Proteasomal degradation of Sfp1 contributes to the repression of ribosome biogenesis during starvation and is mediated by the proteasome activator Blm10

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ABSTRACT The regulation of ribosomal protein (RP) gene transcription is tightly linked to the nutrient status of the cell and is under the control of metabolic signaling pathways. In Saccharomyces cerevisiae several transcriptional activators mediate efficient RP gene transcription during logarithmic growth and dissociate from RP gene promoters upon nutrient limitation. Repression of RP gene transcription appears to be regulated predominantly by posttranslational modification and cellular localization of transcriptional activators. We report here that one of these factors, Sfp1, is degraded by the proteasome and that the proteasome activator Blm10 is required for regulated Sfp1 degradation. Loss of Blm10 results in the stabilization and increased nuclear abundance of Sfp1 during nutrient limitation, increased transcription of RP genes, increased levels of RPs, and decreased rapamycin-induced repression of RP genes. Thus we conclude that proteasomal degradation of Sfp1 is mediated by Blm10 and contributes to the repression of ribosome biogenesis under nutrient depletion.

INTRODUCTION The proteasome is an essential protease in the cytoplasm and nuclei of eukaryotic cells. It consists of two entities: a central proteolytic core (the 20S proteasome in higher eukaryotes or the core particle [CP] in Saccharomyces cerevisiae) and a regulatory or activating complex. The CP has a barrel-shaped topology formed by four stacked rings (two inner β and two outer α rings), composed of seven subunits each. The β rings harbor three different proteolytically active subunits with different specificities: trypsin-like, chymotrypsin-like, and postacidic activity (Kisselev et al., 2006). The active sites are sequestered within the interior of the CP barrel (Groll et al., 1997). Access to the proteolytic chamber is controlled by an adjustable gate and is mediated by proteasome regulators/activators (Foerster et al., 2003). Three activator families have been described: the conserved regulatory particle (19S or PA700), the PA28 family (REG, 11S regulator) (Li and Rechsteiner, 2001), and the conserved Blm10/PA200 proteins (Ustrell et al., 2002; Schmidt et al., 2005), providing a variety of different proteasomal subpecies, which most likely target different groups of proteasomal substrates. The regulatory particle consists of 19 subunits, among them six paralogous ATPases (Finley, 2009). Opening of the CP gate by the RP is achieved by insertion of ATPase C-termini into specific pockets at the CP surface (Smith et al., 2007; Gillette et al., 2008; Stadtmueller et al., 2009). PA28 activators are characterized by a hepta- or hexa-oligomeric ring structure of ~200 kDa (Rechsteiner and Hill, 2005). The C-termini of the subunits of PA28 are inserted into the same α subunit pockets as the C-termini of proteasomal subunits of seven subunits each. The β rings harbor three different proteolytically active subunits with different specificities: trypsin-like, chymotrypsin-like, and postacidic activity (Kisselev et al., 2006). The active sites are sequestered within the interior of the CP barrel (Groll et al., 1997). Access to the proteolytic chamber is controlled by an adjustable gate and is mediated by proteasome regulators/activators (Foerster et al., 2003). Three activator families have been described: the conserved regulatory particle (19S or PA700), the PA28 family (REG, 11S regulator) (Li and Rechsteiner, 2001), and the conserved Blm10/PA200 proteins (Ustrell et al., 2002; Schmidt et al., 2005), providing a variety of different proteasomal subspecies, which most likely target different groups of proteasomal substrates. The regulatory particle consists of 19 subunits, among them six paralogous ATPases (Finley, 2009). Opening of the CP gate by the RP is achieved by insertion of ATPase C-termini into specific pockets at the CP surface (Smith et al., 2007; Gillette et al., 2008; Stadtmueller et al., 2009). PA28 activators are characterized by a hepta- or hexa-oligomeric ring structure of ~200 kDa (Rechsteiner and Hill, 2005). The C-termini of the subunits of PA28 are inserted into the same α subunit pockets as the C-termini of the ATPases. However, C-terminal docking of the PA28 subunits does not trigger gate opening. Instead, internal segments known as the PA28 activation loops induce a structural rearrangement of the gate region, which allows substrate entry (Whitby et al., 2000).
Blm10 in S. cerevisiae and its human orthologue PA200 are large ~245-kDa proteins composed of HEAT repeats, which associate with the CP/20S gate region (Ortega et al., 2005; Schmidt et al., 2005; Iwanczyk et al., 2006; Sadre-Bazzaz et al., 2010). In mammalian cells PA200 was found exclusively in a complex with the proteasome (Blickwedehl et al., 2008). We have demonstrated recently that in rapidly growing yeast cells Blm10 is part of a mature proteasome hybrid complex, where Blm10 occupies one end of the core cylinder and the regulatory particle the other end (Schmidt et al., 2005). Blm10–CP devoid of regulatory particles was not detected in unfractonated lysates, suggesting that the dominant species of Blm10-containing proteasomes is the hybrid complex. Blm10–CP complexes, purified after the dissociation of the regulatory particle from the hybrid complex, exhibit elevated CP peptidase activity (Schmidt et al., 2005; Iwanczyk et al., 2006; Li et al., 2007; Lehmann et al., 2008). Both hybrid complex formation and activation of the proteasome peptidase activity have also been described for PA200–proteasome complexes (Ustrell et al., 2002; Blickwedehl et al., 2008), suggesting that Blm10/PA200 proteins might represent a novel conserved monomeric proteasome activator family. A recent structural analysis of Blm10–CP complexes showed that similar to RP and PA28, Blm10 binding to the CP is mediated via its C-terminus. The C-terminal Blm10 residues cause structural alterations within the gate region, resulting in a partially open, disordered gate in a molecular mechanism that appears to be similar to the C-terminal docking of the proteasomal ATPases, but different from PA26 C-terminal binding to the CP, which per se does not induce gate opening (Sadre-Bazzaz et al., 2010). Furthermore, a pore within Blm10 of 13–22 Å has been detected. Whether these structural characteristics of Blm10–CP complexes are sufficient to promote protein degradation remains to be established.

The cellular functions of Blm10 are poorly understood. A proposed role for Blm10/PA200 in DNA repair (Febres et al., 2001; Ustrell et al., 2002) was not confirmed in subsequent studies in yeast and in mammalian cells (Schmidt et al., 2005; Khor et al., 2006; McCulloch et al., 2006). In S. cerevisiae Blm10 binding to the proteasome occurs at a late stage during proteasome maturation (Fehlker et al., 2003; Li et al., 2007; Marques et al., 2007), which initially suggested a possible function in proteasome assembly. Loss of BLM10, however, does not result in a significant defect in proteasome assembly (Marques et al., 2007) or in gross changes in mature proteasome populations (Schmidt et al., 2005). Double mutants that combine loss of BLM10 and an assembly-defective proteasomal regulatory particle mutant show defects in 20S maturation, while either single mutant does not (Marques et al., 2007). Thus activator binding to immature CP complexes (either the regulatory particle or Blm10) appears to be an integral part of CP maturation, with Blm10 and the regulatory particle playing redundant roles during this process.

The regulation of ribosome abundance and output is crucial for the maintenance of the energy economy within a cell and thus for cell growth and size. It is estimated that ribosome biosynthesis accounts for ~70% of total transcription and ~25% of the total translation in rapidly growing yeast cells (Warner, 1999; Rudra et al., 2005). Thus ribosome function and biogenesis have to be tightly correlated with the cellular nutrient status. This process is mediated by the activity of the major metabolic signaling pathways, the target of rapamycin kinase (TORC1), and cyclic AMP–dependent kinase A (PKA) pathways. Inactivation of TORC1 or PKA in yeast in response to limiting nutrients results in rapid repression of ribosomal genes and inhibition of translation (Powers and Walter, 1999; Wullschleger et al., 2005). Ribosome biogenesis requires the activity of all three nuclear RNA polymerases, which mediate the expression of ribosomal proteins (RPs), rRNAs, and accessory proteins, which assist in ribosome assembly. In S. cerevisiae factors controlling ribosomal gene expression include Rrn3, regulating Pol I–driven genes (Claypool et al., 2004; Ma11, which acts upon Pol III–mediated transcription (Upadhya et al., 2002); and the Pol II–directed transcriptional regulators Rapi, Ifn1, Hml1, Hmo1, and Sfp1 (Fingerman et al., 2003; Jorgensen et al., 2004; Marion et al., 2004; Martin et al., 2004; Schwalder et al., 2004; Wade et al., 2004; Zhao et al., 2006; Berger et al., 2007). Although TORC1/PKA signaling pathways have been implicated in the regulation of some of these factors, the precise mechanisms of gene activation and repression are not completely understood.

In this report we provide evidence for a regulatory function of proteasome-mediated turnover of Sfp1 in the repression of ribosome biogenesis upon nutrient depletion. Additionally, we demonstrate that proteasome-dependent Sfp1 turnover is mediated by the proteasome activator Blm10.

RESULTS

BLM10 deletion results in resistance to sublethal doses of cycloheximide

To gain insight into the cellular functions of Blm10, we performed a screen for loss-of-function phenotypes of cells deleted for BLM10. We found that blm10Δ cells exhibit resistance to sublethal doses of the translational inhibitor cycloheximide (CHX) (Figure 1A). The same phenotype has been associated with proteasome CP and regulatory particle mutants (crl mutants) defective in the turnover of ubiquitin conjugates (Gerlinger et al., 1997), derived from a screen for CHX-resistant yeast mutants (McCusker and Haber, 1988). Similar observations have been made in Arabidopsis thaliana (Kurepa et al., 2010). To corroborate that CHX resistance is a general phenotype of proteasome loss-of-function mutants, we tested the growth of the proteasomal ATPase mutants rpt1S, rpt2RF, and rpt3R (Rubin et al., 1998) under the same conditions. The respective point mutations prevent nucleotide binding to the ATPase subunits due to a point mutation within the Walker A motif, yet still allow cell growth. The ATPase mutants showed similar CHX resistance as loss of BLM10 (Figure 1A, bottom).

Ubp6 is a negative regulator of proteasome function, and ubp6Δ cells are characterized by proteasome hyperactivity (Hanna et al., 2003, 2006). In contrast to proteasome hypomorphs such as the ATPase mutants, loss of UBp6 results in strong CHX sensitivity (Figure 1A, middle). Interestingly, loss of UBp6 confers resistance to a second translation inhibitor, hygromycin B (Hanna et al., 2003). The same opposing phenotype between CHX and hygromycin B is observed for loss of BLM10 or for the ATPase mutants rpt1S, rpt2SF, and to a lesser extent rpt3R (Figure 1A, right), yet sensitivity and resistance to the drugs are inverted as compared with ubp6Δ. We conclude that proteasome loss-of-function mutants (ATPase mutants) confer CHX resistance and sensitivity to hygromycin B, while proteasome hyperactivity (ubp6Δ) results in the opposite phenotypes. It appears surprising that proteasome mutants are sensitive to one class of translation inhibitors but resistant to another. However, both inhibitors are chemically and mechanistically different. Hygromycin B is an aminoglycoside, which causes miscoding (Sutcliffe, 2005) and accumulation of misfolded proteins, explaining the sensitivity of mutants with reduced proteasomal activity. CHX, on the other hand, is a glutarimide antibiotic, which prevents initiation of translation, elongation, and the removal of the nascent chain from the ribosome. It furthermore inhibits polysome breakdown and reassembly (Pestka, 1971). Thus CHX-treated cells are characterized
by reduced protein synthesis. Increased proteasome activity induced by loss of UBP6 aggravates sensitivity to CHX, while proteasome loss of function alleviates the phenotype.

Because loss of BLM10 results in the same phenotype as the proteasome hypomorphic ATPase mutants (CHX resistance and sensitivity to hygromycin B), we conclude that Blm10 positively regulates proteasome activity, providing evidence for its role as a proteasome activator.

**BLM10 mutations, which prevent CP binding, exhibit a loss-of-function phenotype**

Recently it was demonstrated that Blm10 interaction with the CP involves C-terminal docking of Blm10 to conserved binding pockets located at the upper surface of the CP barrel (Sadre-Bazzaz et al., 2010). To investigate whether the CHX-resistant phenotype is related to the property of Blm10 to bind to the proteasome, we constructed a series of C-terminal BLM10 deletions in which either the last residue (BLM10ΔC1), the last two residues (BLM10ΔC2), or the last three residues (BLM10ΔC3) were removed. All C-terminal mutants exhibited a CHX-resistant phenotype (Figure 1B), arguing for a model in which Blm10 function is linked to its association with the proteasome. To corroborate that the mutations affect Blm10 binding to the proteasome, we purified Blm10–CP complexes and resolved them on native gels. While the deletion of the last residue of Blm10 still allowed complex formation, deletion of the last two or three residues abolished Blm10 association with the CP (Figure 1C). Interestingly, although deletion of the last residue did not prevent proteasome binding (Figure 1C), it caused a loss-of-function phenotype (Figure 1B), which could indicate that the C-terminus of Blm10 might have additional roles beyond CP binding. The same observation, intact complex formation plus a loss-of-function phenotype, is evident for a C-terminal chimera, where the last seven residues of Blm10 were exchanged against the C-terminal residues of PA26 (Figure 1, B and C).

**Loss of Blm10 affects the abundance of RPs after the diauxic shift**

We hypothesized that the CHX-resistant phenotypes upon loss of BLM10 or inactive ATPases might be a consequence of improved ribosome function, for example, due to increased ribosome levels. The control of ribosome abundance is a highly regulated process that is sensitive to the metabolic status of the cell. S. cerevisiae growth is characterized by three defined metabolic phases, as highlighted in Figure 2A (Gasch and Werner-Washburne, 2002; Herman, 2002). Initially, yeast cells grow logarithmically and generate ATP by fermentation (logarithmic [log] phase). On nutrient depletion, ATP is generated by oxidative metabolism. This switch from fermentation to oxidative metabolism is known as the “diauxic shift” and is marked by flattening of the growth curve (Figure 2A), indicative of a reduced growth rate. After several days in the postdiauxic shift (PDS) phase, cells arrest in G0, also known as the stationary (stat) phase in yeast. Both ribosome biogenesis and abundance decrease strongly when cells pass the diauxic shift (PDS) or are treated with the Tor inhibitor rapamycin (Powers and Walter, 1999). To test for potential alterations in ribosome abundance in bml10Δ cells, we analyzed the steady-state levels of two RPs, Rpl3 (Figure 2B, top left) and Rpl30 (top right), in the different metabolic phases. As expected, RP levels are significantly reduced in PDS and stat phase (Figure 2B) in wild-type (WT) cells. A longer exposure of Figure 2B is shown in Supplemental Figure 1. Strikingly, after the diauxic shift and in stat phase, BLM10-deleted cells exhibit elevated steady-state levels of RPs (Figure 2B).

Because Blm10 activates the proteasome, a potential explanation for increased RP levels upon BLM10 deletion after the diauxic shift is a participation of Blm10-proteasomes in RP turnover. To test this hypothesis, we performed CHX chase experiments in the presence of lethal doses of the drug (Kornitzer, 2002). Incubation with high doses of CHX blocks protein synthesis, allowing for determining the rate of protein turnover. Ribosomes are very stable complexes, with an estimated half-life of several days (Warner, 1999). BLM10 deletion did not affect RP turnover (Figure 2C). Our findings are in agreement with a recent report that demonstrates that ribosome turnover is achieved via autophagy in a process involving the deubiquitinating enzyme Ubp3/Bre5 (Kraft et al., 2008).
phases. RP gene transcription is strongly repressed after the diauxic shift in WT cells (Figure 3A; Brauer et al., 2005). Loss of BLM10 did not affect RP gene transcription in log phase (Figure 3B), in agreement with unchanged RP levels observed under the same conditions (Figure 2B). After the diauxic shift, however, RP gene transcription was elevated in the absence of BLM10 (Figure 3C).

RP gene transcription is rapidly down-regulated upon TORC1 inhibition by rapamycin (Powers and Walter, 1999). To investigate whether Blm10 function is required for this process, we examined rapamycin-induced RP gene repression in the presence or absence of BLM10. Rapamycin-induced RP gene down-regulation was significantly attenuated in blm10Δ cells (Figure 3D), suggesting that Blm10 is required for correct TORC1-mediated RP gene repression.

The transcription of BLM10 responds to metabolic changes

Our data suggest a regulatory function of Blm10-mediated proteasomal degradation under nutrient deprivation. We reasoned that this role might be reflected in the transcriptional profile of BLM10 expression. Proteasome protein levels increase under nutrient deprivation (Fujimuro et al., 1998). Blm10 expression has not been
investigated so far. We therefore tested the expression of BLM10, regulatory particle (RPN11 and RPT2), and CP subunits (PRE1 and PRE4) via qRT-PCR during growth in complete media (Figure 4A). While BLM10 and proteasome subunit expression remained constant and at a basal level during log growth, expression of all genes tested increased after the diauxic shift (Figure 4B, 10 h and 22 h), that is, when nutrients become limiting.

Rapamycin induces an artificial starvation response even under optimal nutrient conditions. In contrast to the expression of proteasome subunits, BLM10 mRNA levels were elevated rapidly after 1 h of rapamycin addition (Figure 4C). The latter observation is corroborated by a genome-wide study that analyzed the transcriptional response to rapamycin in yeast. BLM10 was found to be among the genes most strongly induced by rapamycin (Hardwick et al., 1999). Blm10 forms a proteasome subpopulation in log phase (Schmidt et al., 2005). Because the expression of proteasomal genes is less responsive to rapamycin, the divergent regulation of proteasome subunits versus BLM10 expression might indicate a redistribution of proteasome populations during nutrient depletion.

The CHX-resistant phenotype of blm10Δ cells is lost upon SFP1 deletion

SFP1-deleted cells exhibit a strong growth defect in the presence of low doses of CHX (Fingerman et al., 2003), while BLM10-deleted cells exhibit CHX resistance (Figures 1A and 5D). To investigate an epistatic relationship between the two genes, we constructed double mutants and tested them for growth on low doses CHX. The growth advantage of blm10Δ in the presence of low doses of CHX is lost in the absence of SFP1 (Figure 5D). Considering the results shown above, we propose that the CHX-resistant phenotype of BLM10-deleted cells originates from Sfp1 stabilization.

Proteasome-mediated Sfp1 degradation requires Blm10

To corroborate that Sfp1 turnover is mediated by proteasomes and is dependent on Blm10, we performed CHX chase experiments (Kornitzer, 2002) in rapidly growing cells. The experimental approach involves lethal doses of CHX, which blocks translation and transcription, which

FIGURE 4: BLM10 expression is up-regulated after the diauxic shift or in the presence of rapamycin. (A) Schematic of cell sampling (marked by arrows) during growth in YPD for the qRT-PCR analysis shown in (B). (B) Expression profile of proteasome subunits and BLM10 during growth in YPD. mRNA abundance of CP subunits (PRE1 and PRE6), regulatory particle subunits (RPN11 and RPT2), and BLM10 was analyzed in WT (yMS268) via qRT-PCR at the time points indicated by arrows in (A). C1 values were normalized to ACT1 expression levels. Values for each gene are presented relative to the 4-h time point. Data are reported as mean ± SEM. P-values for Blm10 expression are presented. A single asterisk indicates a P-value < 0.05; a double asterisk indicates P < 0.01. (C) Expression profile of PRE1, PRE6, RPN11, RPT2, and BLM10 in WT cells in the presence of 50 ng/ml rapamycin, which was added at the 4-h time point. Values were obtained as in (B) and at the time points indicated in (A).
Regulated Sfp1 turnover by Blm10-proteasomes

Inhibits cell growth in a manner that the lethal CHX dose used for the chase experiment indeed inhibits cell growth by specific transporters. To investigate the effect of MG132, a proteasome inhibitor, on cell cycle and results in a growth arrest (McCusker and Haber, 1988; Supplemental Figure 2). In the absence of new synthesis, the half-life of a protein can be determined. We found that in WT cells Sfp1 has a short half-life of ~35 min, indicating continuous turnover of Sfp1 (Figure 6A). In the presence of MG132, a proteasome inhibitor, Sfp1 half-life increased to ~108 min (Figure 6B). Thus Sfp1 degradation is mediated by the proteasome. A similar increase in Sfp1 half-life was detected in blm10Δ cells, demonstrating that Sfp1 degradation is executed most likely by Blm10-proteasomes (Figure 6C). Because the addition of MG132 did not increase Sfp1 half-life further in Blm10-deleted cells (Figure 6D), our data argue for a model in which Sfp1 degradation might be specifically mediated by Blm10-proteasomes, but not by other proteasome complexes. Although MG132 is readily taken up by yeast cells, it is also rapidly exported from the cell by specific transporters. To investigate the effect of MG132, a pdr5Δ deletion strain, a gene that codes for an export pump had to be used (Fleming et al., 2002). To ascertain that the lethal CHX dose used for the chase experiment indeed inhibits cell growth in a pdr5Δ strain background, we tested the strains used in Figure 6, A–D, for sensitivity to CHX. The pdr5Δ strains are exquisitely sensitive to CHX, independent of the presence or absence of Blm10 (Supplemental Figure 3).

Blm10 has been implicated in proteasome assembly (Fehlker et al., 2003; Li et al., 2007; Marques et al., 2007). In consequence, impaired Sfp1 turnover could potentially be explained by a general impairment of proteasome structural integrity upon loss of Blm10. To compare the proteolytic capacity of proteasomes in WT and blm10Δ cells, we tested the turnover of a general proteasome substrate, Ubc6 (Walter et al., 2001; Ravid et al., 2006). Loss of Blm10 did not influence the turnover of Ubc6 (Figure 7, A and B), whereas deletion of the proteasome-related transcription factor Rpn4, which results in reduced proteasome abundance (Xie and Varshavsky, 2001; Schmidt et al., 2005), effectively inhibited Ubc4 turnover. Thus, in the absence of Blm10, proteasomes are fully functional, arguing against impaired proteasome assembly in blm10Δ cells. The result additionally corroborates a model in which Blm10-proteasomes might target a subgroup of proteasome substrates (Schmidt et al., 2005). To...
further investigate the interaction between Sfp1 and Blm10-proteasomes, we mapped their physical interaction by CP immunoprecipitation (Figure 7D, right) in the absence or presence of BLM10. We found that Sfp1 copurifies with the proteasome in both WT and blm10Δ cells, indicating that Sfp1 interaction with the proteasome is not mediated by direct interaction of Sfp1 with Blm10. However, in blm10Δ cells the Sfp1 protein level, which copurified with the CP, was elevated, demonstrating that Blm10 positively affects the regulated turnover of Sfp1 at the proteasome.

**Loss of BLM10 leads to increased ribosome function**

It is well established that changes in Sfp1 levels affect ribosome abundance. The sfp1Δ cells show reduced levels of 80S ribosomes and higher-order polyribosomes compared with WT cells (Fingerman et al., 2003), while elevated Sfp1 level results in increased transcription of RP genes (Jorgensen et al., 2004). To test whether the elevated RP levels we observed upon Sfp1 stabilization in the absence of BLM10 lead to an increase in functional ribosomes, we compared the sucrose gradient profiles of ribosomal subunits and polyribosomes in WT, blm10Δ, sfp1Δ, and SFP1–overexpressing cells. Loss of SFP1 results in a reduced pool of 80S ribosomes and higher-order polyribosomes (Figure 8C), as previously described (Fingerman et al., 2003). Cells overexpressing SFP1, on the other hand, exhibit increased 80S ribosomes and polyribosomes. Consistent with the data reported above, BLM10 deletion results in a profile similar to SFP1 overexpression (Figure 8B).

**Sfp1 accumulates in the nucleus in BLM10-deleted cells**

Sfp1 function appears to be controlled by differential localization. While the protein is localized predominantly to the nucleus if nutrients are abundant, it is found evenly distributed in the cell after the diauxic shift or upon rapamycin treatment (Jorgensen et al., 2004; Marion et al., 2004). A current model for the repression of RP gene transcription under nutrient-limiting conditions involves the dissociation of Sfp1 from RP gene promoters, followed by nuclear export of the protein. We demonstrate here that Sfp1 is degraded after the diauxic shift, and if degradation is abrogated due to loss of Blm10 or upon proteasome inhibition, the protein is stabilized and the repression of RP gene transcription is attenuated. The higher levels of RP mRNA in blm10Δ cells suggest that a significant fraction of Sfp1 must remain functional and bound to RP gene promoters even under repressive conditions. To test Sfp1 localization in BLM10-deleted cells, we tagged the C-terminus of Sfp1 with green fluorescent protein (GFP) and performed live-cell fluorescence microscopy. As reported previously in WT cells (Marion et al., 2004; Lempiainen et al., 2009; Singh and Tyers, 2009), the predominant nuclear localization of Sfp1 during log growth (Figure 9A, top) is lost in PDS (Figure 9B, top). In blm10Δ log phase cells, Sfp1 is also detected predominantly in the nucleus (Figure 9A, bottom). In PDS, however, loss of BLM10 results in a high fraction of Sfp1 retained in the nucleus (Figure 9B, bottom, and 9C). The higher Sfp1 levels in blm10Δ cells after the diauxic shift are also reflected in increased intensity of Sfp1-GFP fluorescence if the cells are imaged with identical exposure times (Supplemental Figure 4).

**DISCUSSION**

Nutrient depletion in *S. cerevisiae* results in a rapid cellular response characterized by a metabolic switch from fermentation to oxidative metabolism (Herman, 2002). The major targets of this metabolic

**FIGURE 7:** Loss of BLM10 does not lead to a general impairment of proteasome function (A and B). Turnover of the proteasome substrate Ubc6 was determined in WT (UBC6-HA3 pdr5Δ [yMS792]) or in blm10Δ (UBC6-HA3 blm10Δ pdr5Δ [yMS1089]). (C) Ubc6 is stabilized in rpn4Δ cells. Turnover of the proteasome substrate Ubc6 in rpn4Δ (UBC6-HA3 rpn4Δ pdr5Δ [yMS1364]) strain. Pgk1 immunodetection was used as a loading control (bottom). (D) Sfp1 interacts with Blm10-proteasomes. For CP pull-down cells from yMS1189 and from yMS1190 containing protein A–tagged Pre1 and carrying SFP1–HA3 in the presence (yMS1189) or in the absence (yMS1190) of BLM10 were harvested in logarithmic (log) phase, lysed, and subjected to immune precipitation. The samples were separated by SDS–PAGE and probed with anti-HA (top) or anti-CP (bottom) antibodies.

**FIGURE 8:** Polyribosome profiles of cells with varying Sfp1 levels. Polyribosomes profiles were recorded for WT (A), blm10Δ (B), sfp1Δ (C), and SFP1–overexpressing cells (TEVpSFP1) (D).
transcription involves several transcription factors and activators. The transcription of these genes is rapidly repressed (Brauer et al., 2004). Under favorable nutrient conditions, Sfp1 is localized to the nucleus and binds to RP gene promoters (Marion et al., 2004). Nutrient depletion results in its relocalization to the cytoplasm. Mutations in putative Sfp1 TORC1 phosphorylation sites partially abrogate its nuclear localization (Lempiainen et al., 2009), and nuclear accumulation of Sfp1 is prevented in the presence of rapamycin (Marion et al., 2004; Singh and Tyers, 2009). Thus TORC1-dependent phosphorylation is thought to mediate localization and promoter binding of Sfp1. As loss of SFP1 results in the mislocalization of two other critical factors for RP gene transcription, Fhl1 and Ifh1 (Jorgensen et al., 2004), Sfp1 function and localization appear to play a central role in the regulation of RP gene transcription.

Here we report that in rapidly growing cells Sfp1 has a short half-life of ~38 min. Sfp1 turnover is impeded by proteasome inhibition or upon loss of the proteasome activator Blm10. Thus Sfp1 levels are regulated by proteasomal degradation in a process that is mediated by Blm10. Loss of BLM10 results in increased RP gene transcription, increased RP levels, increased abundance of active ribosomes, and resistance to low doses of CHX. Furthermore, blm10Δ cells exhibit increased nuclear abundance of Sfp1 during nutrient depletion, suggesting that nuclear export of Sfp1 per se is not sufficient to terminate its function. In summary, our results argue for a functional participation of proteasomes in the repression of ribosome biogenesis during nutrient depletion through degradation of Sfp1. Interestingly, proteasome recruitment to RP genes has been demonstrated recently (Auld et al., 2006). It is therefore tempting to speculate that Sfp1 turnover might occur at the actively transcribed gene, potentially to remove transcriptional complexes after transcription initiation or during the termination of transcription.

The proteasome has different functions in eukaryotic cells: 1) clearance of abnormal proteins to avoid proteotoxicity, 2) a metabolic function by providing building blocks for protein synthesis under starvation conditions, and 3) a regulatory signaling function exerted by the temporally controlled elimination of specific proteins. Ribosomes and proteasomes represent two macromolecular complexes with opposite functions to maintain proteostasis. Thus it could be expected that the first two functions largely determine the relationship between both complexes. Indeed, the proteasome degrades defective ribosomal products (DRiPs) and thus prevents the accumulation of damaged proteins produced by the ribosome (Schubert et al., 2000). Proteasome levels are also up-regulated under starvation conditions to supply amino acids for protein synthesis (Lecker et al., 2006). However, there is increasing evidence that proteasomes also exhibit regulatory functions during ribosome biogenesis. In genome-wide analyses in S. cerevisiae, proteasome subunits were detected at promoters of highly transcribed genes in general, and of RP genes in particular (Auld et al., 2006). Heterozygous proteasomal gene deletions result in a large cell phenotype indicative of increased ribosome function (Jorgensen et al., 2004), which in the light of the present study is most likely caused by impaired Sfp1 degradation. Furthermore, proteasome inhibition in mammalian cells revealed dysregulated ribosome maturation such as the accumulation of 90S preribosomes and altered nucleolar morphology (Stavreva et al., 2006). A significant portion of RPs is

FIGURE 9: Impaired Sfp1 localization in BLM10-deleted cells after the diauxic shift. (A) SFP1-GFP (yMS928) and SFP1-GFP bml10Δ (yMS929) cells were grown in synthetic complete media. Sfp1 localization was visualized in log phase via live-cell fluorescence. Differential interference contrast (DIC) images are shown on the right. (B) Sfp1 localization in PDS phase cells was analyzed in SFP1-GFP (yMS928) and SFP1-GFP bml10Δ (yMS929) as in (A). (C) Quantification of cells with nuclear Sfp1 localization in log and PDS in WT (yMS928) and bml10Δ (yMS929) from 10 independent fluorescent micrographs with ~500 cells each, using ImageJ software 1.42q for visualization.
degraded by the proteasome before ribosome formation in mammalian cells (Lam et al., 2007). Proteasome-mediated degradation of Sfp1, as reported in this study, adds an additional layer to the complex interaction between the proteasome and ribosome biogenesis and function by providing a regulatory mechanism for the repression of RP gene transcription under nutrient limitation.

The precise impact of Blm10 on proteasome function is unknown. Previous reports suggest that Blm10 might play a role in proteasome assembly (Fehlker et al., 2003) and thus might represent a proteasome chaperone required for correct proteasome biogenesis. Several lines of evidence, however, point to a function beyond maturation. 1) bmr10Δ cells do not exhibit assembly defects (Marques et al., 2007). 2) In contrast to the nine identified proteasome assembly chaperones, which either dissociate or are degraded after correct structure formation (Bedford et al., 2010), Blm10 copurifies with mature proteasomes and forms a stable proteasome subpopulation (Schmidt et al., 2005; Lehmann et al., 2008). 3) Different from proteasome chaperones, Blm10 binds to the CP gate region, a critical regulatory entry point for proteasome substrates (Schmidt et al., 2005; Iwanczyk et al., 2006). 4) On Blm10 binding, the CP gate region undergoes structural changes resulting in partial gate opening, as evident from the Blm10–CP crystal structure (Sadre-Bazzaz et al., 2010). 5) Blm10 activates the proteasomal peptidase activity (Schmidt et al., 2005; Iwanczyk et al., 2006; Lehmann et al., 2008; Sadre-Bazzaz et al., 2010). These observations are in line with typical characteristics of proteasome activators, such as the RP or the PA28 activator family.

The data reported here provide additional support for a potential proteasome activator function of Blm10 and suggest that Blm10 promotes the degradation of specific proteasome substrates. Loss of BLM10 results in the stabilization of Sfp1. Furthermore, treatment of bmr10Δ cells with proteasome inhibitors does not result in additional stabilization of Sfp1, arguing for a Blm10–proteasome–specific mechanism. In contrast, turnover of the proteasome substrate Ubc6 is unaffected by loss of BLM10, while Ubc6 is stabilized in rpm4Δ cells, which are characterized by low proteasome levels. These findings argue for fully functional proteasomes in the absence of Blm10 and against a major role of Blm10 in proteasome assembly.

If Blm10 functions as an activator for proteasomal protein degradation, then the interaction between Blm10 and the CP should involve opening of the CP gate. Indeed, an open or partially open gate has been observed in Blm10−CP cryo-electron microscopy and crystal structures (Iwanczyk et al., 2006; Sadre-Bazzaz et al., 2010). Previous studies suggest two distinct gate-opening mechanisms. C-terminal docking of PA26 is not sufficient for gate opening but requires a second internal loop structure. The C-termini of the RP ATPases, on the other hand, directly structurally alter the gate but requires a second internal loop structure. The C-termini of the RP ATPases, on the other hand, directly structurally alter the gate but requires a second internal loop structure.

MATERIALS AND METHODS

Strains, media, growth conditions, and chemicals
All strains and plasmids used in this work are listed in Table 1. They were obtained using standard genetic techniques. Unless otherwise noted, strains are isogenic to BY4741 or BY4742 (Brachmann et al., 1998) and are S288C derived. DY93 and DY106 and their parental strain SUB62 were kind gifts from Daniel Finley (Rubin et al., 1998).

Complete gene deletion, promoter exchange, or tag integration were constructed at the genomic locus by homologous recombination using standard techniques (Longtine et al., 1998; Goldstein and McCusker, 1999). Primer sequences are available upon request. Unless otherwise noted, strains were grown at 30°C in yeast peptone dextrose (YPD) and were harvested at OD

Bloom-proteasome purification and in situ assay to determine CP peptidase activity
For the purification of WT and mutant Blm10–CP complexes, cells from yMS31, yMS568, yMS569, yMS570, and yMS573 containing protein A-tagged Pre1 were collected and lysed using French press cell disruption. Purification was performed as described previously (Schmidt et al., 2005). Briefly, the cleared lysate was batch incubated with IgG Affinity gel (MP Biomedicals, Solon, OH), and the beads were collected and washed. Proteasome complexes were eluted with tobacco etch virus (TEV) protease (Invitrogen, Carlsbad, CA). Subsequently, the purified complexes were first resolved on 3.5% acrylamide native gels (Schmidt et al., 2005), followed by an in-gel activity assay with the fluorogenic proteasome substrate Suc-LLVY-AMC as described previously and silver staining of the gel (Schmidt et al., 2005).

Phenotypic analysis of gene deletion
Strains were grown overnight in YPD and diluted in 96-well plates to a density of 6 × 10⁴ cells per well followed by fivefold serial dilutions. They were spotted onto YPD plates in the absence or presence of CHX or hygromycin B. The concentration is indicated in the respective figure legend.

Growth curves
 Cultures were inoculated overnight in YPD. In the morning the culture was diluted to an OD₆₆₀ ≈ 0.1. Subsequently, four aliquots of 150 μl of the culture were added to an HC2 plate. Growth was recorded in a Bioscreen C MB machine (Growth Curves USA, Piscataway, NJ) for 48 h at 30°C under continuous shaking and with absorbance readings at 600 nm every 30 min.

Immunoprecipitation
For the proteasome CP pull-down cells (strains yMS1189, yMS1190, and untagged control BY4742) were harvested in log
phase, resuspended in lysis buffer (50 mM Tris, pH 8, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA) supplemented with protease inhibitor cocktail (complete, Roche, Indianapolis, IN), 10 mg/ml pepstatin A and 1 mg/ml antipain, and drop-frozen in liquid nitrogen. Frozen yeast cells were lysed in an MM301 grinding mill (Retsch, Haan, Germany) following the manufacturer’s protocol. Cell extracts were cleared at 11,000 rpm for 30 min at 4°C and filtered through cheesecloth (WVR International, San Diego, CA). The supernatants were mixed with rabbit immunoglobulin G resin (MP Biomedicals, Solon, OH) and incubated for 2 h at 4°C. The resin was washed at 500 rpm for 2 min at 4°C and washed five times with cold lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled for 5 min, and the supernatants were collected by centrifugation (500 rpm, 3 min). The proteins were separated

### Table 1: Strains used in this study.

| Strain       | Genotype                                                                 | Source                       |
|--------------|---------------------------------------------------------------------------|------------------------------|
| SUB62        | Mata lys2–801 leu2–3, 2–112 ura3–52 his3–A200 trp1–1(am)                  | Finley et al., 1994          |
| BY4741       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0                                        | Brachmann et al., 1998       |
| BY4742       | Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0                                        | Brachmann et al., 1998       |
| DY106        | Mata lys2–801 leu2–3, 2–112 ura3–52 his3–A200 trp1–1(am)                 | Rubin et al., 1998           |
| DY62         | Mata lys2–801 leu2–3, 2–112 ura3–52 his3–A200 trp1–1(am)                 | Rubin et al., 1998           |
| DY93         | Mata lys2–801 leu2–3, 2–112 ura3–52 his3–A200 trp1–1(am)                 | Rubin et al., 1998           |
| yMS31        | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE1EVPROA::His3                        | Schmidt et al., 2005         |
| yMS63        | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 blm10Δ::natMX                           | Schmidt et al., 2005         |
| yMS94        | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE1EVPROA::His3 blm10Δ::natMX         | Schmidt et al., 2005         |
| yMS131       | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 blm10Δ::natMX                           | This study                   |
| yMS222       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubp6Δ::KanMX                            | This study                   |
| yMS268       | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0                                       | This study                   |
| yMS524       | Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0                                         | This study                   |
| yMS565       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BLM10ΔC1::KanMX                         | This study                   |
| yMS566       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BLM10ΔC2::KanMX                         | This study                   |
| yMS567       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BLM10ΔC3::KanMX                         | This study                   |
| yMS568       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE1EVPROA::His3 BLM10ΔC1::KanMX       | This study                   |
| yMS569       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE1EVPROA::His3 BLM10ΔC2::KanMX       | This study                   |
| yMS570       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE1EVPROA::His3 BLM10ΔC3::KanMX       | This study                   |
| yMS573       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PRE1EVPROA::His3 BLM10PA26C::KanMX     | This study                   |
| yMS598       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 BLM10PA26C::KanMX                       | This study                   |
| yMS792       | Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 UBC6-HA3::KanMX pdr5Δ::HphMX             | This study                   |
| yMS908       | Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SFP1-HA3::KanMX                         | This study                   |
| yMS909       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SFP1-HA3::KanMX blm10Δ::natMX           | This study                   |
| yMS928       | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SFP1-GFP::KanMX                        | This study                   |
| yMS929       | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SFP1-GFP::KanMX blm10Δ::natMX           | This study                   |
| yMS957       | Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SFP1-HA3::KanMX pdr5Δ::HphMX             | This study                   |
| yMS958       | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SFP1-HA3::KanMX blm10Δ::natMX pdr5Δ::HphMX | This study                   |
| yMS1011      | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spf1Δ::KanMX                           | This study                   |
| yMS1012      | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spf1Δ::KanMX blm10Δ::natMX              | This study                   |
| yMS1013      | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spf1Δ::KanMX                           | This study                   |
| yMS1089      | Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 UBC6-HA3::KanMX pdr5Δ::HphMX blm10Δ::natMX | This study                   |
| yMS1090      | Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 kanMX::TEVpSFP1                        | This study                   |
| yMS1092      | Mata lys2–801 leu2–3, 2–112 ura3–52 his3Δ200 trp1–1(am) SFP1-HA3::KanMX | This study                   |
| yMS1093      | Mata lys2–801 leu2–3, 2–112 ura3–52 his3Δ200 trp1–1(am) SFP1-K219R SFP1-HA3::KanMX | This study                   |
| yMS1189      | Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SFP1-HA3::KanMX PRE1EVPROA::His3         | This study                   |
| yMS1190      | Matα his3Δ1 leu2Δ0 ura3Δ0 SFP1-HA3::KanMX PRE1EVPROA::His3 blm10Δ::natMX | This study                   |
| yMS1364      | Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 UBC6-HA3::KanMX pdr5Δ::HphMX rpn4Δ::natMX | This study                   |
by SDS–PAGE, followed by immunodetection for Sfp1-HA and for CP.

**Gel electrophoresis and immunoblotting**

Cells from WT and mutant strains were harvested in the respective growth phases and stored at −80°C. Cells were disrupted by alkaline lysis as described previously (Kushnirov, 2000). Protein concentration was determined using a Bradford protein assay (Bio-rad, Hercules, CA). Equal protein amounts were subjected to SDS–PAGE and immunodetection. Antibodies used were anti-CP (BIOMOL, Plymouth Meeting, PA), anti-Rpr13, and anti-Rip30, kindly provided by Jonathan Warner, and anti-HA 12C5 (Roche, Indianapolis, IN). Anti–phosphoglycerate kinase 1 (Pgk1) (Invitrogen, Carlsbad, CA) was used as a loading control. Signals were detected via enhanced chemiluminescence using a kit (Pierce, Rockford, IL).

**Protein degradation assay (CHX chase)**

Protein turnover was determined using a CHX chase assay (Ravid et al., 2006). To analyze proteasome-dependent degradation, log phase cultures were supplemented with 40 μM for Sfp1 and 75 μM for Ubc6 of MG132 or vehicle (dimethyl sulfoxide [DMSO]) for 3 h or 30 min, respectively, at 30°C before the addition of 0.2 mg/ml CHX. Aliquots were harvested at the times indicated and either immediately frozen at −20°C (Figure 6) or lysed directly (Figure 7). After alkaline lysis (Kushnirov, 2000), equal protein amounts were subjected to SDS–PAGE and immunodetection using an anti-HA antibody to detect Sfp1 or Ubc6 levels. The immunoblots were scanned and band intensity was quantified using ImageJ 1.42q.

**qRT-PCR**

Total RNA was isolated after an enzymatic digest of the outer cell wall with zymolase (Seikagaku, Tokyo, Japan) using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Subsequently, 1 μg RNA was treated with DNase (Invitrogen), followed by reverse transcription using the High Capacity cDNA kit (Applied Biosystems, Carlsbad, CA). TaqMan primers and probes were designed using the software Primer3Plus (Untergasser et al., 2007). The sequences are available upon request. In an Applied Biosystems 7900HT instrument, 5 ng cDNA was subjected to qRT-PCR. The reactions were performed in 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 1 min) after an initial activation with 50°C for 2 min and 95°C for 10 min. Negative controls were run simultaneously for each reaction. Data were analyzed using ABI Prism 7900 SDS 2.1v (Applied Biosystems) 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 individual figure legends, is given by 2−ΔΔCt. All reactions were performed in quadruplicates. Error bars indicate the mean ± SEM of at least three independent experiments. Statistical significance of the obtained data was determined with the independent-samples t-test analysis using SPSS version 16 software (SPSS, Chicago, IL). P-values < 0.05 were considered statistically significant.

**Polyribosome profile analysis**

For polyribosome preparation, 50 ml cultures of BY4741, yMS63, yMS1013, and yMS1090 were grown to log phase (OD660 0.8–1.0), and CHX was added to a final concentration of 100 μg/ml. The cells were chilled immediately on ice. After centrifugation at 5000 rpm for 5 min at 4°C, the cell pellets were washed once with 10 ml ice-cold LHB buffer (0.1 M NaCl, 0.03 M MgCl2, 0.01 M Tris, pH 7.4, 100 μg/ml CHX, 200 μg/ml heparin) and resuspended in 0.5 ml cold LHB buffer. A 700-μl volume of glass beads was added, and the cells were vortexed 1 × 1 min in a BeadBeater. The lysates were spun down briefly to reduce foam and diluted with LHB to a final volume of 1.5 ml. After centrifugation in a microcentrifuge for 10 min at maximum speed and 4°C, A260 was measured, and 10 OD260 units were loaded onto a sucrose gradient (11 ml 10–50% sucrose in 0.05 M Tris-Ac, pH 7.0, 0.05 M NaH2PO4, 0.012 M MgCl2). The gradients were centrifuged in a Beckman SW41 rotor at 40,000 rpm for 2.5 h, and A260 of gradient fractions was read using an ISCO UA-5 absorbance detector.

**Microscopy**

yMS928 and yMS929 carrying C-terminally GFP-tagged Sfp1 were grown overnight in synthetic dextrose at 30°C to PDS. Cells were either redivited in the morning to an OD660 nm 0.1 and grown for additional 3 h to obtain log phase cultures or were harvested immediately. Live-cell fluorescence of the strains was monitored using a fluorescence microscope (Olympus BX61) at the Albert Einstein Imaging Facility with a 60× NA 1.4 objective (PlanApo). Fluorescence or differential interference contrast (DIC) images were captured with a cooled CCD camera (Sensicam QE, Cooke, Romulus, MI) using IPLab 4.0 software. Images were processed using ImageJ software 1.42q.

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Regulated Sfp1 turnover by Blm10-proteasomes

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