viability (Fig. 7A, right panel), which we attribute to reduced turnover of Dnm1. fis1Δ cells, which are known to be exquisitely sensitive to death stimuli, were used as controls (48). These observations are consistent with the reduced viability of Blm10 mutants after treatment with H2O2 or menadione (Fig. 2C), both of which are known to induce programmed cell death in yeast.

Nutrient-dependent Blm10 Nuclear Export—During logarithmic growth, Blm10 is localized predominantly to the nucleus (10, 16), consistent with the mostly nuclear localization of yeast proteasomes under those conditions (49). A nuclear localization of Blm10, however, is not easily reconciled with the degradation of a cytoplasmic target such as Dnm1. Recently, it was reported that proteasomes exit the yeast nucleus when cells pass the diauxic shift (50), and reports in the fission yeast Schizosaccharomyces pombe demonstrate a cytoplasmic localization of the proteasome in G0 cells (51). Because our data indicate that Blm10 is involved in Dnm1 turnover in the cytoplasm, we hypothesized that Blm10 might also exit the nucleus.
Mitochondria are dynamic organelles that continuously fuse and divide, and the machineries that govern mitochondrial fission and fusion are conserved from yeast to human (52). The dynamic nature of the mitochondrial network is essential for aligning the capacity of the organelle with the energetic and metabolic requirements of eukaryotic cells (52). Mitochondrial network adaptations are especially evident in the sperm flagellum, where fused mitochondria are wrapped around the base of the flagellum (53), or during mitosis, where the mitochondrial network is highly fragmented (54). Regulated mitochondrial fission and fusion also play a role in organelle transport, e.g. in neurons, in quality control of mitochondria, and in mitochondrial inheritance during cell division. In addition, mitochondrial fragmentation is linked to the execution of programmed cell death programs (55). Thus, proteins involved in regulating mitochondrial fission and fusion are crucial factors for the health of eukaryotic cells. Here, we report that Blm10 proteasome-mediated proteolysis of the fission protein Dnm1 impacts mitochondrial morphology and is required for correct mitochondrial function under normal growth conditions and under conditions that induce mitochondrial stress.

Dnm1 and its human ortholog Drp1 are large dynamin-related GTPases (39, 44) that cooperate with adaptor proteins to promote mitochondrial fission. Dnm1/Drp1 proteins share the classical dynamin property to drive membrane fission (58). It is thought that mitochondrial fragmentation provides a quality control mechanism for the organelle by isolating defective, depolarized mitochondria, which facilitates their removal from the cell via mitophagy (28, 59). This hypothesis is supported by reports demonstrating that knockdown of Drp1 in mammalian cells leads to impaired mitochondrial function and loss of mitochondrial DNA (26). Loss of Drp1 in Caenorhabditis elegans results in embryonic lethality (60), and mutations in human Drp1 are associated with a lethal birth syndrome characterized by microcephaly, abnormal brain development, optic atrophy, and persistent lactic acidemia (61). Drp1/Dnm1-mediated mitochondrial fragmentation is furthermore an early event during apoptosis and programmed cell death (35, 48).

Many mechanistic details have been identified that regulate Drp1 function in mammalian cells. They can be grouped into two main categories, post-translational modification and regulated turnover (33, 62). Several kinases and phosphatases are known to impact Drp1 activity and recruitment to the mitochondria (62). Furthermore, ubiquitination and sumoylation regulate Drp1 function. Sumoylation stabilizes Drp1, presumably through an antagonistic effect of sumoylation and ubiquitination (63). The ubiquitin E3 ligase MARCH5/MITOL polyubiquitinates Drp1 but does not trigger Drp1 degradation (64). Polyubiquitination by the E3 ligase Parkin, however, regulates proteasomal degradation of Drp1 (65). Ubiquitin-independent Blm10 proteasome-mediated degradation of Dnm1 adds an additional control mechanism to this list. At present, it is unknown whether the impact of Blm10 on fusion is conserved in mammals. Interestingly, loss of PA200, the mammalian ortholog of Blm10, in mice results in reduced male fertility caused by impaired spermatogenesis (20), and PA200 expression is strongly elevated in testes (9, 15). Mitochondria in sperm cells reveal a specific adaptation to the increased energy demand of mobile cells, i.e. a highly fused mitochondrial network (53). It is therefore tempting to speculate that the increased PA200 levels in testes are required for increased degradation of Drp1 and thus to down-regulate the fission process.

Mitochondrial morphology is determined by the balanced activity of the fission and fusion machineries. We demonstrate here that Blm10 proteasome activators mediate the turnover of Dnm1 to counteract mitochondrial fission, but they have no impact on fusion. However, a recent study demonstrated that proteasome-mediated degradation of the mitochondrial fusion protein Mfn2 promotes stress-induced mitochondrial fission

**DISCUSSION**

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Mitochondrial morphology is determined by the balanced activity of the fission and fusion machineries. We demonstrate here that Blm10 proteasome activators mediate the turnover of Dnm1 to counteract mitochondrial fission, but they have no impact on fusion. However, a recent study demonstrated that proteasome-mediated degradation of the mitochondrial fusion protein Mfn2 promotes stress-induced mitochondrial fission
Regulated Turnover of Dnm1 by Blm10 Proteasomes

and apoptosis in mammals (47), and in yeast, UPS-mediated regulation of the Mfn2 ortholog Fzo1 impacts mitochondrial dynamics (32, 34). Thus, proteasomal degradation can promote fission through increased degradation of Mfn2/Fzo1 or it can inhibit fission by reducing Dnm1/Drp1 levels as concluded from the data presented here. The decision whether fission or fusion is promoted by proteasome-mediated degradation must rely on additional signals. Proteasomal turnover of Mfn2 is induced by JNK-mediated phosphorylation in the presence of doxorubicin (47). Whether phosphorylation of Dnm1/Drp1 is involved in regulating proteasome-mediated turnover of Dnm1/Drp1 is not known.

Based on the data reported here, we propose an additional mechanism for differential regulation of fission and fusion via proteasomal degradation, differential regulation via different proteasome subpopulations. We propose that proteasomal regulation of mitochondrial fusion is executed by the 26 S proteasome through degradation of Mfn2/Fzo1, whereas Dnm1/Drp1 degradation is mediated by Blm10/PA200 proteasomes. A specific role for Blm10 in regulating mitochondrial fission is supported by several observations. We did not observe impaired Fzo1 degradation in cells lacking BLM10. BLM10 expression is up-regulated under conditions, which induce oxidative metabolism resulting in increased oxidative damage to mitochondria. Furthermore, even though Dnm1 is stabilized in rpm4Δ cells (Fig. 5A), which are characterized by a general reduction in proteasome capacity (46), these cells do not exhibit impaired mitochondrial function or generate petite colonies at high frequency, nor do they exhibit increased oxidative damage to mitochondria (Fig. 8). Thus, reduced proteasome activity primarily impacts mitochondrial fusion, whereas loss of BLM10 specifically enhances fission.

Cells with a CP binding-incompetent Blm10 mutant, lacking the last three residues of the 240-kDa protein, exhibit impaired Dnm1 degradation similar to complete loss of the protein and show the same phenotypic characteristics as bim10Δ cells. These data suggest that the impact of Blm10 on Dnm1 turnover requires CP binding and activation. Mechanistically, it is still unclear how Blm10 promotes proteasomal substrate degradation. A co-crystal structure of reconstituted Blm10-CP complexes showed that Blm10 binding to the CP results in an at least partially open gate (22). The conformations of the N-terminal residues of four of the α subunits are superimposable with the open gate conformation of PA26-CP complexes (5). The remaining three N termini could not be (19) from the closed gate conformation. Our previous reports showed that Blm10 mediates proteasomal turnover of the transcription factor Sfp1, and in vitro Blm10 accelerates CP-mediated degradation of Tau-441 (14) indicating that the structural changes of the CP gate upon Blm10 binding are sufficient to promote protein entry and turnover. Both Sfp1 and Tau are characterized by large unstructured domains. We also found that ATP or ubiquitination is not required for Blm10-mediated proteasome activation. These observations led to a model in which Blm10 might specifically promote the degradation of unstructured proteins. This hypothesis is supported by a recent study demonstrating Blm10- and PA200-mediated degradation of core histones. Sfp1 and Tau histones also contain large unstructured domains (15). Using an algorithm, which predicts unfolded domains in proteins (66), we detected a relatively large unfolded region of ~150 amino acids in Dnm1 (Fig. 9), and we propose that this region might provide a recognition motif for Blm10 proteasome-mediated degradation.

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