Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain

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Substrates destined for degradation by the 26 S proteasome are labelled with polyubiquitin chains. Rpn11/Mpr1, situated in the lid subcomplex, partakes in the processing of these chains or in their removal from substrates bound to the proteasome. Rpn11 also plays a role in maintaining mitochondrial integrity, tubular structure and proper function. The recent finding that Rpn11 participates in proteasome-associated deubiquitination focuses interest on the MPN+ (Mpr1, Pad1, N-terminal)/JAMM (JAB1/MPN/Mov34) metalloprotease site in its N-terminal domain. However, Rpn11 damaged at its C-terminus (the *mpr1-1* mutant) causes pleiotropic effects, including proteasome instability and mitochondrial morphology defects, resulting in both proteolysis and respiratory malfunctions. We find that overexpression of WT (wild-type) *RPN8*, encoding a paralogous subunit that does not contain the catalytic MPN+ motif, corrects proteasome conformations and rescues cell cycle phenotypes, but is unable to correct defects in the mitochondrial tubular system or respiratory malfunctions

associated with the *mpr1-1* mutation. Transforming *mpr1-1* with various *RPN8–RPN11* chimaeras or with other *rpn11* mutants reveals that a WT C-terminal region of Rpn11 is necessary, and more surprisingly sufficient, to rescue the *mpr1-1* mitochondrial phenotype. Interestingly, single-site mutants in the catalytic MPN+ motif at the N-terminus of Rpn11 lead to reduced proteasome-dependent deubiquitination connected with proteolysis defects. Nevertheless, these *rpn11* mutants suppress the mitochondrial phenotypes associated with *mpr1-1* by intragene complementation. Together, these results point to a unique role for the C-terminal region of Rpn11 in mitochondrial maintenance that may be independent of its role in proteasome-associated deubiquitination.

Key words: deubiquitination, mitochondria, MPN (Mpr1, Pad1, N-terminal) domain, proteasome, Rpn11, ubiquitin.

INTRODUCTION

Most cellular proteins that need to be removed in a timely or regulatory manner are covalently labelled by polyubiquitin chains, and targeted to the proteasome for degradation [1]. Ubiquitination is reversible, due to multiple deubiquitinating enzymes that process these chains or remove them from the tagged substrate [2,3]. The proteasome consists of a 20 S core particle (CP) to which one or two 19 S regulatory particles (RPs) attach, one at either end. Proteolysis takes place within the 20 S CP, while the 19 S RP binds polyubiquitinated substrates, unfolds and translocates them into the 20 S CP for proteolysis [4]. The 19 S RP also removes ubiquitin from the target substrate, recycling ubiquitin even as the substrate is hydrolysed by the 20 S CP [5]. The 19 S RP can be separated further into two subcomplexes: the lid and the base. The base attaches to the outer surface of the 20 S CP, and probably unravels the substrate, simultaneously with gating the channel into the proteolytic chamber [6,7]. The base may also be pivotal in anchoring polyubiquitin substrates, either directly or indirectly, during this process [8,9]. Attachment of the lid to the base is required for proteolysis of ubiquitin–protein conjugates, but not of unstructured polypeptides [10,11]. Interestingly, both the lid and the base have been found to contribute independently to 19 S RP deubiquitination activity. The subunits responsible for this deubiquitinase activity have been identified as Rpn11 in the lid and Ubp6 in the base [11–14].

Both the lid and the evolutionarily related CSN (COP9 signalosome) are composed of eight subunits, six containing a PCI (proteasome/COP9/eIF3) domain, while the other two (Rpn11 and Rpn8 in the case of the lid) contain an MPN (Mpr1/Pad1/N-terminal) domain [15]. Rpn11, the most highly conserved subunit of the lid, is situated within a crosspatch cluster encompassing Rpn5, 11, 9 and 8 [16]. A similar structure is present among the synonymous subunits of the CSN. The central position of Rpn11 within the lid structure and its proposed enzymic role as a deubiquitinase are key features for understanding the diverse phenotypes associated with Rpn11. For instance, Rpn11 plays an important role in the transcriptional response to UV irradiation [17]. Moreover, Rpn11 is also involved in mitochondrial tubular organization and functioning, cell cycle progression and response to cellular damage [18,19]. Underscoring its apparent enzymic role, *RPN11* and its orthologues exhibit dominant phenotypes upon overexpression. For example, high dosage of Rpn11 orthologues in human or *Schizosaccharomyces pombe* cells confers multidrug and UV resistance [20–23]. Overexpression of Rpn11 in *Schistosoma* results in stabilization of c-Jun [24]. In *Saccharomyces cerevisiae*, overexpression of *RPN11* can suppress a mutation in the importin- α , *srp1* [25].

Rpn11 belongs to a subset of MPN-domain proteins that harbour an MPN+ or JAMM (JAB1/MPN/Mov34) motif. The MPN+ motif is defined by a consensus sequence E-HxHx₇Sx₂D (where 'x' denotes 'any amino acid') with similarity to the active

Abbreviations used: CHX, cycloheximide; CSN, COP9 signalosome; CP, core particle; DAPI, 4,6-diamidino-2-phenylindole; DASPMI, 2-(4-dimethylaminostyryl)-N-methylpyridinium iodide; GFP, green fluorescent protein; JAMM, JAB1/MPN/Mov34; MMS, methyl methane sulphonate; MPN, Mpr1/Pad1/N-terminal; RP, regulatory particle; WT, wild-type; Ub, ubiquitin.

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Table 1 Yeast strains used in the present study

Strains	Genotype	Source
WT-CMY	Mat a, his3 Δ -200, ade2-101, leu2 Δ 1, ura3-52, lys2-801, trp1 Δ 62, bar1::HIS3	CMY826 (from Carl Mann)
CMY- <i>mpr1-1</i>	Mat a, his3 Δ -200, ade2-101, leu2 Δ 1, ura3-52, lys2-801, trp1 Δ 62, bar1::HIS3, <i>mpr1-1</i>	The present study
CMY- <i>mpr1-1</i> [rho ^o]	Mat a, his3 Δ -200, ade2-101, leu2 Δ 1, ura3-52, lys2-801, trp1 Δ 62, bar1::HIS3, <i>mpr1-1</i> [rho ^o]	The present study
WT-BY	Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; RPN11::kanMX4 [pYC-RPN11]	MY71 in [28]*
<i>rpn11H</i>	Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; RPN11::kanMX4 [pYC-rpn11H]	[28]
<i>rpn11S</i>	Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; RPN11::kanMX4 [pYC-rpn11S]	[28]
<i>rpn11D</i>	Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; RPN11::kanMX4 [pYC-rpn11D]	[28]
<i>rpn11C</i>	Mat a; his3 Δ 1; leu2 Δ 0; met15 Δ 0; ura3 Δ 0; RPN11::kanMX4 [pYC-rpn11C]	[28]

* Based on Y25683 from EUROSCARF; described in [28].

site of zinc metalloproteases. Recent structure determination of an archaeal member of the MPN+ family, AfJAMM, identified a zinc active site chelated to the histidine and aspartate residues of the MPN+ motif, with a water molecule probably serving as the fourth ligand and active-site nucleophile [26,27]. These properties lie behind the assertion that members of this family could be hydrolytic enzymes for removal of ubiquitin or ubiquitin-like proteins from their targets. Thus Rpn11 partakes in removal of ubiquitin from substrates bound to the proteasome [11–13,28], and the CSN subunit Csn5/Jab1 is responsible for removal of the ubiquitin-like modifier Rub1/Nedd8 from the cullin subunit of cullin-based E3 ubiquitin–ligase complexes [29,30]. Indeed, single-site mutations in MPN+ residues of Rpn11 caused a decrease in proteasome deubiquitination, resulting in marked proteolysis defects [11,13,28]. In contrast, simultaneous substitution of both histidine residues located in this motif (the *rpn11AXA* mutant) was non-viable [12]; likewise, double substitution of both these histidine residues could not rescue RNA-interference treatment of *DmS13/rpn11* in insect cells [31]. Equivalent mutations in Csn5, the MPN+ component of the CSN, abolished the ability of this complex to remove the ubiquitin or ubiquitin-like Rub1 modification from their target proteins [27,30]. This catalytic motif is highly conserved within some MPN domain proteins such as Rpn11 and Csn5, but is not present in others, such as the proteasomal subunit Rpn8, suggesting that the latter are not catalytically active but may play a structural role in their respective complexes.

Although the above results clearly point to a function of Rpn11 in proteolysis, they do not easily explain its participation in maintaining mitochondrial integrity that stems from regions outside the MPN domain. For instance, many studies involving Rpn11 in yeast have been obtained by taking advantage of the *mpr1-1* mutant: a nonsense mutation in the C-terminal region that results in a truncated Rpn11 protein lacking the last 31 amino acids [18,19]. This *mpr1-1* mutant exhibits pleiotropic phenotypes, such as a temperature-sensitive cell cycle arrest at the G₂–M phase with severe abnormalities in the bud, and disruption of the mitochondrial tubular network [18,19]. Interestingly, these mitochondrial defects could be dissociated from defects in the cell cycle by extragenic suppressor mutations [19]. So far, this has been the only case in which a mutation in a proteasomal subunit was shown to cause defects in mitochondrial morphology and function.

In the present study, we have investigated the role of the Rpn11 lid subunit in mitochondrial tubular organization and in protein degradation, and attempt to map regions of the protein essential for either function. We find that overexpression of WT (wild-type) *RPN8* is able to partially rescue the proteolysis phenotypes of *mpr1-1*, but not the mitochondrial defects. We also constructed chimaeric proteins by exchanging the MPN domain of Rpn8 with

that of Rpn11. Only chimaeric constructs containing the natural C-terminal region of Rpn11 could suppress the mitochondrial defects. Rpn11 mutants defective in their MPN+ catalytic motif were able to rescue most *mpr1-1* phenotypes. These results point to a new function of the C-terminus of Rpn11 in addition to the deubiquitinating function.

EXPERIMENTAL

Yeast strains, media and genetic strains, plasmids and media

The yeast strains and plasmids used in the present study are listed in Tables 1 and 2. All strains used in this study were derived from the S288C strain. Y25683 (*MATa/MAT α ; his3 Δ 1/his3 Δ 1; leu2 Δ 0/leu2 Δ 0; met15 Δ 0/MET15; LYS2/lys2 Δ 0; ura3 Δ 0/ura3 Δ 0; RPN11::kanMX4/RPN11) was a derivative of BY4743 obtained from EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis; Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany), and was used to generate haploid strains to study complementation of *rpn11* null. In brief, the diploid strain was transformed with a *URA3*-marked centromeric plasmid expressing WT *RPN11* under its own promoter, pYC-*RPN11*, and sporulated to obtain a haploid strain in which pYC-*RPN11* complements the lethal phenotype of the chromosomal knockout of *RPN11* [28]. The resulting haploid strain is referred to in this study as WT-BY, and was later used for introduction of plasmids expressing single-site mutations or chimaeras by the shuffle-in/shuffle-out method. Strains harbouring the *mpr1-1* truncation mutation in the *RPN11* gene were based on the S288C derivative strain CMY826, referred to herein as WT-CMY, which was obtained from Carl Mann (CEA/Saclay, Gif-sur-Yvette, France).*

Standard methods for cloning procedures by PCR were performed according to the manufacturer's instructions. Plasmids for expression of *RPN8* or *RPN11* from either *ADHI* or *RPN11* promoters were generated by PCR amplification from genomic yeast DNA by using the primers 5'-GCCC AAG CTT CCG AAG AAA TGA TGG-3' and 5'-GTCC CCC GGG CTC GAG TTG ATT GTA TGC TTG-3' to obtain the *ADHI* promoter, or the primers 5'-GC CCA AGC TTC AAG TTT ACG ATG GCG C-3' and 5'-GTC CCC CCG GGC TCG AGT ATG TCT CGT CTT TCT TG-3' to obtain the *RPN11* promoter; each was cloned into the *HindIII/XhoI* sites.

RPN8- and *RPN11*-coding regions were also generated by PCR amplification from genomic yeast DNA by using the primers 5'-GTC CCC CGG GCT CGA GAT GGA ACG ACT ACA GAG AT-3' and 5'-GTA CCG AGC TCT AGA CCA TTC ATT CCA TTA ACT-3' for *RPN11* or 5'-GTC CCC CGG GCT CGA GAT GTC TCT ACA ACA-3' and 5'-GTA CCG AGC TCT AGA GTG GAC GAG AAT AGA GG-3' for *RPN8*, and cloning into the

Table 2 Plasmids used in the present study

Plasmid	Vector	Description	Reference
pYE-RPN11	YEplac181	ADH1 promoter, RPN11 gene	The present study
pYE-RPN8	YEplac181	ADH1 promoter, RPN8 gene	The present study
pYC-RPN8	YCplac111	RPN11 promoter, RPN8 gene	The present study
pYE-CH _{R8-R11}	YEplac181	ADH1 promoter, CH _{R8-R11} gene	The present study
pYE-CH _{R11-R8}	YEplac181	ADH1 promoter, CH _{R11-R8} gene	The present study
pYC-CH _{R8-R11}	YCplac111	RPN11 promoter, CH _{R8-R11} gene	The present study
pYE-RPN11-GFP	YEplac181	ADH1 promoter, RPN11-GFP gene	The present study
pYE-CH _{R8-R11} -GFP	YEplac181	ADH1 promoter, CH _{R8-R11} -GFP gene	The present study
pYC-RPN11	YCplac111	RPN11 promoter, RPN11 gene	M134 in [28]
pYC-rpn11C	YCplac111	RPN11 promoter, rpn11C116A gene	M135 in [28]
pYC-rpn11H	YCplac111	RPN11 promoter, rpn11H111A gene	M143 in [28]
pYC-rpn11S	YCplac111	RPN11 promoter, rpn11S119A gene	M144 in [28]
pYC-rpn11D	YCplac111	RPN11 promoter, rpn11D122A gene	M145 in [28]
pYE-HsRPN8	pFL61	PGK1 promoter, HsRPN8 gene	The present study
pYE-POH1	pFL61	PGK1 promoter, POH1 gene	The present study
pGEM-ScRPN8	pGEM-T Easy	ScRPN8 gene	The present study
pYC-ScRPN8	YCplac111	ScRPN8 gene	The present study
pYE-ScRPN8	YEplac181	ScRPN8 gene	The present study
pYX232-mtGFP	pYX232	Mitochondrial-import sequence-GFP gene	[50]

XhoI/SacI sites. Tagging of different clones at the C-terminus with GFP (green fluorescent protein) was performed between the *BglII/BamHI* mismatch.

Construction of chimaeric proteins

The synthesis of chimaeric DNA was achieved by inserting a *SalI* restriction site at the end of the MPN-domain-coding region of each gene. The *SalI* site was inserted by single-site silent substitutions in *RPN11* or *RPN8*, and generated using PCR site-directed mutagenesis on plasmids with the *LEU2* marker for selection (either centromeric YpLac111 or 2 micron). The *SalI* site was added at the end of the MPN-domain-coding region by using the overlapping primers of *RPN8* (5'-CCG CTA TTA TTA ATT GTC GAC GTC AAA CAA CAA GGT GTT G-3' and 5'-CAC CTT GTT GTT TGA CGT CGA CAA TTA ATA ATA GCG G-3') or of *RPN11* (primers 5'-GTT GCT GTC GTT GTC GAC CCT ATT CAA TCC G-3' and 5'-GAT TGA ATA GGG TCG ACA AcG ACA GCA ACA G-3'). The area which encodes the N-terminus of each of the proteins was switched with the one from the other by 'cutting and pasting' with the restriction enzymes *XhoI* and *SalI*.

Yeast culture media

YPD (1 % bactopectone, 1 % yeast extract and 2 % glucose) and YPG (1 % bactopectone, 1 % yeast extract and 2 % glycerol) media were used as rich media. WO medium (0.17 % yeast nitrogen base, 0.5 % ammonium sulphate and 2 % glucose) was used as minimal medium. All media were supplemented with 2.3 % Bactoagar (Difco) for solid media; WO medium was supplemented with the appropriate nutritional requirements according to the phenotype of the strains. MMS (methyl ethane sulphonate) was used at the concentration of 0.01 % or 0.03 % in YPD medium. Canavanine was added to minimal medium with other nutritional requirements, but without arginine, at the concentrations of 1, 3, 5, 7 and 10 µg/ml. Cycloheximide (CHX) was used at concentrations of 0.05, 0.1 and 0.5 µg/ml.

UV-sensitivity test

Strains were grown at the concentration of 2×10^7 cells/ml, plated on YPD medium and irradiated at 0, 30, 60, 90 and 120 J/m²

(200 cells/plate; three plates for each point). Plates were incubated at 28 °C in the dark for 3 days and colonies were counted.

Canavanine sensitivity

Strains were streaked on 3 µg/ml canavanine minimal media plates without arginine and grown at 30 °C for 2 days.

Isolation of human suppressors of the *mpr1-1* mutation

The *mpr1-1* strain was transformed with the human library (generously provided by Anna Chelstowska, University of Warsaw, Poland). From 40000 transformants, 32 were able to grow on glucose and glycerol media at 34 °C and 11 were able to grow only on glucose medium at 34 °C. After checking that the growth of the transformants was associated with the presence of a plasmid, the inserts were sequenced. The transformants with a wild-type phenotype contained the *POH1* gene (human homologue of *RPN11*; [32]); transformants able to grow only on glucose medium at 34 °C contained the human proteasomal *RPN8* gene.

The suppressor gene *RPN8* obtained from the human library was identified using the M13 reverse primer. The primers used for *RPN8* amplification were *RPN8*-5' (5'-CCT CCA TTA GGG CCT AAC-3') and *RPN8*-3' (5'-GGA CGA GAA TAG AGG CG-3').

Microscopy

For DAPI (4,6-diamidino-2-phenylindole) staining, cells were harvested in exponential phase, and fixed with 1 % formaldehyde for 30 min. DAPI was added at a concentration of 1 µg/ml and cells were observed by fluorescence microscopy. The vital dye DASPMI [2-(4-dimethylaminostyryl)-N-methylpyridinium iodide] was used at a final concentration of 10^{-6} M; cells were then observed by fluorescence microscopy and photographed directly from the culture.

Protein gel electrophoresis and immunoblotting

SDS/PAGE was usually performed using 12 % (w/v) separating gels, unless stated otherwise. For immunoblotting, proteins were wet-transferred to nitrocellulose membranes, and blotted with appropriate antibodies. For in-gel proteasome visualization, rapidly

Figure 1 Overexpression of the *RPN8* gene suppresses the cell cycle defect of the *mpr1-1* mutant

(A) Temperature-sensitivity of *mpr1-1*. The *mpr1-1* mutant transformed with the [pYE-ScRPN8] plasmid (right panel) and *mpr1-1* (transformed with an empty plasmid for comparison; left panel) were grown in glucose medium at 28 °C to the exponential-growth phase, and then shifted for 5 h to the non-permissive temperature (34 °C) and stained with DAPI. Pictures were taken from [*rho*^o] cells to better appreciate the undivided nucleus of the *mpr1-1* strain that migrates into the daughter cell. (B) Rounded mitochondria in *mpr1-1* at the permissive temperature. Congenic WT-CMY, *mpr1-1* and *mpr1-1* harbouring [pYE-ScRPN8] were transformed with the pYX232-mtGFP plasmid expressing the GFP gene with a mitochondrial import sequence. Cells were cultured on glucose medium at 28 °C to the exponential-growth phase, harvested, stained with DAPI and observed by fluorescence microscopy.

prepared whole-cell extracts were resolved by non-denaturing PAGE, as published previously [32].

RESULTS

Multicopy *RPN8* suppresses the cell cycle, but not mitochondrial defects of *mpr1-1*

The *mpr1-1* mutation results in a truncated Rpn11 protein [18]. As previously reported, the *mpr1-1* mutant is slow growing in the 24–28 °C temperature range, and sensitive to higher temperatures; a shift to 34 °C induces a cell cycle arrest at the G₂–M phase, resulting in lethality. Figure 1(A) shows highly aberrant cell morphology following this temperature shift, in particular elongated buds with an undivided nucleus (Figure 1A, left panel). We screened for possible multicopy suppressor genes present in homologous or heterologous libraries. One interesting suppressor gene we found in both human and *S. cerevisiae* libraries was *RPN8*. Overexpression of *RPN8* suppresses the temperature-induced cell cycle arrest of *mpr1-1*, and allows for growth at the non-permissive temperature (Figure 1A, right panel). In addition to the cell cycle phenotype, *mpr1-1* cells have malformed and malfunctioning mitochondria. Even at the lower permissive temperature, *mpr1-1* harbours multiple spherical mitochondria (Figure 1B, centre panel), which differ from the elongated tubular mitochondria present in WT cells (left panel). These mutants are unable to grow on a non-fermentable carbon source at 34 °C. Overexpression of *RPN8* does not suppress this phenotype (Figure 1B, right panel).

Figure 2 Suppression of *mpr1-1* phenotypes by Rpn8, and Rpn11-Rpn8 chimaeras

(A) Schematic representation of various constructs used to study the *mpr1-1* mutant. The MPN domain depicted in black covers most of the N-terminal region of Rpn11 and Rpn8. The MPN+ motif embedded within the MPN domain of Rpn11 is indicated by a large box, with the key residues highlighted in bold, and by underlining. Rpn11, *mpr1-1* and the CH_{R11-R8} chimaera all contain this MPN+ motif. The C-terminal regions of Rpn11 and Rpn8 diverge significantly, and are shown either in white or by grey stripes respectively. The missense mutation in *mpr1-1* leads to a truncation of residues 277–285, highlighted by the grey-shaded box. Numbers indicate the total amino acids in each construct. (B) Growth phenotypes: *mpr1-1* was transformed with an empty plasmid (1 in the pie chart), pYE-RPN11 (2), pYE-RPN8 (3), pYC-RPN8 (4), pYE-CH_{R8-R11} (5), pYC-CH_{R8-R11} (6), pYE-CH_{R11-R8} (7) or pYE-HsRPN8 (8). Transformed strains were grown at 28 °C, streaked on to YPD or YPG plates and grown at 34 °C for 3 additional days. As mentioned in the text, constructs 3–8 alone do not rescue an *rpn11* null, while construct 2 rescues the null creating a WT strain.

Domain mapping of Rpn11

The suppressive effects of Rpn8 are not easy to explain; both *RPN11* and *RPN8* are essential genes, the products of which are present at approximately stoichiometric levels in the 19 S RP [32,33]. How then does an overdose of Rpn8 correct phenotypes associated with Rpn11? Rpn8 is a paralogue of Rpn11; both contain an MPN domain at their N-terminal region (Figure 2A). Interaction between those two proteins was shown previously (perhaps by physical interaction between these domains [16,34]), although it should be emphasized again that, in contrast with Rpn11, the MPN domain of Rpn8 does not contain the catalytic MPN+ motif; furthermore, the C-terminal parts of the two

Figure 3 Mitochondrial structure in *mpr1-1* and suppressed strains

mpr1-1 was transformed with the constructs described in Figure 2, grown at 28 °C to the mid-exponential phase, stained with DASPMI, and mitochondria were observed by fluorescence microscopy. A WT-like mitochondrial tubular network is visualized in *mpr1-1* only upon transformation with plasmids bearing either the WT *RPN11* (B) or the CH_{R8-R11} chimaera (E).

proteins do not share obvious similarities. In order to verify specific roles for these domains within Rpn11 or Rpn8, we created chimaeras by interchanging their N-terminal MPN domains (Figure 2A): CH_{R11-R8} (N-terminal region of Rpn11 with C-terminus of Rpn8) and CH_{R8-R11} (N-terminal region of Rpn8 with C-terminus of Rpn11). These chimaeras were tested for complementation and had no ability in rescuing the *rpn11* null, either at natural abundance or when overexpressed (results not shown). However, both chimaeric proteins can rescue some of the phenotypes associated with *mpr1-1*, when expressed in the *mpr1-1* mutant strain.

Chimaeras of Rpn11 with Rpn8 in either combination rescue the temperature-sensitivity of *mpr1-1*, allowing for growth at the restrictive temperature (Figure 2B, left panel). Overexpression of *RPN8*, or of its human orthologue *hsRPN8*, does so as well. However, rescue of the respiratory defects associated with *mpr1-1* is much more stringent. Thus Rpn8 supports growth of *mpr1-1* on glucose-containing medium, but not on the non-fermentable carbon source, glycerol, while multicopy CH_{R8-R11} allows for growth on glycerol (Figure 2B, right panel). This result suggests that properly functioning mitochondria require the C-terminal region of Rpn11. In order to corroborate this observation, we analysed the ability of various constructs to repair mitochondrial structural defects observed in *mpr1-1* (Figure 3). Indeed, the C-terminal part of Rpn11 is necessary for the maintenance of a correct mitochondrial tubular network (Figure 3E). To conclude, a WT copy of the C-terminal region of Rpn11 is required for proper mitochondrial function, even if this domain is detached from the catalytic domain in its N-terminal region.

The C-terminal domain of Rpn11 functions independently of the N-terminal metalloprotease MPN+ motif

Having observed that the C-terminal part of Rpn11 is necessary for the maintenance of a correct mitochondrial tubular network, we wished to investigate the relative phenotypes associated with Rpn11 domains. Single-site mutants in the MPN+ sequence E-HxHx₇Sx₂D of Rpn11 exhibit general proteolytic defects, accumulate polyubiquitinated proteins and are temperature-sensitive [28], yet contain a normal-looking tubular mitochondrial network (Figure 4B). We transformed *mpr1-1* with plasmids expressing these point mutations in Rpn11, and found that they are able to rescue the growth of *mpr1-1* at the restrictive temperature of 34 °C and allow for utilization of glycerol as a carbon source as well (Figure 4A). Such intragene complementation of two slow-growing and temperature-sensitive mutants may indicate that the two domains of Rpn11 function independently, and there is no need for an intact molecule bearing both a functional MPN+ domain and the C-terminal part. We may deduce that the participation of Rpn11 in mitochondrial function may be independent of its deubiquitination activity.

Mutants in the C- and N-terminal regions of *rpn11* exhibit distinct phenotypes

To distinguish between the relative roles of the C- and N-terminal regions of Rpn11 we compared additional phenotypes. Low levels of amino acid analogues such as canavanine are known to promote accumulation of damaged proteins, which must be removed by the proteasome. Thus many proteasome mutants are sensitive

Figure 4 Intragenic complementation of *mpr1-1* and *rpn11* MPN mutations

Suppression of the mitochondrial defect of *mpr1-1* by plasmids expressing single amino acid substitutions in the MPN+ motif of Rpn11. **(A)** The *mpr1-1* mutant was transformed with the following plasmids (counter-clockwise from the top right): an empty plasmid, WT *RPN11* (pYC-RPN11), *rpn11C116A* (pYC-rpn11C), *rpn11H111A* (pYC-rpn11H), *rpn11S119A* (pYC-rpn11S) and *rpn11D122A* (pYC-rpn11D). Plates were incubated at the non-permissive temperature (34 °C) for 2 days. Independently, both *mpn* mutants and *mpr1-1* are thermosensitive and lethal at 34 °C [28]. It should be noted that of the *mpn* mutations, *rpn11H111A* is the most severe [28]; therefore, unsurprisingly, it is slightly less efficient in rescuing *mpr1-1*, although it too rescues temperature sensitivity (left panel) and allows for growth on glycerol (right panel). **(B)** Normal tubular mitochondrial network in *mpn* mutants. The *rpn11* point mutants were cultured to the mid-exponential growth phase at 28 °C and stained with DASPMI for visualization of mitochondria. The *rpn11* point *mpn* mutants exhibit normal mitochondrial morphology.

to low levels of canavanine [16,35]. As expected, the MPN+ mutant *rpn11D122A* is sensitive to 3 µg/ml canavanine in the growth medium, although surprisingly *mpr1-1* behaves similarly to WT and is insensitive at these levels (Figure 5A). This result corroborates the conclusion that the effects of *mpr1-1* are inherently different from those of mutations in the MPN+ motif. Underscoring this conclusion, mutants in the MPN+ motif were found to be sensitive to UV damage and exposure to low levels of

the translation inhibitor CHX, while *mpr1-1* was not (Figures 5B and 5C). Sensitivity to low levels of CHX is reported to be a consequence of depleted levels of ubiquitin that occur when degradation of ubiquitin is faster than synthesis [36,37]. Thus sensitivity to low levels of CHX (as shown in Figure 5C) can be a good indication of malfunctions in the ubiquitin-proteasome pathway, in contrast with sensitivity at high concentrations that reflect general translational arrest. Overall, the phenotypes of *rpn11*^{MPN} mutants are typical of mutants in the ubiquitin-proteasome pathway, whereas those of *mpr1-1* are pleiotropic. Curiously, both MPN+ mutants and *mpr1-1* are sensitive to the alkylating agent MMS (Figure 5D). This sensitivity can be rescued by intragenic complementation of *rpn11*^{MPN} and *mpr1-1* mutant copies, suggesting that different regions of Rpn11 participate differently to MMS adaptation. In contrast with suppression of the temperature-sensitivity of *mpr1-1*, Rpn8 cannot rescue the MMS sensitivity of *mpr1-1* (Figure 5D), suggesting a requirement of an intact C-terminus of Rpn11 in the MMS-damage response, but surprisingly not for the UV-damage response.

Suppression of proteasome structural defects does not rescue *mpr1-1* mitochondrial phenotypes

To verify that the chimaeric proteins are indeed expressed and can be stably incorporated into proteasomes, we analysed the migration pattern of tagged Rpn11 constructs. Fractionation of whole-cell extract using a glycerol gradient separates proteasomes from lower-molecular-mass proteins in their native state. Upon overexpression, tagged Rpn11 or CH_{R8-R11} chimaeras are incorporated into the proteasome, but are also found in low-molecular-mass fractions (Figure 6). Highly abundant Rpn8 behaves similarly (results not shown). Incorporation of these tagged proteins into assembled proteasomes is similarly efficient in WT and *mpr1-1* strains (Figure 6). Finding two pools of Rpn11 indicates that excess Rpn11 can exist in a stable form that is not associated with the proteasome, and may explain the documented phenotypes observed upon overexpression of this subunit (see the Introduction).

A slight shift towards lower-molecular-mass fractions was found for proteasomes in *mpr1-1* that were not covered by rescue plasmids (Figure 6, bottom panel). This observation agrees with a report showing structural defects in proteasomes purified from *mpr1-1* [12]. To test whether a proteasome structural defect is the source of other *mpr1-1* phenotypes, we studied proteasome conformation in various *rpn11* strains. Crude cell extracts from strains grown at the permissive temperature were resolved by non-denaturing PAGE, and proteasomes were visualized by fluorogenic peptide overlay (Figure 7A). The overwhelming majority of proteasomes from exponentially growing WT yeast are found as doubly capped or singly capped 26 S holoenzymes with nearly undetectable levels of free 20 S CP, in agreement with previous results [38]. In contrast, proteasomes that were rapidly resolved from whole-cell extracts of the *mpr1-1* strain are almost exclusively present as 'lidless' base-CP complexes (Figure 7A). An elevation in temperature promotes proteasome dissociation, resulting in increased levels of singly capped base-CP complexes and 20 S CP over the larger proteasome conformations (Figure 7B). Quite unexpectedly, these defects in proteasome structure are almost completely rescued by highly abundant Rpn8 or the chimaeric protein CH_{R8-R11}, even though neither carries a functional MPN+ domain. It should be noted that no structural defects are observed for proteasomes purified from mutants in the MPN+ motif of Rpn11 [28], supporting a largely functional, rather than a structural, role for the MPN+ motif itself. An additional observation regarding proteasomes from the *mpr1-1*

Figure 5 Comparative growth phenotypes and stress response of *mpr1-1* and MPN+ mutants of *rpn11*

(A) Canavanine-sensitivity of WT-CMY, WT-BY and the congenic mutants *mpr1-1* and *rpn11D* respectively. The same number of cells were spotted on to a YPD plate and on a minimal medium plate supplied with the required amino acids supplemented with 3 μ g/ml of canavanine. (B) UV-sensitivity of WT-CMY ($\blacktriangle$), WT-BY ($\bullet$), the *mpr1-1* mutant ($*$) and the point mutant *rpn11D* ($\blacklozenge$). The strains were grown at a concentration of 2×10^7 cell/ml, plated on to YPD medium and irradiated at 0, 30, 60, 90 and 120 J/m² (200 cells/plate; three plates for each point). Plates were incubated at 28 °C in the dark for 3 days and colonies were counted and averaged to determine residual viability compared with untreated cells. Comparisons should be made between each mutant and its congenic WT (see the Experimental section). (C) CHX = sensitivity of WT-CMY, WT-BY, the *mpr1-1* mutant and the point mutant *rpn11D*. Serial dilutions of starting culture containing the same number of cells were spotted on to the YPD plate or on to YPD plates containing 0.05, 0.1 or 0.5 μ g/ml CHX. (D) MMS-sensitivity of the *mpr1-1* mutant transformed with the empty plasmid for control, or with pYC-RPN11, pYE-RPN8 or pYC-*rpn11D* plasmids, as well as the MMS-sensitivity of the *rpn11D* mutant strain and its congenic WT strain WT-BY. Cultures containing identical number of cells were spotted at 10-fold dilutions on to YPD medium or on to YPD medium containing 0.03 % MMS.

strain is that they appear naturally repressed compared with WT (Figure 7). Lower levels of peptidase activity were measured for proteasomes prepared from *mpr1-1* whole-cell extract (controlled for equal proteasome levels as determined by protein staining and immunoblotting), in agreement with diminished peptidase activity observed for purified proteasomes from *mpr1-1* [12].

It would be expected that such dramatic structural and functional proteasomal defects would give rise to general proteolytic phenotypes. Indeed, slight accumulation of polyubiquitinated pro-

teins is detected in *mpr1-1*, even at the permissive temperature (Figure 8A). Accumulation is greatly pronounced following a shift to the restrictive temperature for *mpr1-1* cells (Figure 8B), in agreement with the marked decrease in intact proteasome levels (Figure 7B). That the *mpr1-1* mutant does not show sensitivity to canavanine (Figure 5A) is surprising in light of these results, as it suggests that the proteolysis defect of *mpr1-1* may be limited to a subset of proteins, rather than a general effect. Overexpression of Rpn8 almost completely rescues the

Figure 6 Chimaeric proteins are incorporated into the proteasome and are present in non-associated forms

Glycerol-gradient fractionation of yeast whole-cell extract was analysed for proteasome activity and the Rpn11 migration pattern. Peptidase activity indicates migration of WT proteasome (top panel). Fractions were also immunoblotted with anti-GFP for detecting the relevant constructs. Our antibodies were not sensitive enough to detect naturally abundant GFP-tagged Rpn11 constructs; however, upon overexpression of Rpn11-GFP, it can be found both in proteasome-associated and free (low-molecular-mass) fractions. Similarly, CH_{R8-R11}-GFP expressed from high-copy plasmids is found incorporated into proteasomes, as well as in low-molecular-mass fractions. Migration of proteasomes in *mpr1-1* that were not covered with an Rpn11-expressing construct was determined with anti-Rpt1. A slight shift towards lower-molecular-mass fractions is observed for proteasomes in *mpr1-1*, which is corrected for upon expression of Rpn11 or Rpn11 chimaeric proteins (see also Figure 7).

Figure 7 Proteasome structural defects in *mpr1-1* are temperature-sensitive and can be suppressed by RPN8

(A) Cell extracts were prepared from exponentially growing WT or *mpr1-1* strains, or from other mutant strains that were brought to an identical cell density at the permissive temperature of 26 °C for *mpr1-1*. Extracts were clarified by centrifugation, and samples containing identical quantities of total protein were separated by non-denaturing PAGE (native gels). Proteasomes were visualized by the fluorogenic peptide overlay assay. Proteasome holoenzymes in WT are found as a mixture of symmetric doubly capped (RP₂CP) and asymmetric singly capped (RP₁CP) conformations. 26 S proteasomes in *mpr1-1*, however, are found almost exclusively in lidless forms, whether singly or doubly capped. Slightly higher levels of dissociated free 20 S CP are also evident. 20 S CP is easily visualized upon activation of the CP by SDS (right panel). (B) At 30 °C, dissociation of *mpr1-1* is greatly pronounced. Almost no doubly capped form is present, and levels of singly capped base-CP complex are diminished. This structural defect can be suppressed to a great extent by overexpression of RPN8, and to a lesser degree by CH_{R8-R11}. As a control, expression of WT RPN11 fully rescues the *mpr1-1* proteasome structural defect.

accumulation of polyubiquitinated proteins, probably because of Rpn8 correcting proteasome structural defects (Figures 6–8). Independently, accumulation of polyubiquitinated substrates was

Figure 8 RPN8 can suppress general proteolysis defects associated with *mpr1-1*

(A) Accumulation of polyubiquitin (polyUb) conjugates in *mpr1-1* at the permissive temperature can be rescued by overexpression of RPN8. Naturally abundant *rpn11D* (containing the WT C-terminus, but not a functional MPN+ motif) can also rescue polyubiquitin-conjugate accumulation, but to a lesser extent than multicopy RPN8. (B) Same as (A), after a 5 h shift to the restrictive temperature for *mpr1-1*. As expected, due to the heat-shock damage, polyubiquitinated conjugates accumulate even in WT to a greater extent than at the lower temperature. Accumulation in *mpr1-1* is especially pronounced, but can be suppressed to a great extent by overexpression of RPN8 or naturally abundant *rpn11D*. The *rpn11D* mutant alone also accumulates polyubiquitinated substrates (right panel); however, this accumulation is not additive with accumulation due to the *mpr1-1* mutation (left previously). The bottom panels (marked with an asterisk) indicate an equal protein loading in the lanes, and the positions of molecular-mass markers are shown on the left.

also observed for MPN+ mutants [28]. Interestingly, expression of *rpn11D122A* partially corrects proteolysis defects in *mpr1-1* (Figure 8B), indicating that subunits lacking a functional MPN+ motif can rescue proteolysis defects associated with *mpr1-1*.

Table 3 Summary of Rpn11 phenotypes

The Table shows a general summary of the phenotypes observed for the *rpn11* mutants detailed in this study.

Mutant	Region	Proteasome structural defects	Growth phenotypes							Growth on glycerol	Mitochondria structural defects
			PolyUb-conjugate accumulation	Sensitivity to temperature	Can	CHX	UV	MMS			
<i>mpr1-1</i>	C-terminus*	+	+	+	—	—	—	+	—	+	
<i>mpn</i>	N-terminus†	—	+	+	+	+	+	+	+	—	
<i>mpr1-1 [mpn]</i>	Intragenic complementation‡	—	+/—	—	—	—	—	—	+	—	
<i>mpr1-1 [Rpn8]</i>	Extragenic complementation§	—	—	—	—	—	—	+	—	+	

* The *mpr1-1* mutant results in a frameshift and truncation in the final (C-terminal) 30 amino acids of Rpn11.

† A general summary of phenotypes common to single-site substitutions at key residues that define the MPN+ putative metalloprotease motif (the signature EHxHSD consensus sequence) of Rpn11.

‡ Most phenotypes associated with either mutant are suppressed upon intragenic complementation of naturally abundant *mpn*-mutated *rpn11* expressed in an *mpr1-1* background.

§ Extragenic complementation of *mpr1-1* by overexpression of *RPN8*. Proteasomal and proteolytic defects are corrected, but not mitochondrial or respiratory defects.

We conclude that *mpr1-1* and MPN+ mutants do not influence proteolysis in the same manner.

DISCUSSION

The tubular organization and cellular distribution of mitochondria is a highly dynamic process that is shaped by a tight equilibrium between fusion and fission events [39,40]. The ubiquitin-proteasome pathway may influence mitochondrial structure and function in multiple ways. For instance, it has long been documented that breakdown of some mitochondrial proteins is an ATP- and ubiquitin-dependent process, attributed to the proteasome [41]. Targets of proteasomal proteolysis could be proteins normally situated in the outer mitochondrial membrane. A fraction of proteasomes may even be involved in the quality-control system on the outer membrane of the mitochondria, similar to its documented role in ER-associated degradation. Alternatively, ubiquitination could be involved in targeting or degrading nuclear-encoded proteins on their way to be imported into the mitochondria [42,43]. Underscoring the link between the ubiquitin system and the mitochondrial network, aberrant mitochondrial morphology and inheritance have been observed in mutants encoding E3 ubiquitin ligases. Examples include Rsp5, an E3 that is also involved in endocytosis and cytoskeleton remodelling [44], or the F box protein Mdm30, which may be responsible for targeting the mitochondrial fusion protein fusion protein Fzo1 for ubiquitination [45]. At the other end of the ubiquitin system, a deubiquitinating enzyme Ubp16 has been localized to the mitochondria outer membrane, although its purpose there is unknown at present [46]. Finally, a connection between the proteasome itself and mitochondria structure can be inferred from phenotypes associated with mutations in two proteasome subunits. Mitochondrial defects caused by a mutation in the mitochondrial protease Yme1 are suppressed by a mutation in the *rpt3* locus that encodes an ATPase subunit of the proteasome [47]. Since Yme1 is localized to the inner membrane of the mitochondria, indirect participation of the ubiquitin-proteasome pathway is suggested in this case. In a more direct example, mutations in the extreme C-terminal region of the proteasome lid subunit Rpn11 (the *mpr1-1* mutant) cause mitochondria malformation and malfunction [18,19].

In the present study, we observed pleiotropic effects of Rpn11 in both proteolysis and mitochondrial integrity. On the one hand, mutations in the MPN+ motif at the N-terminal domain cause proteolytic defects resulting from reduced deubiquitinating activity associated with this domain. On the other hand, mutations at the C-terminal region lead to both proteasome and mitochondria

malfunctions. Interestingly, we now show that proteasome structural defects and general proteolytic defects associated with *mpr1-1* can be suppressed, for the most part, by high amounts of another lid subunit, Rpn8 (Figures 1, 2, 5 and 8, and Table 3). The suppressive effects of Rpn8 are not easy to explain; both *RPN11* and *RPN8* are essential genes, the products of which are present at approximately stoichiometric levels in the 19 S RP. As mentioned above, Rpn8 does not contain the catalytic MPN+ motif and is not expected to participate in deubiquitination. We can speculate that high levels of Rpn8 could stabilize Rpn11-containing lid precursors, such as the Rpn5/11/9/8 cluster [16]. Nevertheless, it is surprising that repairing proteasome conformations and proteolysis defects by expressing high levels of Rpn8 is insufficient to correct defects in the mitochondrial tubular system observed in *mpr1-1* (Figures 1 and 2). Rpn8, or other constructs that do not contain the C-terminal region of Rpn11, cannot correct for phenotypes associated with mitochondrial structure and respiratory function (Table 3). In contrast, expression of mutant or chimaeric proteins that contain the natural C-terminal region of Rpn11 does suppress the mitochondrial phenotypes by intragenic complementation (Figure 2 and Table 3). Overall, these results point to a unique role for the C-terminal region of Rpn11 in mitochondrial maintenance, beyond the function of Rpn11 as a deubiquitinase that emanates from its N-terminal domain.

A dual role for Rpn11 could explain how the mitochondrial effects of the *mpr1-1* mutation (which are not common to other proteasomal mutants) can be dissociated from phenotypes associated with other *rpn11* mutants. The situation regarding Rpn11 is reminiscent of the pleiotropic roles executed by two distinct domains of another 19 S RP subunit, Rpn10. On the one hand, Rpn10 has been shown to bind polyubiquitin chains via the ubiquitin-interacting motif present in its C-terminal region. On the other hand, Rpn10 independently participates in stabilizing the structure of the 19 S RP via a von Willebrand factor A domain in the N-terminal portion of the protein [16]. Pertinent to its function, Rpn10 is found in two pools: proteasome-bound and 'free form' [48]. The free form has been proposed to shuttle polyubiquitinated substrates to the proteasome, whereas the proteasome-bound form is pivotal for stabilizing lid-base interactions [9,10,16]. In this context, suppression of cell-cycle and temperature-sensitivity phenotypes of *mpr1-1* by *RPN8* overexpression, but not of mitochondrial ones, is in agreement with the possibility that the two functions ascribed to Rpn11 could be carried out independently (possibly, although not certainly, by two separate pools of Rpn11 molecules; Figures 5–8).

That *mpr1-1* cells are viable, despite finding the majority of proteasomes in a lidless configuration, questions whether a tight interaction between lid and base is necessary for proteasome function. Clearly, all lid subunits are essential (with the exception of Rpn9), and are found at approximately stoichiometric levels in purified proteasomes [32,33,49]. The lid is also required for proper degradation of both mono- and poly-ubiquitinated substrates *in vitro* [10,11]. Nonetheless, under certain conditions the lid can detach rather easily from proteasomes, suggesting that *in vivo* dynamic association of the lid with the proteasome takes place [10,12,34,49]. It is not unreasonable to surmise that the lid has independent functions as well. Finding stable pools of Rpn11 that are not associated with the proteasome (Figure 6) supports such a possibility, and may explain the documented phenotypes observed upon overexpression of this subunit.

The recent revelation that Rpn11 participates in proteasome-associated deubiquitination focuses interest on the MPN+ metalloprotease site in its N-terminal MPN domain. However, in the present study we show that the role of Rpn11 in mitochondria maintenance does not necessarily invoke these properties of Rpn11. In particular, the observation that the *mpr1-1* mutant is insensitive to conditions typically associated with defects in the proteasome or other components of the ubiquitin system, such as exposure to amino acid analogues, low levels of CHX or low UV dosage (Table 3), points to a distinct role of Rpn11. At this stage, it is unclear whether Rpn11 functions independently in mitochondrial maintenance, or whether it serves to link mitochondria with proteasomes for unique proteolytic needs. One hypothesis is that the C-terminal part of Rpn11 could partake in contacting proteins at the mitochondrial surface, or serves to recruit a subpopulation of proteasomes to the mitochondria. For instance, levels of Fzo1, a protein localized to the outer membrane and involved in mitochondrial fusion, are regulated by ubiquitination [45]. Whether the proteasome in general, and Rpn11 in particular, partake in regulating Fzo1 levels should be a topic of future research.

We are grateful to Anna Chelstowska for many helpful discussions, and to Noa Reis for suggestions and technical assistance. Anna Chelstowska generously provided the human library. Many thanks to Benedikt Westermann for the mitochondrial GFP plasmid, and to Allen Taylor of the anti-ubiquitin antibody. This work was partially supported by Center of Excellence BEMM, MIUR 2003 University of Rome "La Sapienza" and FIRB (code RBNE01KMT9) to L. F., and grants from the Israel Science Foundation (ISF), the German-Israeli Binational Foundation (GIF) and the Wolfson Foundation for research on ubiquitin to M. G.

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Received 5 January 2004/9 March 2004; accepted 12 March 2004

Published as BJ Immediate Publication 12 March 2004, DOI 10.1042/BJ20040008