A nonproteolytic proteasome activity controls organelle fission in yeast

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Summary

To understand the processes underlying organelle function, dynamics and inheritance, it is necessary to identify and characterize the regulatory components involved. Recently in yeast and mammals, proteins of the membrane fission machinery (Dnm1-Mdv1-Caf4-Fis1 in yeast and DLP1-FIS1 in human) have been shown to have a dual localization on mitochondria and peroxisomes, where they control mitochondrial fission and peroxisome division. Here, we show that whereas vacuole fusion is regulated by the proteasome degradation function, mitochondrial fission and peroxisomal division are not controlled by the proteasome activity but rather depend on a new function of the proteasomal lid subunit Rpn11. Rpn11 was found to regulate the Fis1-dependent fission machinery of both organelles. These findings indicate a unique role of the Rpn11 protein in mitochondrial fission and peroxisomal proliferation that is independent of its role in proteasome-associated deubiquitylation.

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Key words: Mitochondria, Peroxisomes, Fission, Proteasome

Introduction

Mitochondria are ubiquitous and essential organelles whose morphology and activity adapt to physiological states of the cell. They form a branched tubular network in the cell periphery and as a result of constant fission and fusion of individual mitochondria, form a dynamic mitochondrial network (Shaw and Nunnari, 2002; Yaffe, 1999). Most of the components of the mitochondrial fusion and fission machineries have been identified and are highly conserved between yeast and mammals. In the yeast *Saccharomyces cerevisiae*, at least six proteins seem to constitute the fusion and fission core machineries (Shaw and Nunnari, 2002). Mitochondrial fusion is controlled by the outer mitochondrial membrane GTPase Fzo1 (Hermann et al., 1998), the outer mitochondrial membrane protein Ugo1 (Sesaki and Jensen, 2001) and the dynamin-related GTPase Mgm1 located in the intermembrane space (Wong et al., 2000). Deletion of either of the genes encoding either of these proteins results in the loss of mitochondrial fusion and because of the on-going fission, mitochondria fragment into small pieces (Jensen et al., 2000). Mitochondrial fission is mediated by the dynamin-related GTPase Dnm1 (Otsuga et al., 1998), the adaptor proteins Mdv1 and Caf4 (Griffin et al., 2005; Tieu and Nunnari, 2000) and the outer membrane tail-anchored (TA) protein Fis1 (Mozdy et al., 2000). Based on genetic and biochemical approaches, a model has emerged for the assembly of these fission proteins. Fis1 recruits Dnm1 to mitochondrial membranes in concert with Mdv1 and Caf4. In this process, Mdv1 is suggested to act as an adaptor protein that is important in the activation of the protein machinery and Caf4 seems to be essential for establishing polarity (Griffin et al., 2005; Schauss et al., 2006). Mutation or deletion of either of these fission proteins leads to highly interconnected, often net-like, mitochondria. Reflecting the equilibrium between fusion and fission processes, cells defective in both fusion and fission proteins harbor wild-type-like mitochondrial networks (Bleazard et al., 1999; Cerveny et al., 2001; Fekkes et al., 2000; Mozdy et al., 2000; Sesaki and Jensen, 1999; Sesaki and Jensen, 2001; Tieu and Nunnari, 2000).

In yeast and mammals, all the components of the mitochondrial fission machinery (Dnm1-Mdv1-Caf4-Fis1 in yeast and DLP1-FIS1 in human) were found to colocalize with peroxisomes and mediate both mitochondrial and peroxisomal fission (Kobayashi et al., 2007; Koch et al., 2005; Kuravi et al., 2006; Motley et al., 2008; Nagotu et al., 2008a). Peroxisomes and mitochondria are metabolically linked organelles that crosstalk and cooperate. They are also both dynamic organelles that have been shown to frequently change size and shape and to move in a motor-dependent manner along cytoskeletal tracks throughout the cell. The classical model of peroxisome biogenesis predicts that peroxisomes grow by uptake of newly synthesized matrix and membrane proteins from the cytosol and multiply by division (Lazarow and Fujiki, 1985). However, findings also suggest that peroxisomes can be formed de novo from the endoplasmic reticulum (ER) or a subdomain of the ER (Hoepfner et al., 2005). Recently, it was demonstrated in wild-type *S. cerevisiae* cells, that mature peroxisomes, which do not fuse, multiply by fission of pre-existing peroxisomes driven by two dynamin-related proteins (Vps1 and Dnm1) and do not form de novo (Motley and Hettema, 2007). Only in cells temporarily devoid of peroxisomes, can these organelles form de novo, but this process is slow and independent of the dynamin-related proteins (Motley and Hettema, 2007). *S. cerevisiae* contains only few small peroxisomes per cell under most conditions of growth. Proliferation of peroxisomes and induction of the fatty acid β-oxidation machinery is required for growth on fatty acids as the sole carbon

source. In the past few years, it has been shown that fission of elongated peroxisomes, driven by the fission machinery Fis1/Mdv1/Caf4/Dnm1, contribute to peroxisome abundance under oleate induction (Kuravi et al., 2006; Motley et al., 2008). It is still an open question how the dual targeting of the fission components is mediated, and whether organelle-specific factors exist that regulate the assembly of the division machineries of both organelles.

Regulation of the mitochondrial and peroxisomal dynamics during cell cycle or metabolic changes is poorly understood. However, the ubiquitin-proteasome system (UPS) has been found to have a role in mitochondrial morphology and dynamics. Fisk and Yaffe showed that a mutated form of ubiquitin produces mitochondrial aggregation (Fisk and Yaffe, 1999). More recently, a role for the 26S proteasome in degradation of the fusion protein Fzo1 has been reported in response to the α-factor during nonvegetative growth (Neutzner and Youle, 2005). However, there is no evidence for ubiquitylation and no ubiquitin ligase has been implicated in this process. In vegetative growth conditions, Cohen and co-workers reported ubiquitylation of Fzo1 at mitochondria and its subsequent degradation by the 26S proteasome (Cohen et al., 2008). Genes of the UPS were identified in a systematic screen of essential genes required for the maintenance of proper mitochondrial morphology (Altmann and Westermann, 2005). It has also been demonstrated that the proteasomal protein Rpn11 is essential for maintaining a correct cell cycle and normal mitochondrial morphology and physiology (Rinaldi et al., 2008; Rinaldi et al., 2004; Rinaldi et al., 2002).

The 26S proteasome is a multicatalytic protease that degrades polyubiquitylated proteins into short peptides (Glickman and Ciechanover, 2002). In addition to its role as a protease, the proteasome also functions nonproteolytically in a variety of cellular processes, including transcription (Ferdous et al., 2001; Gonzalez et al., 2002), DNA repair (Reed and Gillette, 2007) and chromatin remodeling (Collins and Tansey, 2006). The 26S proteasome is composed of two subcomplexes: a 20S core particle that carries the catalytic activity and the 19S regulatory particle (RP) (Baumeister et al., 1998). The 19S RP can be further dissociated into two subcomplexes referred to as the base and the lid (Glickman et al., 1998). The base, which mediates a direct contact with the 20S core complex, is made up of six homologous AAA-ATPases (Rpt1-Rpt6), together with two non-ATPase subunits (Rpn1 and Rpn2). The lid of the RP is made of nine non-ATPase subunits (Rpn3, Rpn5-Rpn9, Rpn11, Rpn12 and Rpn15) and contains a deubiquitinase activity. A supplementary subunit Rpn10 connects the base to the lid. The main function of the 19S RP is to recognize ubiquitylated proteins, cleave the ubiquitin moiety and to unfold and insert the substrates into the 20S (Braun et al., 1999; Glickman et al., 1999).

Consistently with the multiple involvements of the proteasome in different cellular processes, mutations in genes encoding the regulatory particle are known to have pleiotropic phenotypes. Among them, a mutation in *RPN11*, called *mpr1-1* and renamed *rpn11-m1* (Rinaldi et al., 2008), shows the phenotypic characteristics generally associated with other proteasomal mutations, such as cell cycle defects and accumulation of polyubiquitylated proteins at the nonpermissive temperature (Rinaldi et al., 1998). In addition and more specifically, *rpn11-m1* exhibits defects in mitochondrial morphology (fragmented mitochondria) at the permissive temperature. Rpn11 belongs to a subset of MPN-domain proteins that harbor a MPN+ or JAMM metalloprotease motif responsible for deubiquitylation of certain substrates (Verma et al., 2002; Yao and Cohen, 2002). Mutations in this catalytic deubiquitinase active site result in reduced proteasome-dependent deubiquitylation, but still allow formation of tubular mitochondria (Rinaldi et al., 2004). However, mutations in the C-terminal 31 amino acids of the protein lead to abnormal mitochondrial morphology (Rinaldi et al., 2008). The involvement of Rpn11 in mitochondrial biogenesis has been further supported by the isolation of extragenic suppressors of *rpn11-m1*, which can dissociate the phenotypes related to cell cycle defects from those of mitochondria (Rinaldi et al., 2002). Our recent studies identified a functional domain formed by a putative α-helix in the C-terminal part of Rpn11 that is necessary for the maintenance of a correct cell cycle. A very short region adjacent to this α-helix was found to be essential for the maintenance of tubular mitochondrial morphology and important for respiration. We showed that the absence of the last 31 C-terminal amino acids of Rpn11 does not affect the mitochondrial fusion process but rather its presence might regulate the mitochondrial fission and/or the tubulation process (Rinaldi et al., 2008).

In the present study, to investigate the molecular function of Rpn11 on the mitochondrial fission process, driven by Fis1/Mdv1/Caf4/Dnm1 and shared with the peroxisomes, we have analyzed more specifically mitochondrial morphology and peroxisome abundance. We found that whereas vacuole fusion is regulated by the proteasome degradation function as previously demonstrated (Kleijnen et al., 2007), the mitochondrial fission and the peroxisomal division processes are not controlled by the proteasome activity but depend on a new function of the lid subunit Rpn11. Together, our results indicate a new role for the Rpn11 protein on the Fis1/Mdv1/Caf4/Dnm1 fission machinery that is independent of its role in proteasome-associated degradation.

Results

Examination of organelle structures in the *rpn11-m1* mutant

It has been previously shown that the *rpn11-m1* mutant strain harbors fragmented mitochondria at the permissive temperature (Rinaldi et al., 1998; Rinaldi et al., 2002). We examined whether other intracellular structures were affected in this strain by staining wild-type and *rpn11-m1* cells with fluorescent probes specific for vacuoles, ER, actin cytoskeleton, mitochondria and peroxisomes. We studied in vivo vacuolar morphology by using the vital stain FM4-64, and observed no difference in the vacuolar morphology between the *rpn11-m1* and wild-type cells at the permissive temperature, indicating that vacuolar fusion is not impaired in the *rpn11-m1* strain (Fig. 1B). ER morphology revealed by a protein fusion between Erg6 and GFP did not seem to be affected in the *rpn11-m1* mutant cells (Fig. 1D), nor did the actin network stained with green phalloidin (Fig. 1E). However, as previously described, mitochondria of the *rpn11-m1* cells were highly fragmented (Fig. 1A) (Rinaldi et al., 2008). Interestingly, labeling the peroxisomal matrix with the red fluorescent protein DsRed containing the C-terminal peroxisomal targeting signal type 1 (PTS1, DsRed-SKL), revealed a large variation in peroxisome abundance in the *rpn11-m1* cells compared with wild-type cells grown overnight in oleate (Fig. 1C). Oleate was chosen as a carbon source for peroxisomal proliferation involving the Fis1-Mdv1-Caf4-Dnm1 apparatus. We observed no strong alteration in peroxisome morphology in *rpn11-m1* cells relative to wild-type cells.

These data reveal that only the mitochondrial morphology and the peroxisome abundance are affected in the *rpn11-m1* mutant at the permissive temperature when compared with the wild-type strain; the vacuole, ER and actin cytoskeleton are not affected.

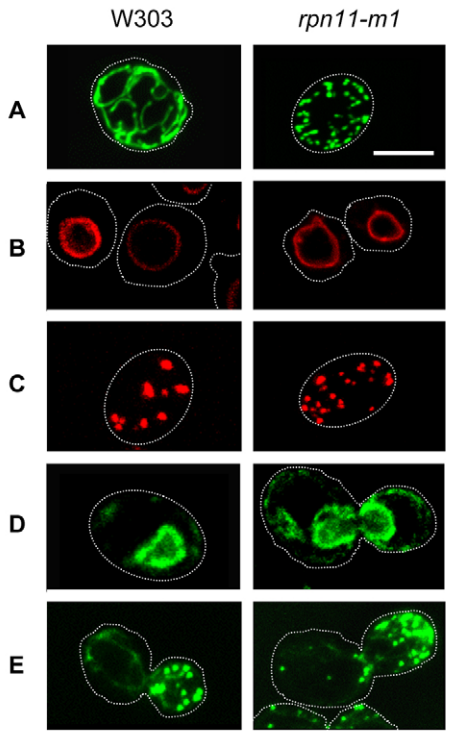

Fig. 1. Organelle morphology defects caused by the *rpn11-m1* mutation at permissive temperature. (A) Wild-type (W303) and *rpn11-m1* yeast cells transformed with the pYX142-mtGFP plasmid expressing the GFP targeted to the mitochondria. (B) Cells stained with the dye FM4-64 in glucose to visualize vacuoles. (C) Cells transformed with the pUG34 DS.Red.SKL plasmid expressing RFP targeted to the peroxisomes. Cells were grown in minimum medium and induced for 14 hours on oleate. (D) Cells transformed with the pERG26-GFP-2 plasmid expressing the Erg26 protein of the ER fused to GFP. (E) Cells stained with AlexaFluor488-phalloidin dye to label the actin network. The white dotted line corresponds to the cell wall. Scale bar: 5 μm.

Rpn11-m1, but not proteasome dysfunction, leads to an increase in peroxisome number

Defects in vacuolar morphology have already been documented in a proteasomal AAA-ATPase mutant strain as a result of impaired vacuolar fusion (Kleijnen et al., 2007). It has been shown that proteasome degradation is required for vacuolar fusion merely by degrading the vacuolar Ypt7 protein when ubiquitylated (Kleijnen et al., 2007). To investigate whether such a proteasomal function is also required for mitochondrial and peroxisomal fission events, we examined mitochondria, vacuole morphology and peroxisome abundance of thermosensitive strains mutated in different proteasomal subunits: *pre1-1pre2-2* in the 20S core particle, *Δsem1* and *rpn11-m1* in the lid and *Δrpn10*, *rpt2RF* and *rpt4R* in the base. An interaction between Pre1 and Pre2 is necessary for formation of the chymotrypsin-like active site in the proteasome (Heinemeyer et al., 1993; Hilt et al., 1993; Hilt et al., 1996; Hilt and Wolf, 1996). Degradation of polyubiquitylated proteins has been shown to be impaired in the strain deleted for *SEM1* (*RPN15*) (Sone et al., 2004). Deletion of *RPN10* was shown to have a more discrete phenotype (resistance to amino acid analogs) and contribute to the turnover of only a subset of proteins (Mayor et al., 2007). *RPT2* and *RPT4* encode two of the six AAA-ATPases within the base of the regulatory particle 19S, which have been proposed to mediate proteasome substrate unfolding before translocation into the core particle. The mutant strain *rpt2RF* shows the strongest phenotype, with a dramatic inhibition of the proteasome peptidase activity whereas *rpt4R* is less affected (Rubin et al., 1998).

All these strains were transformed with a plasmid encoding GFP targeted to mitochondria (Fig. 2B) or with a plasmid encoding the DsRed targeted to the peroxisomes (Fig. 2D). As a control of proteasome degradation inactivation, these strains were also stained with FM4-64 to allow vacuole visualization (Fig. 2C). With the exception of *Δrpn10*, which already showed a defect in vacuolar fusion at the permissive temperature, vacuolar fragmentation was observed in all the mutant strains at the nonpermissive temperature, independently of the proteasomal subunit mutation (Fig. 2C), confirming that in our conditions, the degradation function of the proteasome mutants is impaired, as previously demonstrated (Kleijnen et al., 2007). Altered mitochondrial morphologies were observed after 6 hours at the nonpermissive temperature for *rpt2RF* and *rpt4R* in agreement with earlier work (Altmann and Westermann, 2005), whereas such an effect was not observed for *Δsem1* and *Δrpn10*. Only *rpn11-m1* presented highly fragmented mitochondria at the permissive temperature (Fig. 2B). No increase in peroxisome number was observed in any of the proteasome mutants examined at 36°C compared with numbers at 26°C (Fig. 2D). Strong fragmentation of the mitochondria and a high peroxisome number could be observed only in the *rpn11-m1* cells at the permissive temperature.

We then performed quantitative analyses of peroxisome numbers in these various strains (Fig. 3). When the wild-type and *rpn11-m1* strains were grown in glucose, the distribution and the average number of peroxisomes per cell were similar between these two isogenic strains (Fig. 3A). However, when these strains were grown in oleate, a huge increase in peroxisome number was observed in *rpn11-m1* cells compared with the wild-type cells (Fig. 3B). Up to 17 fluorescent spots per wild-type cell could be detected with an average of 7.5±0.24 peroxisomes per cell. This number was largely increased in *rpn11-m1* cells, where up to 28 peroxisomes per cell could be counted, with an average of 14.34±0.47 peroxisomes per cell. Note that, not only the average number of peroxisomes, but also the frequency distribution of peroxisomes was largely affected in *rpn11-m1* (Fig. 3B). These cells contained at least three peroxisomes instead of one per wild-type cell (Fig. 3B). In the same conditions, the average number of peroxisomes per wild-type cell varied depending on the genetic background of the strains, ranging from 3.7±0.4 for BY4741 to 7.5±0.24 for the W303-1B background. Analyses of the peroxisome number of all the proteasome mutant strains compared with their isogenic parent strain, showed a comparable average number of peroxisomes at the nonpermissive temperature (Fig. 3C). For the *pre1-1pre2-2* strain, the average number of peroxisomes was compared between the permissive and nonpermissive temperatures, because the parent strain was not available. Thus, impairment of the degradation activity of the proteasome disturbs the vacuole fusion process, as previously demonstrated (Kleijnen et al., 2007), but does not affect the peroxisomal abundance on oleate. Altered function of only Rpn11 leads to fragmented mitochondria and an increase in peroxisome number on oleate.

Because mature peroxisomes do not fuse and multiply by fission of pre-existing peroxisomes (Motley and Hettema, 2007), these data strongly suggest a specific involvement of the proteasomal lid subunit Rpn11 on a mechanism that controls the division of mitochondria and peroxisomes.

Rpn11 has a role in the regulation of the peroxisome number independently of its role in the cell cycle

Our observation that a mutation in the C-terminal domain of Rpn11, but not five other mutations in the core (20S) or lid (19S) proteasomal subunits, led to a huge increase of peroxisome number indicates a specific role of Rpn11 on the peroxisomal proliferation process. To study the specific involvement of Rpn11 in the mitochondrial morphology and the peroxisome abundance, we first quantitatively analyzed the number and distribution of peroxisomes in two *rpn11-m1* revertant strains.

Intragenic and extragenic suppressors of *rpn11-m1* able to rescue the cell cycle defect of *rpn11-m1* but not the mitochondrial morphology defect have been previously isolated (Rinaldi et al., 2008). Among them, we selected *rpn11-m1-RevA2* an extragenic suppressor of *rpn11-m1* (mutation not identified) and *rpn11-m1-RevA5* an intragenic mutant. This latter mutant restored the complete open reading frame of *RPN11* but contained seven amino acid changes in the C-terminal domain (Fig. 4A). Both mutants were able to grow at 36°C on glucose. Only *rpn11-m1-RevA2* did not grow on glycerol at 36°C (Fig. 4B), but both mutants still clearly showed highly fragmented mitochondria at 26°C (Fig. 4C).

Quantitative distribution of peroxisomes in oleate-grown cells of the wild type, *rpn11-m1* and the two suppressor strains are shown Fig. 3B and Fig. 4D. The distribution of the peroxisomes was much more scattered for the *rpn11-m1*, *rpn11-m1-RevA2* and *rpn11-m1-RevA5* isogenic strains compared with the wild-type strain. All the mutant cells contained at least three peroxisomes instead of one, as was the case for the wild-type cells. The average number of peroxisomes per cell decreased moderately between *rpn11-m1* (14.37±0.47) and the suppressors strains *rpn11-m1-RevA2* (10.24±0.14) and *rpn11-m1-RevA5* (11.39±1.62) (Fig. 4D). Altogether, these results show that rescuing the cell cycle defect of the *rpn11-m1* cells does not correct the peroxisomal and mitochondrial defects.

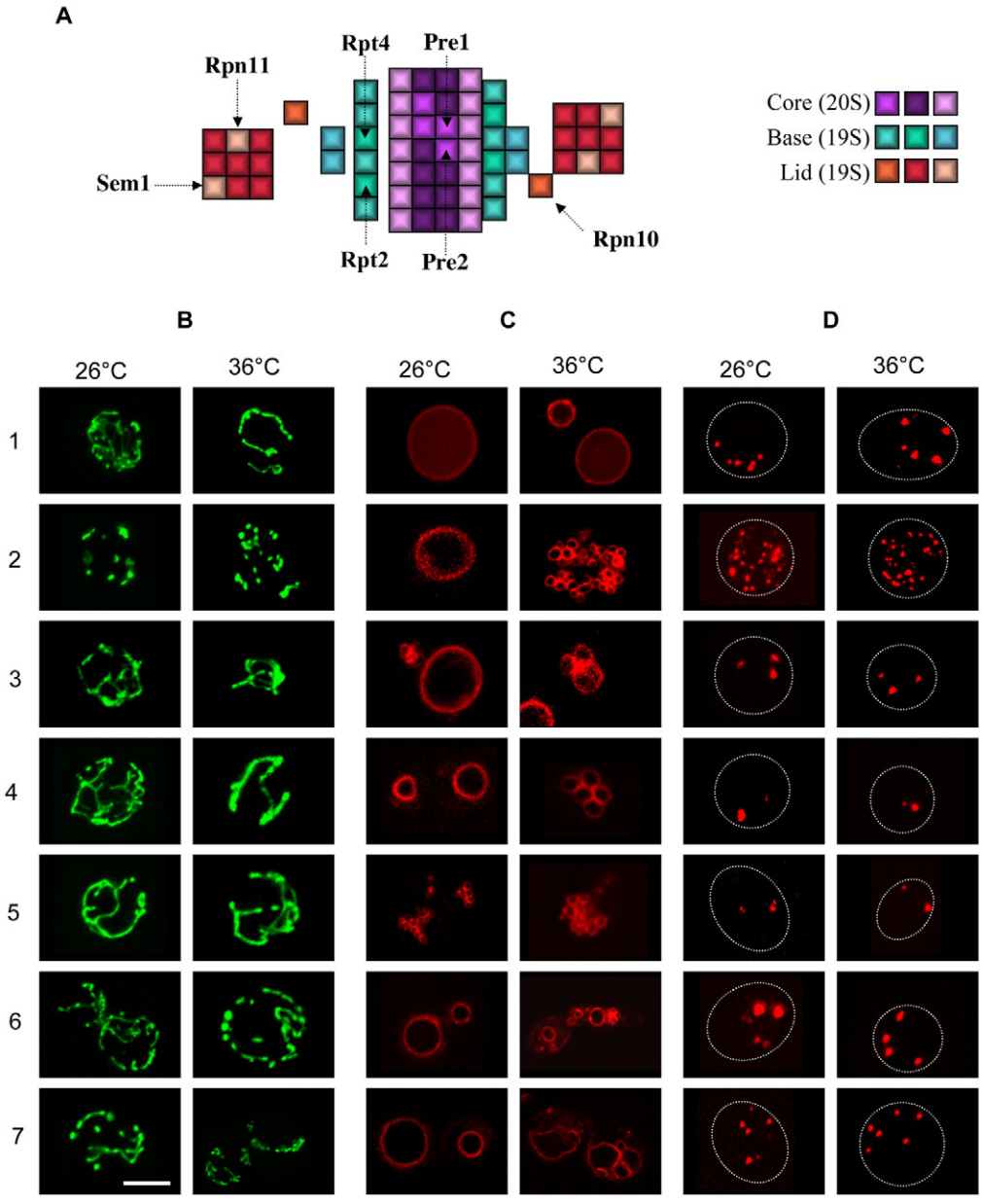

Fig. 2. The mitochondrial and peroxisomal defects are not a consequence of proteasome deficiency. (A) Schematic location of the different subunits examined in the 26S proteasome. (B-D) Mitochondria (B), vacuoles (C) and peroxisome (D) staining in cells grown in oleate. Organelles were observed in (1) wild-type (W303-1B), (2) *rpn11-m1*, (3) *pre1-1pre2-2*, (4) *Δsem1*, (5) *Δrpn10*, (6) *rpt2RF* and (7) *rpt4R* strains either at the permissive temperature of 26°C or the nonpermissive temperature of 36°C. The white dotted line corresponds to the cell wall. Scale bar: 5 μm.

Mutation in the catalytic deubiquitinase domain of Rpn11 does not increase peroxisome number

We then investigated peroxisome abundance in a single-site mutant of the MPN+ deubiquitinase catalytic domain (motif-E-$HxHx_7Sx_2D$) of Rpn11 (*rpn11-S119A*). This mutant strain exhibits general proteolytic defects, accumulation of polyubiquitylated proteins and temperature sensitivity, but contains a tubular mitochondrial network (Rinaldi et al., 2004). The quantitative distribution and the average number of peroxisomes were quantified at the nonpermissive temperature in the *Δrpn11* strain, which expresses the Rpn11 protein with or without the S119A mutation (*rpn11-S119A*) (Fig. 4D). The strain expressing Rpn11-S119A shared a frequency distribution of peroxisomes that was more scattered than the strain producing the wild-type protein. The average number of peroxisomes in cells expressing Rpn11S119A was higher (6.63±0.15) than in those expressing Rpn11 (4.51±0.4), but much lower than in *rpn11-m1* (14.34±0.47) (Fig. 4D; Fig. 3B). Importantly, both strains contained at least one peroxisome per cell, as it was the case for wild-type cells.

These results indicate that the Rpn11 deubiquitinase activity might not be involved in the oleate-induced increase of peroxisome number and suggest a new role for the Rpn11 C-terminal domain in regulating the peroxisome abundance.

Rpn11-m1 does not alter Pex11 expression

To grow on a fatty acid as sole carbon source, proliferation of peroxisomes and induction of the fatty acid β-oxidation machinery is required. Pex11 is a peroxin of the inner surface of the peroxisomal membrane that is the most strongly induced peroxin on oleic acid and is absolutely required for peroxisome proliferation (Marshall et al., 1995). Yeast mutants lacking *PEX11* are unable to increase the peroxisome number, whereas overexpression of Pex11 induces peroxisome division in a multi-step process involving elongation of pre-existing peroxisomes followed by their division (Gurvitz et al., 2001). Therefore, we first asked whether Rpn11-m1 would affect Pex11 expression. We examined Pex11 levels in *rpn11-m1* cells grown in glucose and oleate (Fig. 5). Pex11 was tagged with 3×HA and expressed from its chromosomal locus in both genetic contexts. No variation in Pex11 level was detected in cells grown in glucose between the two genetic contexts compared with the cytosolic marker eRF1. Note that in W303-1B, the concentration of Pex11 is quite high in cells grown in glucose compared with the study of Marshall and colleagues (Marshall et al., 1995). In cells grown in oleate, the Pex11 levels were higher than those in glucose-grown cells, but were at the same level in *rpn11-m1* and wild-type cells. This result shows that Rpn11-m1 does not alter the Pex11 expression.

Rpn11 regulates the Fis1-dependent fission of peroxisomes

It has been previously demonstrated that Vps1 is the major dynamin-related protein that regulates the peroxisome division process in yeast (Hoepfner et al., 2005; Li and Gould, 2003). However, the mitochondrial fission machinery (Fis1-Mdv1-Caf4-Dnm1) was also shown to localize to peroxisomes and control peroxisome division in oleate-grown cells in addition to the Vps1 protein (Kuravi et al., 2006; Motley et al., 2008; Nagotu et al., 2008a; Nagotu et al., 2008b). As the mitochondrial fission apparatus is common to both mitochondria and peroxisomes, we decided to investigate the number of peroxisomes in oleate-grown strains deleted for *VPS1* and/or *DNM1*, *MDV1*, *CAF4* and *FIS1* in the presence or absence of the *rpn11-m1* allele (Fig. 6).

As previously shown, deletion of *VPS1* led to an important decrease in the average number of peroxisomes per cell: 1.28±0.19 compared with 3.72±0.4 for the isogenic wild-type cells (Fig. 6). This number (1.28±0.19 for *Δvps1*) more than doubled in the double mutant strain *Δvps1/rpn11-m1* (2.82±0.04). The frequency distribution of the peroxisomes in *Δvps1/rpn11-m1* cells was much more dispersed, with cells containing up to 11 peroxisomes whereas the *Δvps1* cells contained no more than four peroxisomes (Fig. 6). Deletion of *DNM1* led to an average

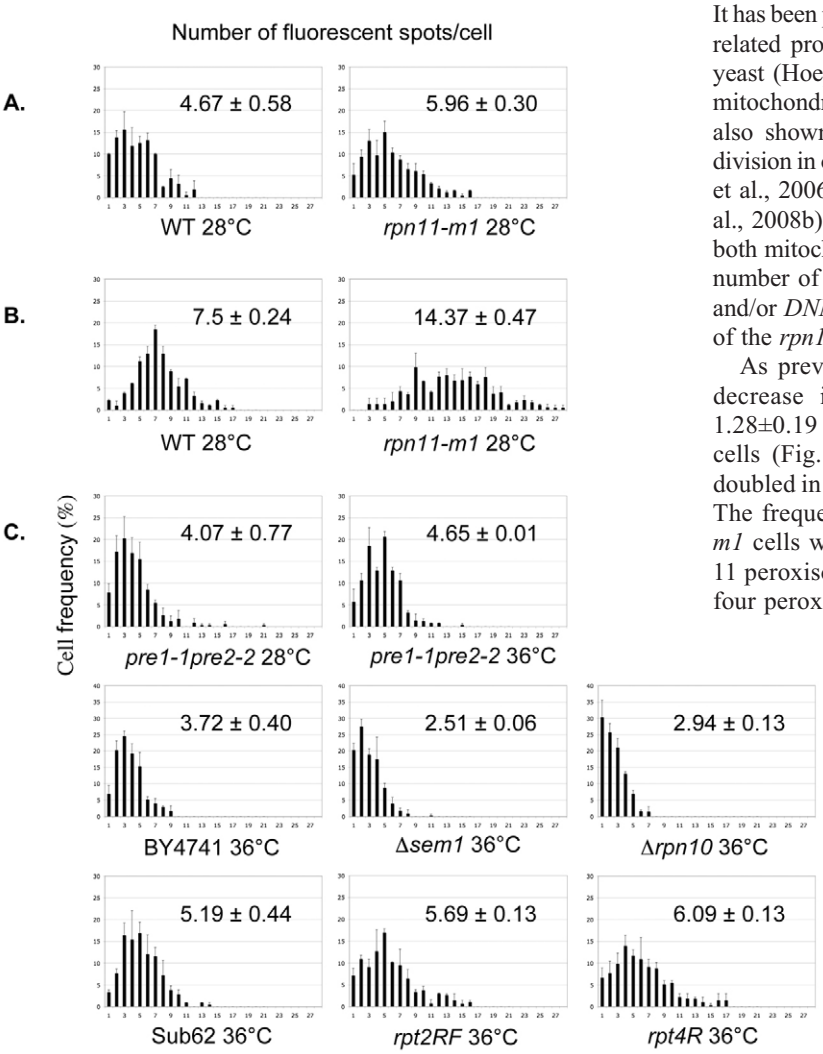

Fig. 3. Rpn11 regulates peroxisome numbers in cells grown in oleate. (A) Distribution of peroxisomes in wild-type and *rpn11-m1* cells grown in glucose. (B) Quantification of peroxisome in wild-type and *rpn11-m1* cells grown in oleate. (C) Quantitative distribution of the peroxisomes in different proteasome mutant cells grown in oleate. For each sample, the number of fluorescent spots was counted from images of two counts of 100 non-budding cells from two independent experiments. The frequency distributions of cells with number of peroxisomes per cell are shown. The average numbers of fluorescent spots per cell observed are presented as means ± s.d. Peroxisomes were labeled with DsRFP-SKL.

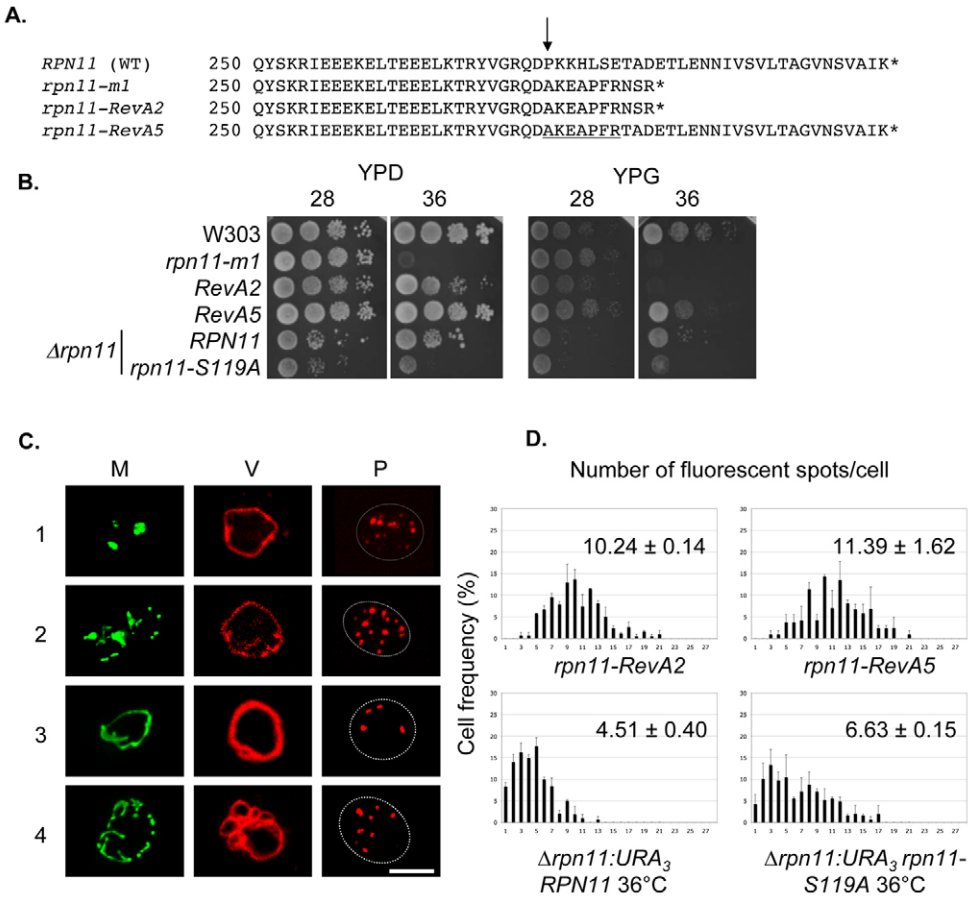

Fig. 4. Integrity of the C-terminal domain but not the deubiquitinase activity of Rpn11 is involved in peroxisome proliferation. (A) Amino acid sequence of the Rpn11 protein and the mutated proteins Rpn11-m1, Rpn11-RevA2 (extragenic revertant) and Rpn11-RevA5 (intragenic revertant). The localization of the *rpn11-m1* mutation (P256A + fs) is indicated with an arrow. The underlined sequence in Rpn11-RevA5 is different from the WT sequence. (B) Phenotype of the wild-type and mutant strains on fermentable (YPD) and respiratory (YPG) medium at 28°C and 36°C. (C) Morphologies of mitochondria (M), vacuoles (V) and peroxisomes (P) in *rpn11-revA2* (1) and *rpn11-revA5* (2) strains at 28°C and *Δrpn11:URA3* /yepLac111 *RPN11* (3) and *Δrpn11:URA*$_3$ /yepLac111 *rpn11(S119A)* (4) strains at 36°C. (D) Frequency distributions of the peroxisomes in mutant cells grown in oleate. The average numbers of fluorescent spots per cell observed in oleate-grown cells are presented as means ± s.d. Peroxisomes were labeled with DsRFP-SKL. Scale bar: 5 μm.

number of 3.43±0.47 peroxisomes per cell, whereas the presence of the *rpn11-m1* allele in this strain showed an average number of 6.67±0.62 peroxisomes per cell. Because Vps1 and Dnm1 are redundant for peroxisome fission, we analyzed the effect of *rpn11-m1* in a *Δvps1 Δdnm1* double deletion strain. In this context, the presence (1.13±0.03) or the absence (1.1 ±0.04) of the *rpn11-m1* allele had no effect on peroxisome number. These results show that the presence of either dynamin-related protein Vps1 or Dnm1 in *rpn11-m1* is necessary and sufficient for the increase in peroxisome number.

Deletion of *FIS1* led to 3.59±0.19 peroxisomes per cells compared with 4.09±0.27 peroxisomes in *Δfis1*/*rpn11-m1* cells (Fig. 6). Thus, the Fis1 protein is required for the *rpn11-m1* increase in peroxisome number. However, deletion of *MDV1* did not decrease the peroxisome number of *rpn11-1* cells. Since Caf4 has been shown to be redundant with Mdv1 for peroxisome fission in glucose (Motley et al., 2008), we also examined peroxisome abundance in a *Δcaf4*/*rpn11-m1* background. Interestingly, those oleate-grown cells presented severe growth defect and abnormal morphologies (giant cells, elongated and multibuds). It was impossible to reasonably compare the peroxisome number in such cells, because we always counted the peroxisome number in healthy nonbudding cells. Altogether, these data indicate that Rpn11 has a role in the Fis1-dependent fission process of peroxisomes.

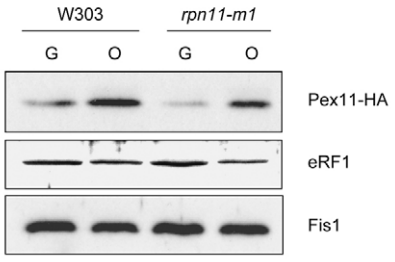

Fig. 5. Rpn11-m1 does not alter Pex11 expression. Wild-type and *rpn11-m1* cells expressing 3×HA-tagged Pex11 protein from its chromosomal locus were grown overnight in glucose (G) or oleate (O). Total cell extracts were immunoblotted for endogenous Pex11-HA and Fis1 proteins. eRF1 is a cytosolic protein used as loading control.

Redirecting Fis1 entirely to peroxisomes in *rpn11-m1* increases peroxisome number in cells grown in glucose

In glucose, most of the Fis1 and Dnm1 proteins were found to predominantly localize to mitochondria and few Fis1 localized to peroxisomes (Kuravi et al., 2006). In *rpn11-m1* cells, mitochondria are highly fragmented when cells are grown in glucose and the increase in peroxisome number in *rpn11-m1* cells was only observed when cells were grown in oleate. To determine whether the increase in peroxisome number in *rpn11-m1* cells was restricted to the oleate inducing conditions, we redirected all Fis1 protein exclusively to peroxisomes and analyzed the peroxisome number and the mitochondrial morphology of cells grown in glucose. Exchanging the C-terminal membrane-anchor sequence of Fis1 with that of the peroxisomal membrane protein Pex15 has been shown to result in

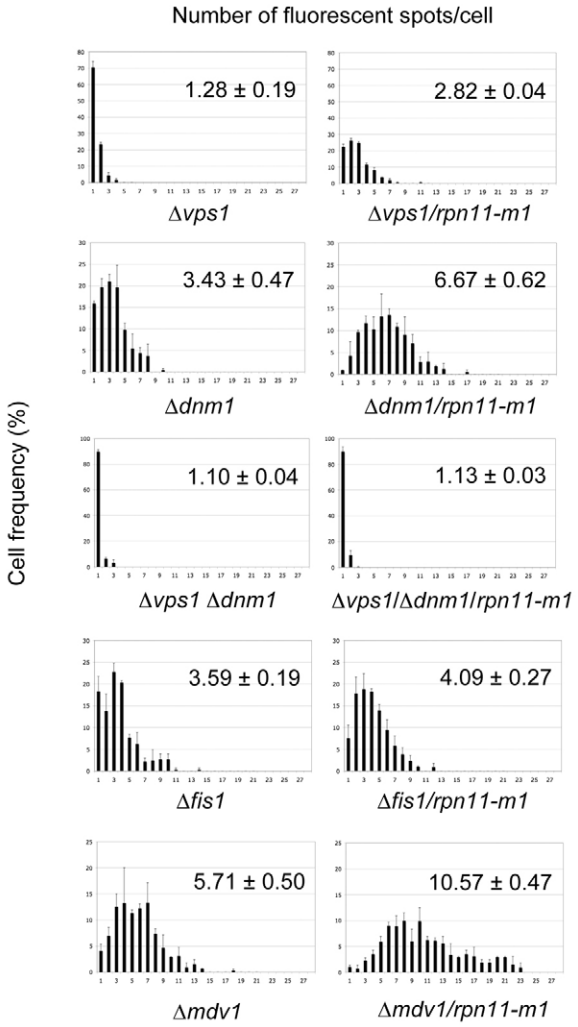

Fig. 6. Rpn11 regulates the Fis1-dependent division process of peroxisomes. (A) Quantitative distribution of peroxisomes in *Δvps1*, *Δdnm1*, *Δvps1/Δdnm1*, *Δfis1* and *Δmdv1* cells with or without the *rpn11-m1* mutated allele grown in oleate. For each strain, fluorescent spots were counted in two counts of 100 non-budding cells from two independent experiments. The frequency distributions of cells with numbers of peroxisomes are shown. The average numbers of fluorescent spots per cell observed are presented as means ± s.d.

an exclusive localization of the fusion protein to peroxisomes (Halbach et al., 2006; Motley et al., 2008). We expressed this chimeric protein in *Δfis1* cells in the presence or absence of the *rpn11-m1* allele and analyzed the peroxisome number and mitochondria morphology in glucose-grown cells (Fig. 7). Expression of the Fis1-Pex15 fusion protein in the *Δfis1* strain did not restore the mitochondrial fission defect of this strain but restored the peroxisome abundance to wild-type levels, as previously shown (Motley et al., 2008). Interestingly, expression of the fusion protein in the double mutant strain *Δfis1/rpn11-m1* led to a huge increase in peroxisome number in glucose. Mitochondria of this strain resemble those of the *Δfis1/rpn11-m1* strain, with elongated and collapsed mitochondria, not the interconnected tubules of the *Δfis1* parent, as previously shown (Rinaldi et al., 2008). These results show that redirecting Fis1 to peroxisomes in the presence of the *rpn11-m1* allele is sufficient to increase the peroxisome fission process when cells are grown in glucose.

Dnm1 and Fis1 are very stable proteins

The increased fission of mitochondria and peroxisomes, which was dependent on the Fis1 protein, prompted us to examine the involvement of Rpn11 on potential post-translational regulation of the fission proteins common to both organelles. The turnover of two fission proteins Dnm1 and Fis1 was examined in *rpn11-m1* cells compared with the wild-type strain. We carried out cycloheximide-chase experiments in both strains at the permissive temperature, in which the Dnm1 protein was expressed as a fusion with GFP from its chromosomal locus. As a control, we also examined the turnover of the fusion protein Fzo1 known to be a substrate of the proteasome. As shown in Fig. 8, the turnover of the Fzo1 protein was comparable in wild-type and mutant cells, indicating that the proteasome is functional at the permissive temperature. In the same conditions, the Dnm1 and Fis1 proteins were very stable, and their turnover was comparable in the two genetic backgrounds. These data indicate that the increased fission observed in *rpn11-m1* is not a consequence of stabilization of the fission proteins Dnm1 and Fis1.

Rpn11 copurifies with mitochondria and peroxisomes

We previously showed that a fraction of Rpn11 and most of the Rpn11-m1 proteins are found in the enriched mitochondrial fraction after cellular fractionation (Rinaldi et al., 2008). To explore a potential dual association of Rpn11 and Rpn11-m1 at both peroxisomes and mitochondria, enriched mitochondrial pellets of strains expressing Rpn11 or its mutated form, tagged with 3×HA at the chromosomal locus, were subjected to further cell fractionation by ultracentrifugation through a sucrose step gradient. Aliquots of each gradient fraction were analyzed for protein content. The data presented in Fig. 9, show that fractions 8, 9 and 10 were further enriched in mitochondria and peroxisomes. Mitochondria appeared more abundant in fractions 8 and 9 whereas fraction 10 contained more peroxisomes. Both Rpn11 and Rpn11-m1 copurified in fractions corresponding to mitochondria and peroxisomes. These data support evidence for the association of Rpn11 and its mutated form to both peroxisomes and mitochondria.

Discussion

Our previous work showed that the C-terminal domain of Rpn11 is involved in the regulation of the mitochondrial morphology independently of the known deubiquitylating activity of this protein (Rinaldi et al., 2008). However, the process (fission and tubulation) regulating the mitochondrial morphology controlled by Rpn11 was not clear. Here, we establish that the Fis1-dependent fission machinery common to mitochondria and peroxisomes is regulated by the proteasomal lid subunit Rpn11. We show that effective regulation of peroxisome fission in oleate inducing conditions does not require the proteolytic activity of the proteasome but rather a novel function of Rpn11 that might be independent of its deubiquitylating activity.

Two recent studies confirmed the connections between proteasome and mitochondria. Very recently, in *S. cerevisiae*, proof for the involvement of the proteasome activity on the mitochondrial morphology was established by showing the ubiquitylation and the proteasomal degradation of the fusion protein Fzo1 (Cohen et al., 2008). A different connection has also emerged with our study on the *rpn11-m1* mutant in the lid subunit Rpn11. We previously showed that the absence of the C-terminal domain of Rpn11 did not affect the mitochondrial fusion process. Here, we pointed out that the division process of peroxisomes, driven by the Fis1 protein,

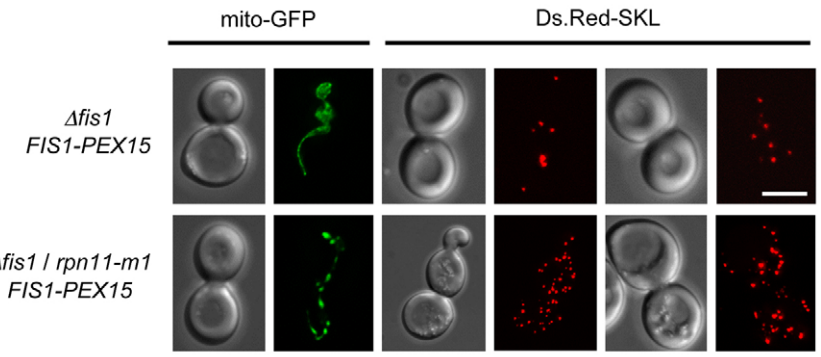

Fig. 7. Redirection of all Fis1 to peroxisomes in *rpn11-m1* increases peroxisome number in glucose. *Δfis1* and *Δfis1/rpn11-m1* cells were transformed with a plasmid expressing the Fis1-Pex15 fusion protein and expressing either GFP targeted to mitochondria or DsRed to label peroxisomes. Cells were grown in glucose medium. Images are flattened *z*-stacks. Scale bar: 5 μm.

is specially affected in *rpn11-m1*. Since Fis1 was shown to have a pivotal role in recruiting other fission components such as Dnm1, Mdv1 and Caf4 at mitochondria and peroxisomes, our results indicate that the huge fragmentation of the *rpn11-m1* mitochondria is also a result of an increased Fis1-dependent fission of mitochondria. This effect is specific for the Rpn11 function, because five other thermosensitive mutants of the proteasome, in the catalytic core, the base or the lid, do not show abnormal peroxisome number in oleate-inducing condition. These data demonstrate that proteasome degradation is unlikely to be involved in the peroxisomal division process induced by oleate.

These results have been further supported by the use of extragenic and intragenic suppressors of *rpn11-m1*. These isogenic revertants have a correct cell cycle and restore the lidless conformation of the proteasome for *RevA5* (data not shown); they still contain abnormal peroxisome abundance and highly fragmented mitochondria. Interestingly, a mutant in the Rpn11 deubiquitinase active site that fails to deubiquitylate proteins (Rinaldi et al., 2004), has a normal peroxisome number under oleate induction, indicating that the catalytic activity of Rpn11 might not be involved in the peroxisomal division process; a property that has already been shown for mitochondrial fragmentation (Rinaldi et al., 2004).

A number of reports have provided indirect links between the UPS and mitochondrial dynamics (Altmann and Westermann, 2005; Durr et al., 2006; Fisk and Yaffe, 1999; Hitchcock et al., 2003; Peng et al., 2003; Rinaldi et al., 2004; Sutovsky et al., 1999; Thompson et al., 2003; Thorsness et al., 1993). Today, the only evidence for involvement of the UPS in directly regulating the mitochondrial fission process is in mammals, where a specific ubiquitin ligase, MARCHV/MITOL, is implicated in ubiquitylating two components of the mitochondrial fission apparatus, DLP1 and FIS1. However, there is a lack of consensus as to whether this ubiquitylation serves to target these factors for proteasomal degradation or facilitates other nonproteolytic functions and/or activity and/or trafficking of these proteins (Karbowski et al., 2007; Nakamura et al., 2006; Yonashiro et al., 2006). Parkin was the other mammalian ubiquitin ligase implicated in the mitochondrial fission process; however, it was recently shown that parkin is selectively recruited to dysfunctional mitochondria to promote and mediate their engulfment and subsequent degradation by autophagosomes (Narendra et al., 2008). The implication of a proteasomal subunit in the mitochondrial fission process in yeast, independently of its role in the degradation function of the proteasome, suggests a nonproteolytic regulation of the fission proteins.

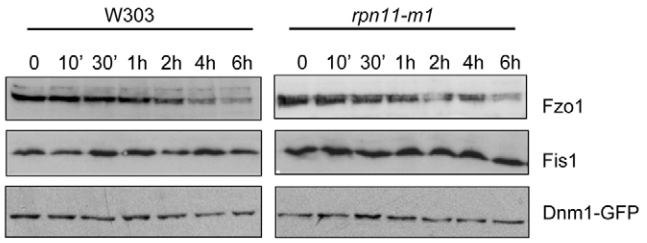

Fig. 8. Dnm1 and Fis1 proteins are stable proteins even in the *rpn11-m1*. Degradation of Dnm1, Fis1 and Fzo1 was assessed in wild-type and *rpn11-m1* strains by treating cells with cycloheximide. Yeast extracts were prepared at the indicated time points and remaining proteins evaluated by immunoblotting.

Rpn11 as a bifunctional protein

The proteasome (26S) was considered for a long time to be a static garbage disposal unit for cellular waste. Because of its modular and dynamic composition, it is now recognized as a multifaceted mediator of many essential cellular processes involving proteolytic and nonproteolytic mechanisms (Demartino and Gillette, 2007). We have already shown that a small fraction of Rpn11 and most of Rpn11-m1 were associated with mitochondria-enriched pellets after cellular fractionation (Rinaldi et al., 2008). A deeper purification of the mitochondrial fraction with sucrose step gradients confirms the mitochondrial localization, but also reveals a peroxisomal localization of these proteins. This suggests that a subpopulation of Rpn11 might interact dynamically with both

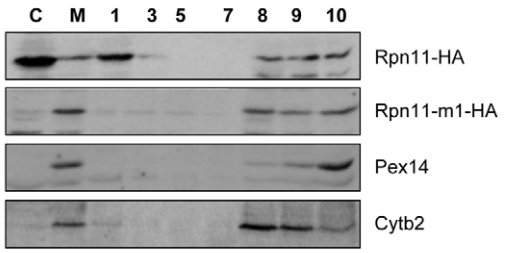

Fig. 9. Subcellular localization of Rpn11 and Rpn11-m1. Organelle pellets (mitochondrial-enriched pellets) from yeast expressing the Rpn11 and Rpn11-m1 proteins tagged with 3×HA at their chromosomal locus were further purified and separated by sucrose equilibrium density gradient centrifugation. Fraction C represents the cytosolic extract before the mitochondrial enriched pellet (M) was collected. Fractions 1 to 10 represent the top to the bottom of the gradient, respectively. Aliquots of each gradient fraction were separated by SDS-PAGE and probed with polyclonal antibodies against cytochrome b2 (mitochondrial intermembrane space) and Pex14 (peroxisomal membrane peroxin). Rpn11-HA and Rpn11-m1-HA were detected with a monoclonal anti-HA antibody.

organelles and that Rpn11-m1 is associated more stably than the full-length protein. These localization data support an important function of Rpn11 at mitochondria and peroxisomes in the fission process of both organelles. We propose that the dissociation of Rpn11 from the proteasome or the independent role of Rpn11 on the Fis1-dependent division machinery allows Rpn11 to act as a 'moonlighting protein' (Gancedo and Flores, 2008). However, we do not know whether Rpn11 acts on the membrane fission process alone or in combination with other lid subunits or in a completely new complex. Experiments are in progress to address this question.

Regulation of the mitochondrial and peroxisomal fission machinery

Peroxisomes have been shown to multiply in response to mitochondrial dysfunction (Butow and Avadhani, 2004; Motley et al., 2008). In glucose, an increased number of peroxisomes are observed in cells impaired in respiration for different reasons (metabolic *atp7* mutant or loss of mtDNA *mgm1* mutant) (Motley et al., 2008). Interestingly, the *rpn11-m1* cells for which we previously showed a defect in the rate of respiration (Rinaldi et al., 2008), do not present an abnormal number of peroxisomes in glucose compared with numbers in wild-type cells. Thus, the huge increase of peroxisome number in *rpn11-m1* in oleate is not a consequence of the mitochondrial dysfunction of these cells and points to a specific regulatory role of Rpn11 on the fission machinery.

Here, we show that *rpn11-m1* mitochondria are highly fragmented in glucose, whereas the peroxisome number was normal in these conditions. The increase in peroxisome number was only observed in oleate. However, by artificially redirecting Fis1 to peroxisomes, we showed that the effect of Rpn11-m1 on peroxisome number was then clearly observed in glucose. Because Fis1 is present predominantly at the mitochondrial surface and very few to peroxisomes when cells are grown in glucose (Kuravi et al., 2006), these results suggest that the Fis1 localization, which has a pivotal role in distribution of Dnm1 for fission, might be differentially controlled in cells grown in glucose and oleate. Furthermore, as the steady state levels of Fis1 in oleate and glucose (Fig. 5) and turnover of Fis1 and Dnm1 (Fig. 8) do not change in *rpn11-m1* cells, these data imply that the Fis1 and/or Dnm1 activity is exacerbated.

The fact that Fis1, and both Vps1 and Dnm1, are required for the *rpn11-m1* increase in peroxisome number is surprising, because Fis1 is thought to act with Dnm1 and not with Vps1. Unfortunately, the absence of the two other fission factors Mdv1 and Caf4 could not be examined because deletion of *CAF4* in the *rpn11-m1* context is deleterious to cells in the peroxisome-inducing condition (oleate). This result indicates that Mdv1 and Caf4 might not be functionally redundant for peroxisome fission in oleate, unlike in glucose (Motley et al., 2008). Thus, the currently accepted fission pathway in cells grown in glucose could somehow differ in peroxisome-inducing conditions. More experiments are needed to address this very interesting question.

Recently, a new model for the assembly of the fission machinery has been proposed in yeast (Wells et al., 2007) that could be conserved in humans (Serasinghe and Yoon, 2008). In this model, Fis1 binds directly to Dnm1 and to Mdv1 instead of Fis1 binding to Mdv1, which subsequently recruits Dnm1 to sites of scissions. Dnm1 binds directly to the concave surface of the Fis1 TPR-like domain and does so independently of Mdv1. Access to this Dnm1-binding site is masked by the N-terminal region (Fis1 arm) of Fis1 itself (Picton et al., 2009; Wells et al., 2007), thus negatively regulating access to the GTPase. In this scenario, the role of Mdv1 and Caf4 is unclear. Upon an unknown stimulus (protein binding, post-translational modification), the Fis1 arm autoinhibition might be relieved to allow Dnm1 binding and fission. It is possible that Rpn11 has a role in this regulatory process in concert with Mdv1, by controlling the relief of the Fis1 inhibitory arm. The mutated protein Rpn11-m1 would hold the Fis1 protein in an active state for Dnm1 binding. However, we cannot exclude the idea that Rpn11 participates in a yet unknown process that would activate directly Mdv1, which could further enhance Dnm1-binding to Fis1. Furthermore, a recent study in humans showed that FIS1 is able to form oligomers, a process that can also be negatively regulated by the FIS1 arm. This oligomerization is proposed to provide a site for DLP recruitment, serving as a template for DLP helical ring assembly and also possibly mediating the initial constriction of the membrane (Serasinghe and Yoon, 2008). It is possible that Rpn11-m1, by allowing oligomerization of Fis1, can recruit Dnm1 but also provides a suitable environment for Vps1 action at these constriction sites.

Finally, our findings indicate that peroxisomes and mitochondria share not only some basic characteristics but also a common regulation of their dynamics. These findings add further evidence that they are much closer than previously assumed, underlying the tight cooperation and crosstalk between both organelles. Interestingly, a lethal defect in peroxisomal and mitochondrial fission in mammals, which appears to be based on a point mutation in the dynamin-related DLP1 gene, has recently been described (Waterham et al., 2007).

Materials and Methods

Strains and growth conditions

The *S. cerevisiae* strains used in this study are listed in supplementary material Table S1. The double mutants used in this study were generated by mating of haploid strains, sporulation, and tetrad dissection except for the *Δvps1Δdnm1* strain (see below). YPD (1% bactopeptone, 1% yeast extract, and 2% glucose), YPG (1% bactopeptone, 1% yeast extract and 2% glycerol) were used as rich media. W0 (0.67% yeast nitrogen base without amino acids and 2% glucose) was used as minimal medium. Oleate induction medium (0.67% yeast nitrogen base without amino acids, 0.1% glucose, 0.1% oleate, 0.05% Tween 40, and 0.1% yeast extract, pH 6.0) was used for peroxisome proliferation experiments. Whenever necessary, media were supplemented with the appropriate nutritional requirements according to the strains. All media were supplemented with 2% bacto agar (Difco) for solid media. Yeast cultures were grown at 26°C, if not indicated otherwise. For oleate induction, cells are grown to log-phase in glucose and then shifted to oleate for 14 hours. When required, cells were further incubated for 6 hours in oleate at the nonpermissive temperature.

Construction of *Δdnm1Δvps1* strain

A URA3 cassette flanked by VPS1 sequences was generated by PCR using the plasmid pFL38 as template, and oligonucleotides VPS1up: 5′-ATGGATGAGCATTTAATTTCTACTATTAACAAGCTTCAGGgtcgggaaacctgtcgtgcc-3′ and VPS1dw: 5′-CTAAACAGAGGAGACGATTTGACTAGCGTTTCTCAATATcgcgttggccgattcattaat-3′ (upper case letters indicate nucleotides homologous to VPS1: 37 bp upstream of the ATG codon for VPS1up and 38 bp downstream of the VPS1 ORF for VPS1dw; lower case letters indicate nucleotides homologous to the plasmid pFL38). The resulting integration cassette was transformed into *Δdnm1* and *Δdnm1/rpn11-m1* strains, and correct integration was verified by PCR using external oligonucleotides.

Construction of PEX11-HA strains

The triple HA-KanMX cassette was generated by PCR using the plasmid pFA6a-3HA-KanMX6 (Longtine et al., 1998) as template, and oligonucleotides PEX11d: 5′-CACATCTATCCTTGGTATGCAAGACATGTGGAAAGCTACAcggatccccgggttaattaa-3′ and L29: 5′-AATTATAAAGAAGGGTCGAATCAAACATAAGCGGAGAATAGaattcgagctcgtttaaac-3′ (upper case letters indicate nucleotides homologous to the C-terminus of PEX11, 41 bp upstream of the stop codon for PEX11d and 40 bp downstream of the PEX11 ORF for L29; lower case letters indicate nucleotides homologous to the plasmid pFA6a-3HA-KanMX6). The resulting integration cassette was transformed into WT and rpn11-m1 strains, and correct integration was verified by PCR using external oligonucleotides.

Fluorescence microscopy and image treatment

For visualization of mitochondria, cells were transformed with pYX142-mtGFP or pYX232-mtGFP plasmid, which expresses GFP fused to a mitochondrial import sequence. For visualization of ER, cells were transformed with the pERG26-GFP-2 plasmid expressing GFP in fusion with the Erg26 protein of the ER. Cells were grown to mid-log phase in complete medium (YPD), washed in 1× PBS, transferred to slides and analyzed by fluorescence microscopy. For observations at high temperature, cells were shifted to 36°C for 6 hours.

For visualization of vacuoles, yeast cells were grown to mid-log phase in rich medium (YPD) at 26°C or 36°C for 4 hours. Cell pellets from 1 ml cultures were resuspended in 50 μl of rich media containing FM4-64 (Molecular Probes, final concentration 10 μM) and incubated at the temperature assay for 1 hour more, in the dark, with gentle shaking. Cells were chased in rich medium for 1.5 hours and resuspended in 1× PBS, transferred to slides and analyzed by fluorescence microscopy.

Actin network was visualized by adding 100 μl of a stationary phase culture (26°C) to 3 ml fresh rich medium (YPD) for 6 hours at the temperature assay. Cells were then fixed in 3.7% formaldehyde directly added to the medium for 30 minutes at room temperature. Cells were then washed twice with 1× PBS and resuspended in 1× PBS. 100 μl of cells were stained with 0.033 μM green-phalloidin (Molecular Probes) for 1 hour at room temperature in the dark and washed in 1× PBS, transferred to slides and analyzed by fluorescence microscopy.

To visualize peroxisomes, yeast strains were transformed with the plasmid pUG34DsRed.SKL expressing RFP fused to a PTS1 peroxisomal import sequence (SKL). Yeast cells were cultured overnight in minimal medium, then transferred in YPD or oleate and incubated overnight at 26°C. For thermosensitive mutants, cells were inoculated overnight in fresh oleate induction medium and shifted to assay temperature for 6 hours.

For quantitative determination of the number of fluorescent spots per cell, cells were fixed in 3.7% formaldehyde directly added to the medium for 20 minutes at 26°C or 36°C. Fluorescent spots were counted in single cells. In each quantification experiment, 200 cells were counted (100 cells from two independent cultures).

The slides were examined with a DMIRE2 microscope (Leica, Deerfield, IL). Filters for GFP (450/490 nm excitation and 500/550 nm emission) and TxRED (542/582 nm excitation and 604/644 nm emission) were used. Images were captured using a CCD camera (Roper Scientific, Tucson, AZ). Metamorph software (Universal Imaging, West Chester, PA) was used to deconvolute *Z*-series and treat the images. The cell wall was reconstructed by manual tracing the contours in the bright-field images.

Cycloheximide chase assay

Yeasts were grown in rich medium (YPD) at 26°C until reaching an OD_{600} of 1. Cycloheximide was added to a final concentration of 100 μg/ml. At the indicated times, 10 ml of the cultures were collected, harvested and TCA protein extraction was performed. Proteins in the samples were resolved on 10% or 16% SDS-PAGE gels, transferred to nitrocellulose membranes and analyzed by immunoblotting using the polyclonal anti-Fis1 (a gift from Doron Rapaport), anti-Fzo1 (a gift from Janet M. Shaw), anti-GFP (Roche) and anti-HA (BABCO) antibodies.

Cellular fractionation

For cell fractionation, yeast cells were grown overnight to mid-log phase (2×10^7 cells/ml) in rich galactose (2%) medium. Enriched mitochondria fraction was generated essentially as described previously (Rinaldi et al., 2008). Briefly, spheroplasts were generated by a 60-minute incubation at 30°C in 20 mM Tris-HCl, pH 7.5, 1.35 M sorbitol, 1 mM EDTA containing Zymolyase 20T at 1.5 mg/g wet weight yeast. Spheroplasts were harvested by centrifugation (10 minutes at 2000 ***g***), washed twice in a solution of 20 mM Tris-HCl, pH 7.5, 1.35 M sorbitol, 1 mM EDTA and carefully resuspended in 1 ml chilled lysis buffer (0.7 M sorbitol, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA) plus protease inhibitors (protease inhibitor cocktail; Roche Mannheim). Cell lysis was performed using 15 strokes of a 5 ml Dounce glass homogenizer, and unlysed cells, nuclei and cell debris were removed by centrifugation at 3000 ***g*** and 4°C for 10 minutes. The supernatant containing the crude yeast cell organelles was centrifuged at 15,000 ***g*** at 4°C for 15 minutes, and the crude organelle fraction was resuspended in a total volume of 500 ml of 0.6 M mannitol, 20 mM HEPES-KOH pH 7.4, 1 mM EDTA plus protease inhibitors. Purity of each organelle fraction was routinely determined by western blot analysis. For the separation of cell organelles, in particular to separate peroxisomes from mitochondria, the crude organelle fraction was layered on top of a sucrose step gradient consisting of 15, 23, 32, 50 and 60% sucrose in 20 mM HEPES-KOH pH 7.4, 1 mM EDTA. The sucrose gradient was centrifuged at 134,000 ***g*** at 4°C for 60 minutes in an SW41 rotor (Beckman) and the gradient was subsequently fractionated from the top. Each fraction (1 ml) was TCA precipitated (13% final) and proteins dissolved in protein loading buffer.

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